EXTRACTION AND CHARACTERISATION OF PROTEIN FRACTION FROM DATE PALM (*Phoenix dactylifera* L.) SEEDS

By

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ABSTRACT

To meet the challenges of protein price increases from animal sources, the development of new, sustainable and inexpensive proteins sources (non-animal sources) is of great importance. Date palm (*Phoenix dactylifera* L.) seeds could be one of these sources. These seeds are considered a waste and a major problem to the food industry. In this thesis we report a physicochemical characterisation of date palm seed protein. Date palm seed was found to be composed of a number of components including protein and amino acids, fat, ash and fibre. The first objective of the project was to extract protein from date palm seed to produce a powder of sufficient protein content to test functional properties. This was achieved using several laboratory scale methods. Protein powders of varying protein content were produced depending on the method used. Most methods were based on solubilisation of the proteins in 0.1M NaOH. Using this method combined with enzymatic hydrolysis of seed polysaccharides (particularly mannans) it was possible to achieve a protein powder of about 40% protein (w/w) compared to a seed protein content of about 6% (w/w).

Phenol/TCA extraction gave the protein powder with the highest protein percentage of 68.24% (w/w) and this powder was used for subsequent functional testing. Several factors were found to influence seed protein extraction such as pH, temperature, the extraction time, the solvent to sample ratio and the solvent concentration. Optimum conditions for extraction were found to be pH 10, 45˚C and extraction time of 60 min. The results showed that use of enzymes to hydrolyse and remove seed polysaccharides improved the extraction of date seed protein. Optimal improvement was obtained using Mannaway, which hydrolyses mannans and galactomannans, which gave a powder with 34.82% (w/w) protein compared to the control of 11.15% (w/w) protein. The proteins in the extracted date seed protein were profiled using LC/MSMS. Three-hundred and seventeen proteins were identified.

The proteins belonged to all major functional categories. The most abundant proteins were glycinin and β-conglycinin, the two major seed storage proteins of plants. The functional properties of extracted date seed protein were investigated using a range of tests.

The thermal properties of date seed proteins were consistent with a powder containing high levels of conglycinin and β-glycinin. The solubility had a similar pH profile to soy protein, but differed in absolute solubility due to differences in non-protein composition. Similarly, water holding and oil holding capacity of date seed protein was lower than for soy protein, probably because of compositional differences. Date seed proteins were able to emulsify oils and had a comparable emulsifying ability and emulsion stability to soy protein isolate. The date seed protein was not a good foaming agent compared to soy protein or whey protein concentrate.

DEDICATION

To:

My dear late Mother,

Fatema

I dedicate

my whole work

to her gracious soul.

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Initially; Praise and thanks be to Allah, Lord of the Worlds.

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ACADEMIC REGISTRY – Research Thesis Submission

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ABBREVIATIONS

%	percent
<	less than
>	greater than
±	plus or minus/error margin
µg	microgramme
µl	microliter
µm	micrometre
1D	one dimensional
2D	two dimensional
ACN	acetonitrile
ADF	acid detergent fibre
ADG	average daily gain
AOAC	Association of Official Analytical Chemists
APS	Ammonium persulfate
a_w	water activity
BSA	Bovine Serum Albumin
CDPS	Crude Date Palm Seed
CHAPS	3- (3-Cholamidopropyl) dimethylammonio -1- propanesulfonate
CHCA	α-cyano-4-hydroxycinnamic acid
cm	centimetre
Da	Dalton
DDPSP	Defatted Date Palm Seed Powder
dH_2O	deionised water
DPF	date palm flesh
DPS	date palm seed
DPSP	date palm seed powder
DPSPI	date palm seed protein isolate
DSC	differential scaning calorimetry
DTT	dithiothreitol
e.g.	for example
EAI	Emulsifying Activity Index
ESI	Emulsion Stability Index
FAO	Food and Agriculture Organization
FFDPSP	Full Fat Date Palm Seed Powder

Fig.	Figure
g	gravitational force constant
GC-MS	Gas chromatography-mass spectrometry
GLC	Gas liquid chromatography
h	hour
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
i.e.	idest/that is
IEF	isoelectric focusing
kDa	kilo Dalton
kg	kilogramme
LC-MSMS	Liquid-chromatography coupled tandem mass spectrometry
Ltd	limited
M	mole
MALDI-TOF	Matrix-assisted laser desorption ionization- time of flight
mg	milligramme
min	minute
ml	millilitre
mM	millimolar
NaOH	Sodium hydroxide
NDF	neutral detergent fibre
nm	nanometer (unit of wavelength)
OAC	Oil Absorption Capacity
˚C	degree celsius
PBS	Phosphate Buffered Saline
pH	decimal logarithm of the reciprocal hydrogen ion activity
Ph/TCA	Phenol/Trichloroacetic Acid
PMF	peptide mass fingerprinting
RF	relative mobility
SDS-PAGE	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis
SPI	Soy Protein Isolate
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic Acid
UAE	United Arab Emirates
UF	Ultrafiltration
UK	United Kingdom

UoE	University of Edinburgh
USA	United States of America
UV	Ultraviolet
V	Volt
v/v	volume to volume ratio
v/w	volume to weight ratio
vol.	volume
W	Watt
w/v	weight to volume ratio
w/w	weight to weight ratio
WAC	Water Absorption Capacity
WHC	Water Holding Capacity
xg	times gravity/gravity force.

PUBLICATIONS ARISING FROM THIS THESIS

Published paper

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CHAPTER ONE

General Introduction

1.1 Introduction

Date palm (*Phoenix dactylifera* L.) is considered the oldest fruit tree in the world. It has been cultivated in North Africa and the Middle East for millennia, although the exact origin of date palm has not been verified (Zaid & de Wet, 2002). Some reports cite that it has been used since 4000 BC in Mesopotamia (Popenoe, 1973), and by the Egyptians since 2000-3000 BC (Manickavasagan et al., 2012). Either the Indian date palm (*P. sylvestris*) or the African date palm (*P. reclinata*) may have been the progenitor of *Phoenix dactylifera* L. (Zaid & de Wet, 2002). Dates play an important role in the social life of the people who live in these regions (El-Sharnouby et al., 2009) both in their diet and medicinally for treating e.g. obesity (Sulieman et al., 2012).

There are more than 2000 different cultivars of date palm known worldwide but only a few of them have been used for their agricultural productivity and fruit quality (Mrabet et al., 2008). Botanically, date palm is a monocotyledonous plant that belongs to the *Arecaceae* (previously known as *Palmaceae* or *Palmae*) family which contains 200 genera and more than 2000 species (Diaz et al., 2003). There are many species of the genus Phoenix, but the species of *Phoenix dactylifera* is the one most cultivated for their edible fruits, whereas other species produce fruits which can be used by animals and birds (Robinson et al., 2012). These species are distributed in tropical or subtropical regions of medial eastern countries, North Africa, southern Asia, North America and Australia (Table 1.1).

The scientific name *Phoenix dactylifera* comes from the Phoenician word, *Phoenix* which means date palm while, *dactylifera* is probably derived from the Greek words daktulos which mean a finger (Biglari, 2009). There are more than 100 million date palm trees throughout the world. Fruits production from the trees starts at an average age of 5 years, with their production lasting up to 60 years (Al-Shahib & Marshall, 2003). They produce an annual average yield of between 400-600 and 100-150 kg/tree for fresh crop and dry crop respectively (Al-Shahib & Marshall, 2003).

The yield and fruit quality of dates are dependant on factors such as pollination, fertilization, water relations and cultivar (Marzouk & Kassem, 2011).

Table 1.1: Some Species of *Phoenix* Genera with their Geographical Distribution (Zaid & de Wet, 2002).

Species	Common Name	Distribution
Phoenix dactylifera L.	Date Palm	Mediterranean countries, Africa and parts of Asia; introduced in North America and Australia
P. atlantica A. Chev.	–	Occidental Africa and Canary Islands
P. canariensis Chabeaud.	Canary Palm	Canary Islands and Cape Verde
P. reclinata Jacq.	Dwarf Palm	Tropical Africa (Senegal and Uganda) and Yemen (Asia)
P. sylvestris Roxb.	Wild Date Palm or Sugar Palm	India and Pakistan
P. humilis Royle.	–	India, Burma, and China
P. hanceana Naudin.	–	Meridional China and Thailand
P. robelinic O'Brein.	–	Sri Lanka, Toukin, Annam, Laos and Thailand
P. farinifera Roxb.	Pigmy Palm	India, Ceylon and Annam
P. rupicola T. Anders.	Rocky Date Palm	India
P. acaulis Roxb.	Dwarf Palm	Bangaladesh and India
P. paludosa Roxb.	Hental or Juliana Palm	Bangaladesh, Tenasherim, Andaman, Nikobaren and Thailand

1.2 Structure and Botanical Description of Date Palm (*Phoenix dactylifera* L.) Tree

Date palm (*Phoenix dactylifera* L.) is a tall tree which reaches a height of 15-20 m before it is cut down due to declining yield, increasing difficulty and danger in reaching the top during pollination, bunch management and harvesting (Shamsi & Mazloumzadeh, 2009). Date palm trees may reach an age of over 100 years (Seelig, 1974).

They produce a fruit called date and botanically consist of roots, trunk, leaves, flowers and fruits as shown in Figure 1.1.

The roots originate from a bulb at the trunk base and have a fasciculated fibrous system similar to a maize plant, with their length reaching 4 m on average and up to 10 m in light soil (Manickavasagan et al., 2012). In a deep loamy soil approximately 85% of date palm roots are distributed in a zone to 2 m deep and 2 m on both lateral sides. These roots can withstand wet soil for months, but if such conditions continue for longer periods they may harm the roots and thus the fruit production (Zaid & de Wet, 2002).

The trunk of date palm, also called a stipe or stem, is brown in colour and is a single vertical cylinder which can extend to 30 m in length. It is covered by leaf bases that are enclosed in fibre which protect the trunk from animals and insects, and in addition reduce loss of water (Manickavasagan et al., 2012; Zaid & de Wet, 2002).

The leaves of date palms have a normal life span of 3–7 years and can reach up to 6 m in length and half a meter in width. The leaf narrows at the midrib and towards both leaf ends. The mature trees have approximately one hundred to one hundred and twenty-five leaves and produce ten to twenty-six new leaves annually. The leaf can support the production of date clusters from 1–1.5 kg under good conditions (Manickavasagan et al., 2012; Zaid & de Wet, 2002). Separate male and female flowers are produced. Flowers are arranged in strands that attach to a rachis forming an inflorescence called a spadix. The spathe encloses the immature inflorescence, which splits longitudinally in order to help the pollination of mature male and female flowers. Pollination can achieve naturally by wind or artificially by hand (Manickavasagan et al., 2012). Artificial pollination in date palm helps improve yield and quality of date fruit (Bechar et al., 1999). In confirmation of this Iqbal et al. (2010) reported that hand pollination gives a better yield than natural pollination. The fruits are called dates only after pollination. The dates develop from one fertilized ovule to form a single carpel whilst other ovules are aborted but remain visible at the fruit calyx. The developing fruit is characterized by a membranous endocarp surrounding a seed. The endocarp is the edible part of the fruit (Zaid & de Wet, 2002).

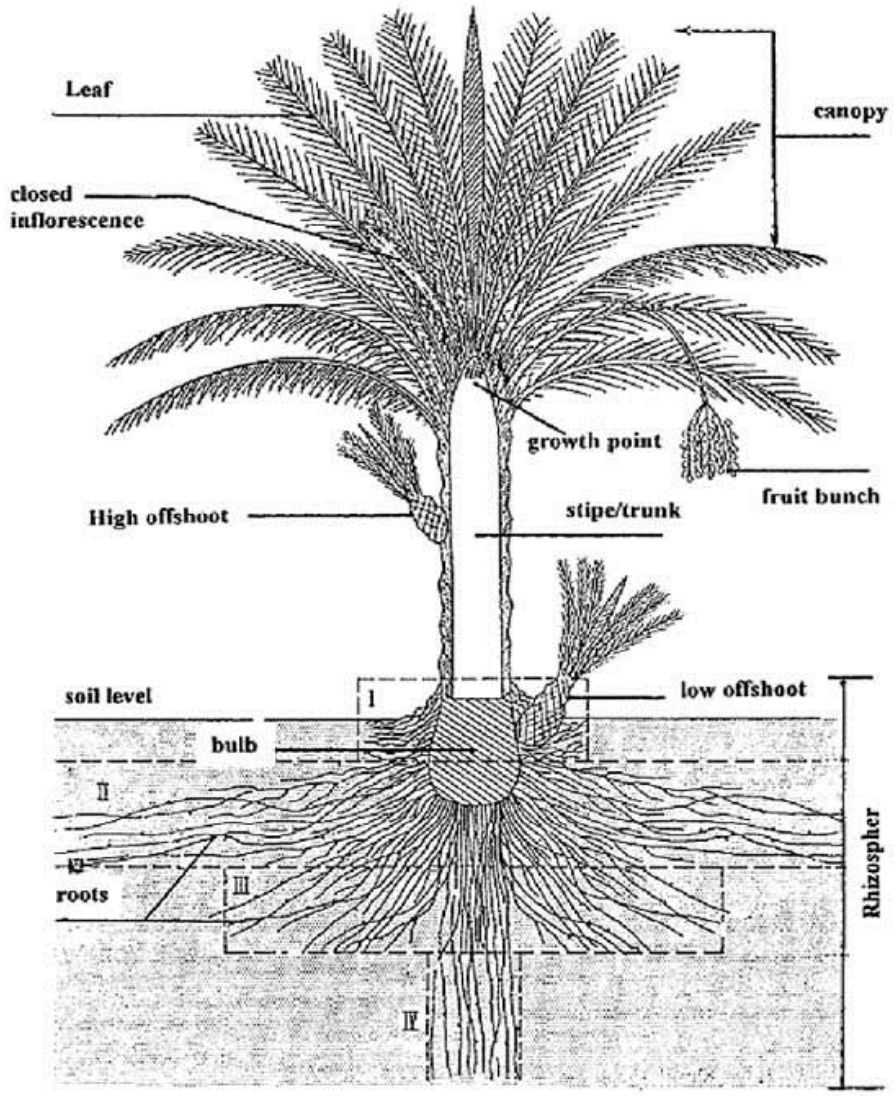

Fig 1.1: Diagram of a date palm with its root system (Zaid & de Wet, 2002).

There are many variations in date fruits that show differences in the shape, size, colour, and chemical composition of fruits which arise due to varietal differences, growing conditions, soil and climatic variations (Al-Yahyai & Kharusi, 2011).

1.3 Distribution and Global Production of Date Palm

Date palm (*Phoenix dactylifera* L.) is an example of a plant crop that is closely connected with human life, It can be cultivated globally in all five continents, but generally is found at latitudes between 39˚ north and 20˚ south. The main areas of date palm production are in the Middle East and North Africa where approximately 90% of the global date crop is produced

(Manickavasagan et al., 2012). The global distribution of date palm (*Phoenix dactylifera* L.) trees is shown in Figure 1.2 which shows data from the Global Biodiversity Information Facility (GBIF). Global production, industrialisation and utilisation of dates are increasing steadily.

The worldwide production of dates has increased from 1.8 million tons in 1961 to 6.9 million tons in 2005 (Chao & Krueger, 2007) and so has nearly quadrupled over the past four decades.

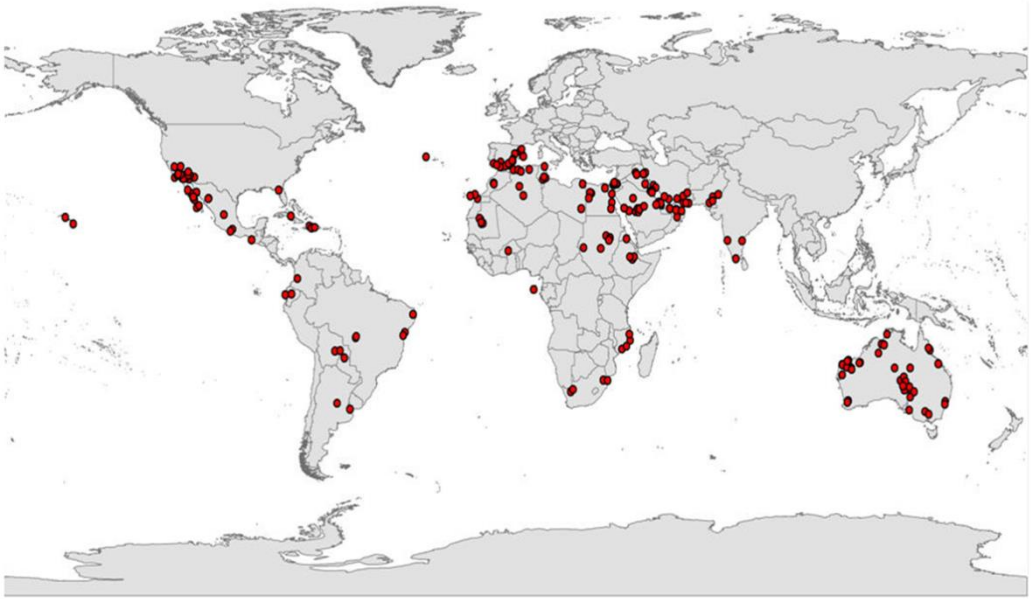

Fig 1.2: Global map distribution of date palm (*Phoenix dactylifera* L.) (Shabani *et al*., 2012).

The highest producer countries for date palm fruits are Egypt, Iran and Saudi Arabia with annual production of 1.3, 1.0 and 0.9 million tons per annum respectively (Abdul Qadir et al., 2011; FAO, 2008) which represents almost half the global production. In the UAE the number of date palm trees has increased from 10 million in 1999 to 43 million in 2010, whilst other countries such as the sultanate of Oman are planning to plant an additional one million date palms by the year 2015 (Shabani et al., 2012). Date palm trees can adapt to salty and alkaline soils with hot and dry climates. It is desired to have a climate of low humidity with a long hot summer over the period of growth and maturity, but the trees can tolerate a climate with a wide range of temperatures from –5˚C up to 50˚C. The optimum temperatures for all stages of maturity range from 21˚C to 27˚C. Date palm fruit can be classified to three categories according to their moisture content at the final stage of ripeness-soft (>30% moisture), semi-dry (20–30% moisture), and dry (<20% moisture) (Kader & Hussein, 2009).

1.4 Stages of Growth and Maturity of Date Palm Fruit

The fruits of date palm are called dates (Al-Yahyai & Al-Kharusi, 2011). After pollination the fruit of date palm grows through five stages of maturity that are characterized by physical changes that traditionally have been described by changes in taste, colour, and texture and chemical changes in the composition of the fruit (Tafti & Fooladi, 2006). These stages are called Hababouk, Kimri, Khalal, Rutab and Tamar stage as shown below (Figure 1.3). The appearance of date fruit in the five stages are shown in Figure 1.3.

Fig 1.3: Stages of growth and maturity of Date Palm fruit (Baliga et al., 2011).

A brief description of each stage of the ripening process follows (Ahmed et al., 1995; Al-Shahib & Marshall, 2003; Fadel et al., 2006; Baliga et al., 2011).

1.4.1 Stage One (Hababouk Stage)

Hababouk stage is considered the slowest stage of growth and starts 4-5 weeks after fertilization. The growth at this stage is very slow and the fruit is round with colour ranging between cream and light green.

1.4.2 Stage Two (Kimri stage)

The second stage is the Kimri stage, which is the longest stage of growth and maturity. It is characterized by a green colour of the fruit, increase in the size and weight of the fruit and rapid accumulation of reducing sugars and total solids. In this stage the fruit contains the highest percentage of moisture, acidity and tannins.

1.4.3 Stage Three (Khalal stage)

At the Khalal stage (third stage), the green colour changes to yellow or red according to variety, moisture content decreases and sucrose content begins to increase.

1.4.4 Stage Four (Rutab stage)

At the fourth stage (Rutab stage), loss of moisture is rapid and there is an increase in enzymatic activity of pectinase enzymes. All the enzymes that can hydrolyse pectic substances are termed as pectinases (Singh et al., 1999; Kapoor & Kuhad, 2002). The increase in activity of these enzymes leads to textural changes and softening of fruits during maturation and ripening; these enzymes are named on the basis of their mode of action such as pectin esterase (PE), pectin methyl esterase (PME) and polygalacturonase (PG). PE enzyme is specific to the hydrolysis of the methyl ester group from polygalacturonic acid. PG enzyme is a depolymerizing enzyme that specifically hydrolyses the α-1,4 glycosidic bonds between galcturonic acid monomers from the non-reducing end of pectic substances yielding the corresponding α-1,4-D-galacturonide and galacturonic acid (Favela-Torres et al., 2006; Ladaniya, 2008). At this stage there is a weight decrease together with conversion of sucrose into invert sugar and the fruit skin becomes brown and softer in texture.

1.4.5 Stage Five (Tamar stage)

In the Tamar stage (the final stage of maturity), the fruit shows a massive decrease in moisture with the decreases from about 84% in the second stage (Kimri stage) to about 24% in the Tamar stage (Ahmed et al., 1995; Al-Shahib & Marshall, 2003), as well as develops a wrinkled pericarp and the characteristic dark brown colour associated with dates. The fruit also loses tannins and acidity leading to loss of their astringency.

1.5 Storage of Dates

During the date harvest season which is spread over 2–3 months in the summer time, it may not always be possible to consume the fresh dates. As a consequence, date factories may receive huge amounts of freshly harvested dates as Tamar batches over a short period of time, which exceeds the markets capacity. Therefore it is necessary to store the dates and then re-market them at a later date (Ismail et al., 2008). Dates can be consumed at Khalal, Rutab or Tamar stage. A few cultivars are not allowed to reach Tamar stage and are commercially sold either as Khalal or Rutab fruit (El-Hadrami & Al-Khayri, 2012).

It is only at the fully mature stage (Tamar) that dates can be stored for future consumption and processing (Al-Yahyai & Al-Kharusi, 2011). Dates can be packed and stored at –3˚C for up to a year, and the shelf life of dates is expected to be up to two years at room temperature. The quality of dates may affected by storage parameters (Biglari, 2009). There are many reports in the literature on the effect of storage on different fruits and vegetables, but, few if any of these studies deal with the effect of storage parameters on date quality (Ismail et al., 2008). Additional studies need to be done to investigate the effect of storage on different parameters of dates, physical attributes and ultimately ideal conditions of storage suitable for the dates.

1.6 Structure of Date Palm Seeds

Date palm is a pitted fruit, and consists of a fleshy pericarp and seed. The seeds are oblong in shape with a ventral groove and range from 0.5 cm to 1.5cm in length. The cell wall of date palm (*Phoenix dactylifera* L.) seed consists almost entirely of linear mannan molecules which confer a fibrillar texture (DeMason et al., 1983) and hardness to protect the seeds against mechanical damage (Reid, 1995; Rodriguez-Gacio, 2012). The seed contains a small embryo which is located in the middle of the seed, and is surrounded by a thick walled endosperm (Figure 1.4). Jones (1969) and Obata (1979) found that the difference between date palm seed endosperm and other seed endosperms is the apparent lack of an active metabolism. Storage polysaccharides are present in many seeds as mannan, glucomannan, or galactomannan, with the mannans being important in date palm seeds (*Phoenix dactylifera* L.) (Buckeridge, 2010).

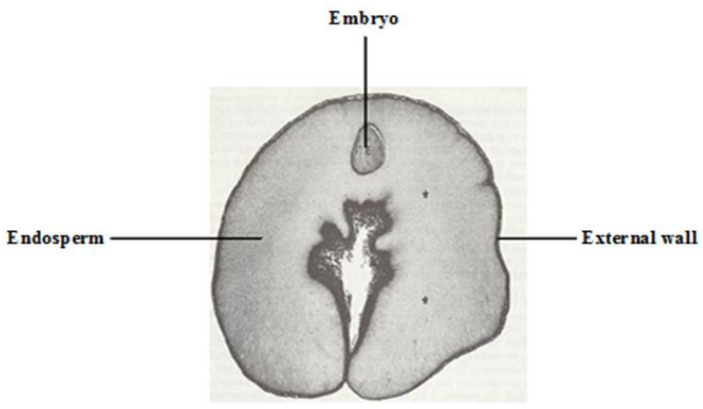

Fig 1.4: Cross section of date palm seed (Demason et al., 1983).

Mannans are a structurally diverse group of polysaccharides that differ in their monosaccharide composition, degree of polymerization and the substitution pattern (Mikkonen, 2009). There is a range of types of mannans found in seeds, bulbs, roots and tubers of different plants as storage polysaccharides (Hagglund, 2002). Mannans can be classified into four subfamilies: linear mannan, glucomannan, galactomannan, and galactoglucomanan (Petkowicz et al. 2001; Moreira & Filho, 2008).

They are comprised of linear chains of the sugar mannose, whilst the other groups contain varying amounts of glucose, galactose or both sugars in addition to mannose. Mannan is a polymer of mannose that can contain more than 10 individual sugars as shown in (Figure 1.5). It has been isolated from several seeds such as date palm (*Phoenix dactylifera* L.), green coffee bean (*Coffea arabica*) and ivory nut (*Phytelephas macrocarpa*) (Hagglund, 2002).

Most plant cell walls are composed mainly of cellulose, hemicellulose and lignin. Hemicelluloses are a diverse group of polysaccharides which includes galactans, xylans and mannans depending on the prevailing sugar type in the main chain (Hagglund, 2002). The endosperm cell walls of date palm seed are composed mainly of a linear 1,4 β-D-mannan with a small proportion of α-1,6-linked D-galactopyranosyl side chains (Meier, 1956 & 1958). This shares the β-1,4 bonding pattern of cellulose and presumably has a similar structural function to cellulose (Sekhar & Demason, 1988). The main function of these thick cell walls is as a site of carbohydrate storage (Meier & Reid, 1982; Demason et al., 1985). The major storage compounds found within the cells of date palm seed are lipids, which are present as numerous small lipid bodies, and proteins found as large protein bodies (Demason et al., 1983; Demason et al., 1985).

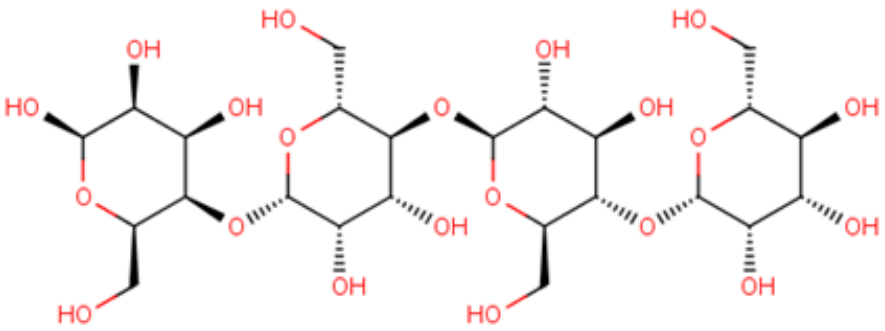

Fig 1.5: Structure of Mannan
(http://www.hmdb.ca/metabolites/HMDB29931)

A wide variety of proteins bodies have been described in the endosperm cells of date palm which differ in size but appear to be similar in composition in all cells (Demason et al., 1983). The endosperm walls consist of over 90% mannan with small amount of cellulose. Cells immediately adjacent to the embryo are free of cytoplasm and these cells have a very thick cell wall which consists mainly of mannan (Meier, 1956; Meier, 1958; Meier & Reid, 1982). Mannan that has been isolated from date palm (*Phoenix dactylifera* L.) is highly insoluble in water. It is responsible for the hardness of date palm seed (Hagglund, 2002). Demason et al. (1983) report that imaging of date seeds with light microscopy and histochemical stains has detected carbohydrates, proteins and lipids and shows the different sized large proteins bodies and a lack of starch in the cytoplasm.

1.7 Physical Characteristics of Dates

An understanding of the physical properties of the fruits and their seeds is very important in designing machines for processing, handling and storage of dates. These properties include length, mass, thickness, width, geometric mean diameter, bulk density, true density, volume, surface area, sphericity, porosity aspect ratio and static friction on different types of surfaces (Abdullah et al., 2011). The fruit of date palms differ in terms of shape, weight and size according to growth conditions. In general, most of the fruits are oblong with average length, width and mass of 40 mm, 20 mm and 9 g (Biglari, 2009).

Sulieman et al. (2012) studied the physical characteristics of five Sudanese date varieties and found that the average of weight, diameter, surface area, length, and flesh thickness were 9.2 g, 16.2 mm, 5.46 cm^2, 46.2 mm and 0.34 cm respectively, and the seed accounted for 12.8% of the total weight of the fruit on a mass basis. Sensory characteristics of date seeds have been studied by Hamada et al. (2002) who found they were light to dark brown in colour, odourless and had a bland taste with slight bitterness.

1.8 Chemical Composition of Fruit from *Phoenix dactylifera* L.

Most dates are consumed at Tamar stage due to their availability all round year and their good storage stability. A few varieties such as Khasab and Hilali do not reach the Tamar stage and are consumed at Khalal and Rutab

stages due to their short shelf lives (Ahmed et al., 1995). The composition of dates is of importance for the quality of the fruit and can be influenced by the variety and degree of ripeness (Manickavasagan et al., 2012).

Most previous studies have focused on the chemical characteristics of date palm fruit which are described below (Yousif et al., 1982; Ahmed et al., 1995; Al-Hooti et al., 1997; Hamada et al., 2002; Al-Shahib & Marshall, 2003; Al-Farsi et al., 2005; Rahman & Al-Farsi, 2005; Ismail et al., 2006; Sahari et al., 2007; Al-Farsi et al., 2007; Khan et al., 2008; Elleuch et al., 2008; Chaira et al., 2009; Rock et al., 2009; Guizani etal., 2010).

1.8.1 Moisture and Total Solids Content

Moisture and total solids content of foods are considered important in the food industry as they influence food preservation, food quality and resistance to deterioration. In addition, determination of moisture is essential to calculate the content of other food components on a uniform basis (i.e. dry weight basis). For date palm fruits the moisture content at the Tamar stage is very important in determining storage stability (Manickavasagan et al., 2012). Microbiologically, dry dates are stable when their water activity (a_w) is below 0.6 (Beuchat, 1981). The relationship between moisture content and a_w at constant temperature has been described theoretically and practically by moisture desorption isotherms (Manickavasagan et al., 2012).

The texture of dates depends on interaction between water and other components such as protein, carbohydrate, lipids and salts. Date texture can be classified according to their moisture content as soft, semisoft, semidry or dry (Manickavasagan et al., 2012). Under storage, dates will absorb water depending on their composition, a_w and the humidity of the air. Different varieties show differences in water sorption which relates to differences in total sugar content and sugar type. As an example fructose has a higher water holding capacity than at the sucrose or glucose (Manickavasagan et al., 2012). The moisture sorption isotherm of dates can be used to determine suitable storage conditions and aid in food product formulations (Myhara et al., 1998). In general, the moisture content of dates decreases as they ripen from Kimri to Tamar stage. Al-Shahib & Marshall (2003) have determined the average of moisture content of 13 varieties of dates in Kimri and Tamar stage and have found that this decreases from 83.6% to 24.2% respectively.

1.8.2 Acidity and pH

Dates have low acidity which ranges from 2.02 to 6.30 g of acid per kg of fresh fruits (Rygg et al., 1953). The main organic acids responsible for the acidity in the Deglet Nour variety were found to be malic and acetic acids (Manickavasagan et al., 2012). These organic acids impede the growth of microorganisms and influence the sensory characteristics of the fruits. Low quality of dates is often associated with high acidity (Jadlhav & Andrew, 1977; Siebert, 1999). The pH of dates tends to be slightly acidic (pH 5–6) which is not suitable for bacterial growth but is suitable for preservation of some B group vitamins and for mould growth. The pH of dates is related to their degree of growth and maturity with pH increasing as the maturity stage changes from Kimri to Tamar stage (Manickavasagan et al., 2012). Rastegar et al. (2012) have reported that the pH values of three date palm cultivars (Shahani, Piarom and Deiry) increases gradually from 5 to 6.8 during four fruit ripening stages (Kimri, Khalal, Rutab and Tamar).

1.8.3 Proteins and Amino Acid Content

Date palm fruit contains a percentage of protein (Pennington, 1989; Ahmed et al., 1995) between 2.3–5.6% protein depending on the stage of maturity. This compares to other fruits such as apples, oranges, bananas, and grapes which contain 0.3%, 0.7%, 1.0% and 1.0% protein respectively (Al-Showiman, 1998). Although the protein content is low, date palm fruit may have a role in human protein nutrition due to their contents of some essential amino acids (Salem & Hegazi, 1971). Ahmed et al. (1995) reported that most date flesh proteins are water soluble albumins.

The seeds of date fruits also contain protein. Stegmann et al. (1987) reported that the seeds of the Sayer cultivar had the highest protein content of 10.6% compared to three other cultivars grown in Iran. A similar study by EI-Shurafa et al. (1982) found that the seed of six cultivars of dates growing in the south of Libya (Tasfert, Taghiat, Adwi, Seloulou, Aspear and Taleesi) contain a maximum protein content of 6.42%. Al-Showiman & Baosman (1992) report that the protein content of seven seeds cultivars (Sheshi, Shaqra, Saqae, Maktomey, Sekkeri, Berni, and Barhey) growing in different regions of Saudi Arabia ranged from 4.79% to 7.50%.

There are twenty three different amino acids present in date palm fruit proteins and most of these amino acids are not found in many popular fruits such as bananas, apples and orange. For example, alanine, glycine, proline, glutamic acid, aspartic acid, serine and threonine are exclusively found in dates. Lysine is present in dates at over 2000 times that found in bananas, nearly 2000 times that in apples and nearly 5000 times that in oranges (Al-Showiman, 1998).

Most of the previous studies on the amino acid composition focused on the flesh of date palm (Hussein & El-Zeid, 1975; Auda et al., 1976; Considine, 1982; El-Sayed & Basseshim, 1982; Anonymous, 1985; Al-Hooti et al., 1998) whereas few studies have looked at the amino acids of date palm seeds. Hussein & El-Zeid (1975) studied the Khalas variety seeds and found they contain at least sixteen amino acids including significant amounts of essential amino acids, and Bouaziz et al, (2008) reported seventeen amino acids were found in the date seeds of the two varieties they studied. Al-Showiman & Baosman (1992) reported that of the nine essential amino acids (histidine, lysine, leucine, threonine, methionine, valine, isoleucine, tryptophan and phenylalanine) eight were found in the seeds of five different cultivars and only tryptophan was not present.

1.8.4 Carbohydrates Content

The most important chemical/nutritional constituent of dates are the carbohydrates (see Figure 1.6).

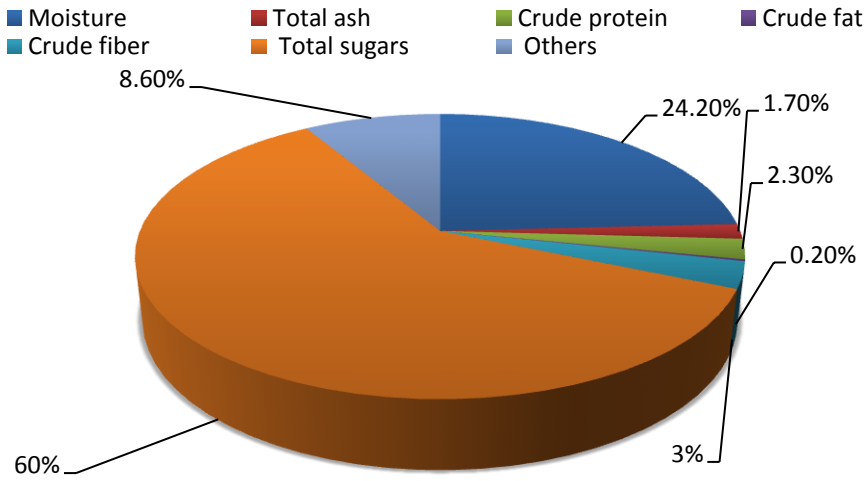

Fig 1.6: Proximate composition of date palm flesh (data is average of 15 varieties of dates; Al-Shahib & Marshall, 2003).

These include simple and complex carbohydrates and provide a good energy source (Manickavasagan et al., 2012). In addition the firmness of the pericarp in dates depends mainly on the content of carbohydrates and fibre (El-Hadrami & Al-Khayri, 2012). The carbohydrates in dates are mainly simple sugars and are a mixture of sucrose, glucose and fructose, with small amounts of polysaccharides such as cellulose and starch (Shinwari, 1993). Mannose and maltose are only present in the seeds (Al-Showiman, 1990). Sucrose is totally inverted into glucose and fructose at Tamar stage (Fayadh & Al-Showiman, 1990; Manickavasagan et al., 2012).

Dates can be classified based on their texture and pliability at the Tamar stage into soft, semidry and dry which is related to increasing sucrose content. In addition, this classification can be based on fibre and moisture contents as well (Barreveld, 1993). The total sugars in date palm fruit increases as the fruit ripening progresses from Kimri to Tamar stage. The total sugar has been found to increase from 3.4% to 7.7% in Kimri stage and from 43.40% to 87.54% in Tamar stage (Al-Shahib & Marshall, 2003; Manickavasagan et al., 2012).

Most of the reports in the literature focus on the concentrations of total sugars in Tamar stage (Ahmed et al., 1995; Al-Hooti et al., 1997; Al-Farsi et al., 2005; Rahman et al., 2005; Ismail et al., 2006; Sahari et al., 2007; Khan et al., 2008; Chaira et al., 2009). Total sugar contents have been reported for Deglet Nour (74.11%) (Guizani et al., 2010), Madjool (66.40%) (Rock et al., 2009), Khanizi (87.54%) (Sahari et al., 2007), and Allig (53.22%) (Ellecuchet al., 2008). For seeds, Al-Farsi et al (2007) reported the total carbohydrates content of seeds of three date varieties from Oman, Mabseli 86.89%, Shahal 86.54% and Um-sellah 83.14%. The polysaccharides of crude date palm seeds contain acidic and neutral fractions, with the acidic fraction comprising almost 5% of the total carbohydrates. The acidic fraction polysaccharides are composed principally of arabinose, rhamnose, galactose and glucose. The neutral fraction approximately 46% of the total carbohydrates, are composed mostly of mannose, glucose, arabinose and rhamnose. The polysaccharides of date seed are comprised mainly of mannose in the form of mannans, galactomannans and glucomannans. These have been isolated from date seed and characterized (Ishrud et al., 2001; Ishrud, et al., 2002) and found to comprise approximately 78–84% of the

total polysaccharides of the seed (Ishrud et al., 2001; Aldhaheri et al., 2004 & Ishrud et al., 2005).

1.8.5 Dietary Fibre Content

Dates are considered a good source of dietary fibre with the content depending on the stage of maturity and the variety. The dietary fibre content of some varieties of dates is given in Table 1.2. Dates contain both soluble and insoluble dietary fibre.

Table 1.2: Dietary Fibre Contents of some Date Varieties.

Variety	Dietary Fibre content (%w/w)
Hayani[a]	3.6
Khalas[b]	10.9
Fard[c]	10.1
Deglet Nour[d]	10.9
Allig[d]	13.5

Data is extracted from (a) El-Zoghbi (1994), (b) Myhara et al. (1999), (c) Al-Farsi et al. (2005); Elleuch et al. (2008).

The insoluble fibre (mainly cellulose, hemicelluloses, pectin and lignin) is the major fraction of dietary fibre in dates (Manickavasagan et al., 2012). The cellulose, hemicellulose and lignin contents in flesh are quoted by Barreveld (1993) as 1.55%, 1.28% and 2.01% (w/w) respectively, whilst Fayadh & Al-Showiman (1990) found that the pectin content ranged from 0.5% to 3.9% (w/w).

1.8.6 Triglyceride Content

Date flesh contains triglycerides that are mainly concentrated in the skin (Manickavasagan et al., 2012). The triglyceride content of date flesh of varieties grown in different regions has been studied by many researchers (Yousif et al., 1982; Ahmed et al., 1995; Al-Hooti et al., 1997; Al-Farsi et al., 2005; Al-Farsi et al., 2007; Sahari et al., 2007; Guizani et al., 2010) who found that the oil content in flesh is low and varies from 0.1% to 3.25% of the fresh weight. The major free fatty acids identified in date flesh oil in

order of abundance are caprylic acid, linoleic acid, lauric acid, pelargonic acid and myristic acid (Manickavasagan et al., 2012).

Date palm seed oil has been extracted and tested by several researchers. Devshony et al. (1992) measured the oil content in four date palm seeds cultivars (Deglet Nour, Zahidi, Medjool and Halawy) as 8.40%, 8.13%, 7.98% and 8.10% (w/w) respectively. Another study by Besbes et al. (2004) found that the oil contents of Deglet-Nour and Allig varieties were 10.19% and 12.67% (w/w) respectively. Besbes et al. (2005) also examined the effects of heating on the quality characteristics of Deglet Nour and Allig variety seed oil. They found that oil is resistant to thermal treatment over long time scales (up to forty hours) and has high oxidation stability. Basuny & Al-Marzooq, (2011) studied the chemical composition of Khalas variety seed oil (9.76% (w/w) of the total seed mass) and found that the total saturated and unsaturated fatty acid contents were 51% and 49% (w/w) respectively. Recently, Abdalla et al. (2012) have measured the content of five fatty acids in the seed oil of two cultivars grown in Sudan (Al-Barakawi & Alqundeila) (Table 1.3).

Table 1.3: Fatty Acid Composition of Seed Oil from two Date Varieties.

	Variety of Date	
Fatty Acid	Composition (w/w of total oil)*	
	Al-Barakawi	Alqundeila
Lauric acid	37.10	0.11
Palmitic acid	9.24	0.42
Stearic acid	1.71	46.93
Oleic acid (omega 9)	32.66	None detected
Linoleic acid (omega 6)	4.33	None detected

*Data from Abdalla et al. (2012).

GLC analysis of date palm seed oil has also shown the presence of other minor fatty acids such as myristoleic, capric, palmitoleic and linolenic acids (Vyawahare et al, 2009).

1.8.7 Mineral Content

Date palm fruit is rich in minerals and has a proportion of potassium 25% greater than is found in bananas (Sulieman et al., 2012). A range of studies have looked at the mineral content of date palm flesh and these have found that potassium is the most abundant mineral with significant quantities of

calcium, sodium, phosphorus, magnesium, iron, zinc, copper and manganese (Ahmed et al., 1995; Al-Farsi et al., 2005; Ismail et al., 2006; Khan et al., 2008; Chaira et al., 2009; and Rock et al., 2009). The mineral content of date palm seed based on these studies is given in Table 1.4.

Table 1.4: Range of Mineral Compositions of a Date Palm Fruit from Different Cultivars.

Mineral	Composition (mg/100 g)
Potassium	400–1160
Calcium	15–90
Sodium	3–290
Phosphorus	60–100
Magnesium	50–90
Iron	0.3–6
Zinc	0.2–2.4
Copper	0.1–2.3
Manganese	0.2–1.2

Data based on Ahmed et al., (1995); Al-Farsi et al., (2005); Ismail et al., (2006); Khan et al., (2008); Chaira et al., (2009); & Rock et al., (2009).

Ali-Mohamed & Khamis (2004) reported the mineral composition of the seeds of six different cultivars of Bahraini date palm seeds (Khalas, Khunaizi, Murzban, Khasaib Asfor, Khawajah, and Khaseeb) analyzed by atomic absorption spectroscopy. Besbes et al. (2004) also reported the mineral contents of two Tunisian cultivars (Deglet Nour and Allig). The results of both of these studies are summarised in Table 1.5.

Table 1.5: Mineral Contents of Date Seeds from Various Cultivars.

Mineral	Average of six Bahraini cultivars* (mg/100 g)	Two Tunisian cultivars* (mg/100 g)
Potassium	486	229, 293
Magnesium	66	51.7, 58.4
Sodium	24	10.4, 10.25
Calcium	10	38.8, 28.9
Manganese	1.5	–
Zinc	1.2	–
Copper	0.5	–
Phosphorus	–	68.3, 83.6
Iron	–	2.30, 2.21

*Data for the Bahraini cultivars from Ali-Mohamed and Khamis (2004) and for the Tunisian cultivars from Besbes et al. (2004).

Since dates are rich in potassium and extremely low in sodium, they are a recommended food for patients with high blood pressure who are advised to consume low sodium diets (Mrabet et al., 2008).

1.8.8 Vitamin Content

The flesh of date palm is an important source of water soluble vitamins. Significant quantities of water-soluble and oil-soluble vitamins such as C, B1 (thiamine), B2 (riboflavin), nicotinic acid (niacin) and vitamin A have been found in date palm fruit (Al-Shahib & Marshall, 2003). Vitamins A and C are found in relatively low concentrations in dried dates (Manickavasagan et al., 2012).

Most of data available in the literature has been for the vitamin content in date palm fruits. Yousif et al. (1982) have found that the vitamin contents (in mg/100g) of four Iraqi date varieties (Hallawi, Sayer, Khadrawi and Zahidi) are 0.073–0.12 for vitamin B1, 0.125–0.16 for B2, 0.039–0.065 for B9 and 2.2–16 for vitamin C. Similar amounts have been reported by Considine, (1982) and Khatob et al. (1982). In addition Sawaya et al. (1982) reported vitamin A at a concentration of 1mg/100g. In date seeds, HPLC and GCMS have identified some oil soluble vitamins such as vitamin E (Abdalla et al., 2012). Vitamin E (tocopherols) have antioxidant properties that can protect the body against oxidation reactions from free radicals, skin damage and aging by UV radiation, inhibit the growth of cancer cells, and lowering cholesterol levels (Sundram et al., 1989; Goh et al., 1994; Abdalla et al., 2012).

1.8.9 Phenolics, Carotenoids and Antioxidant Activity in Dates

Phenolic compounds are present in oil seeds and dates and are responsible for the dark colours and astringent flavours of some fruits (Yada, 2004).

The fruits of date palm are a good source of phenolic compounds and their associated antioxidant activity. Different cultivars of dates have different total phenolic and antioxidant activity and there is a relationship between antioxidant activity and phenolic compounds of various fruits of dates (Siddiq et al., 2012). Date palm contains significant amounts of phenolic acids, flavonoids, carotenoids and steroids (Figure 1.7).

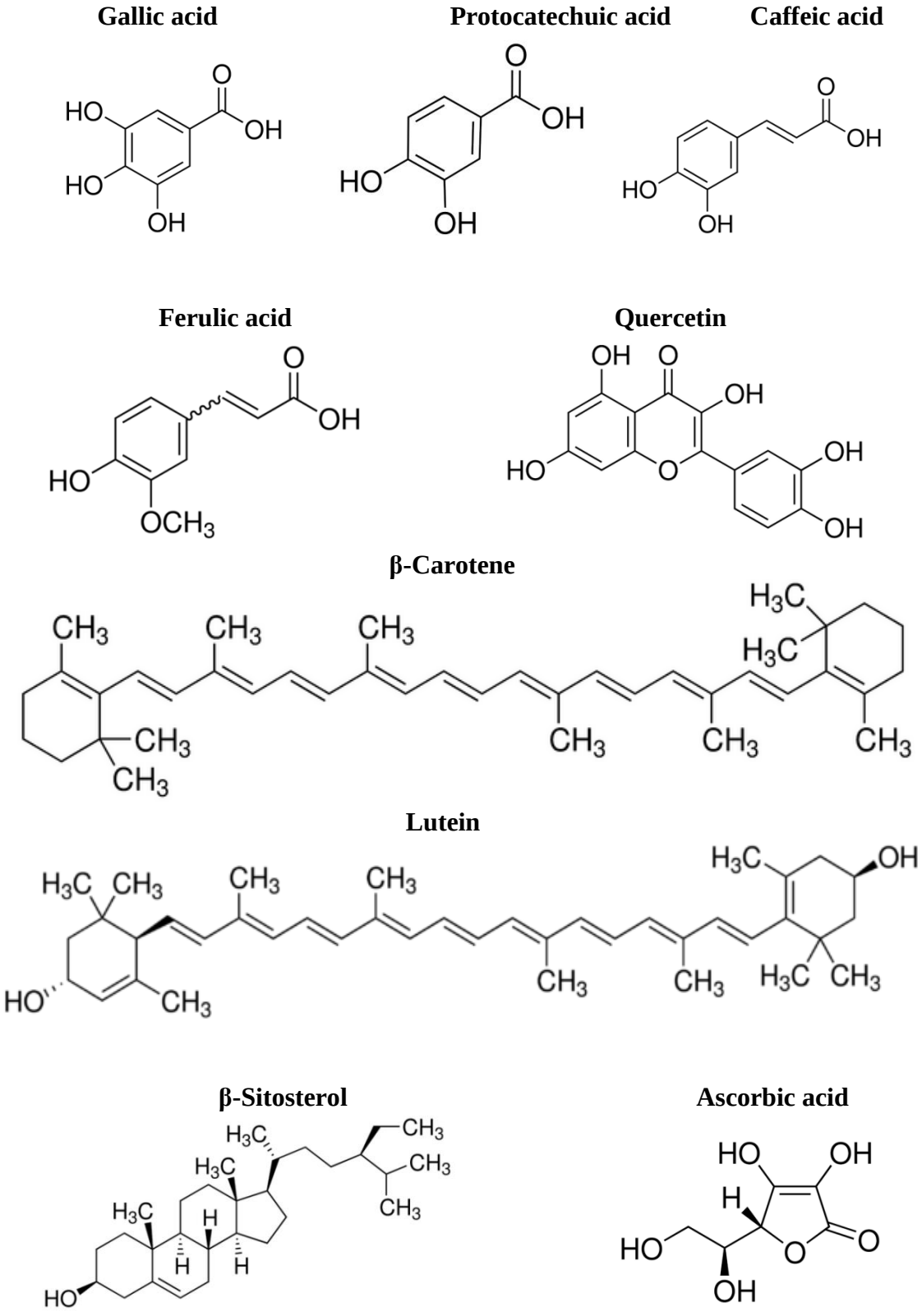

Fig 1.7: Some Phytochemical Constituents of Date Palm (*Phoenix dactylifera* L.) (based on Vyawahare et al., 2009).

There are three main families of phenolic compounds in dates namely flavonols, flavan-3-ols, flavan-3,4-diols; proanthocyanidins and hydroxycinnamates. The hydroxycinnamates are the major group of phenolic

compounds and can be found as derivatives of ferulic, chlorogenic, sinapic and *p*-coumaric acids as well as their esters such as caffeoylshikimic acid derivatives. Flavonoids such as quercetin, kaempferol, luteolin and proanthocyanidins are found in fresh dates (Daayf et al., 2003; El-Hassni et al., 2004; J'Aiti et al., 2009; El-Hadrami & Al-Khayri, 2012). These are antioxidants and act as scavengers of free radicals, metal chelators, anti-mutagens, and signalling agents (Bravo, 1998; Dillard & German, 2000; Vasco, 2009), and responsible for protective effects on human health.

Quercetin has been proven to be an excellent antioxidant that can be used as an anti-inflammatory and anti-proliferative. Quercetin has been used as a food supplementation especially for people suffering from sarcoidosis and hypertension disease (Boots et al., 2008). Kaempferol has a wide range of pharmacological activities, including anti-cancer, anti-microbial, anti-oxidant, anti-inflammatory, anti-osteoporotic, neuroprotective, cardioprotective, anti-diabetic, analgesic and anti-allergenic activities. It exerts cytotoxic effects in many types of cancer and helps the body to fights cancer cells (Lau, 2008; Yoshida et al., 2008; Calderon-Montano et al., 2011).

Luteolin has been shown to inhibit tumour cell proliferation and angiogenesis (Bagli et al., 2004). Proanthocyanidins (water soluble antioxidant) are considered an effective protective antioxidant and cancer inhibitor. Proanthocyanidins are more powerful antioxidants than vitamin E (oil soluble antioxidant). Analysis of six proanthocyanidins has shown they can be up to fifty times more effective than vitamin E (Hoffmann, 2003).

Wu et al. (2004) studied the phenolic profiles of two date varieties (Deglet Nour and Medjol) and found the phenolic contents were 661 and 572 mg of gallic acid per 100 g fresh weight, respectively. Comparative studies with fresh and three dried date cultivars grown in Oman (Fard, Khasab and Khalas) by Al-Farsi et al. (2005) revealed that the phenolic contents ranged from 134–280 mg ferulic acid equivalents per 100 g fresh weight.

Recently, Saafi et al. (2009) measured the phenolic contents of fresh dates of four cultivars (Allig, Deglet Nour, Kentichi and Khouet Kenta) and found phenolic contents ranged from 209.4–447.7mg gallic acid equivalents per 100g fresh weight.

Al-Farsi et al. (2007) reported that the seeds of three native sun dried date varieties from Oman (Mabseeli, Shahal and Um-sellah) have total phenolic compounds ranging from 3102–4430 mg gallic acid equivalents per 100 g fresh weight. Al-Farsi and Lee (2008) examined the phenolic acid composition in the seed of Mabseeli variety. The main phenolic acids were *p*-hydroxybenzoic, protocatechuic, *m*-coumaric, ferulic, *p*-coumaric, vanillic, *o*-coumaric, gallic, and caffeic with concentrations of 9.89, 8.84, 8.42, 6.93, 6.07, 4.07, 3.96, 0.28, and 0.18 mg/100g respectively. The phenolic content in dates can be affected by several factors such as growing conditions, geographic origin, cultivar, maturity of the tested dates, soil type, season, fertilizers, sampling and extraction and conditions of storage (El-Hadrami & Al-Khayri, 2012).

1.9 Enzymes in Dates

Enzymes play an important role in the date ripening process over all stages of growth and maturity of the date fruit with the final product quality highly dependent on the activities of enzymes. Invertase enzyme is responsible for the conversion of sucrose into glucose and fructose. This is related to texture and pliability changes in the fruit. Polygalacturonase and pectinesterase enzymes are both responsible for the conversion of insoluble pectic substances into more soluble pectins, contributing to increasing softness of the fruit particularly during the Rutab stage. The cellulase enzyme complex breaks down cellulose into shorter chain oligosaccharides, increasing the solubility and eventually leading to glucose formation. This can decreased the fibre content of the date fruit. Polyphenol oxidase is responsible for the biochemical changes in the polyphenol components (tannins). These are important in non-oxidative browning reactions of dates (FAO, 1993).

1.10 Nutritional and Medicinal Value of Dates

On the palate, dates have a fleshy mouth feel and a sweet taste. They provide essential nutrients and are claimed to have benefits in human health (Manickavasagan et al., 2012). Al-Farsi et al, (2007) pointed out that date by-products can be considered a good source of dietary fibre, total phenolics and an inexpensive source of natural antioxidants and could be used as a functional food ingredient. The flesh of date palm is also a good source of

dietary fibre with every 100 g of dates providing 34% of the daily recommended amount of dietary fibre (Manickavasagan et al., 2012) which is 25 g/day (Marlett et al., 2002). Dietary fibre is considered good for your health and is claimed to have a preventative effect against many diseases such as diabetes, obesity, hyperlipidaemia, coronary heart disease, hypertension, intestinal disorders, prostate cancers, and colorectal cancer (Kritchevsky, 1988; Johnson & Southgate, 1994; Tariq et al., 2000).

Date palm seed powder has been used in some traditional medicines (Boukouado & Yousfi, 2009). Date palm is considered a medicinal plant in many countries and continues to prove valuable in both traditional and modern medicine (Krentz & Bailey, 2005). Dates are widely used for the treatment of sore throats, bronchial asthma, colds, fever, oedema, cystitis and to counteract alcohol intoxication. It is also useful in treatment of abdominal and liver problems (Barh & Mazumdar, 2008). It can be used against several kinds of tumour and has been found to exhibit a dose dependant anticancer activity (Vyawahare et al, 2009).

1.11 Applications and Benefits of Dates

Different parts of the date palm tree have been used for animal feed, paper or as construction materials (Anwar, 2006; Ata, 2011). The leaves can be used to make manual fans, mats, huts, baskets, bread dishes, large hats, rope, containers and packing material for vegetables and fruits. The dried branches can be used as brooms, sticks or fuel, whilst the trunk is used to make pillars for huts, roofing of small houses as well as making bridges. The rest of the tree parts can be used as fuel (Ata, 2011). Fruits of date palm have been eaten fresh or used by the food industry to make products such as jam, jellies, fruit bar, date syrup, date pies, alcohol, date chocolate, salads, sauces, baking products, breakfast cereals and date confectionery.

It is usually taken as the whole fruit or with milk, yoghurt, or Arabian coffee (El-Shaarawy et al., 1986; Khatchadourian et al., 1987; Al-Hooti et al., 1997; Sablani et al, 2008; Bessbes et al., 2009; Manickavasagan et al., 2012). Almana & Mahmoud, (1994) found that the bread containing 10% coarsely milled date seed fraction gave a product similar to or better than that containing wheat bran, whereas, the fine milled seed fraction caused an increase in bread odour, flavour, colour, uniformity and overall acceptability.

Date by-products, in particular seeds, could be an excellent source of functional food component because they contain a balance of fats, proteins, minerals and carbohydrates.

Date palm seed powders as a coffee substitute have been recently introduced to the market (Rahman et al., 2007), either as the pure powder or in the form of mixture of the date palm seed powder with coffee powder (Al-Farsi et al., 2007). In the past date seeds have been used mainly for animal feeds for camels, cattle, sheep, poultry and fish.

Several reports were conducted on the use of date palm seeds for sheep (Elgasim et al., 1995), fish (Yousif et al., 1996) and rats (Ali et al., 1999). In another study it was found by Hussein et al. (1998) that the addition of date seed to broiler chick diets gave a similar improvement in their body weight to that of a diet containing soybean and/or corn meal. Whereas Khiyami, et al., (2008) report that a mix of date palm flesh and seed wastes with crab shell and shrimp wastes could be a good fertilizer for plants and is an environmentally friendly composting process for treating date waste. Furthermore, the carbon of date seeds has a high efficiency as a filter aid to remove oxidation products from fried oils (El-Anany et al., 2008), and could be used as an organic fertilizer and for generation of biogas by mixing with cattle faeces (Al-Turki et al, 2004).

As mentioned previously the global production of dates in 2005 was around 6.9 million tons (Chao & Krueger, 2007) and the average weight of a date seed is about 10–15% of whole fruit weight (Almana & Mahmoud, 1994; Hussein et al., 1998). From this it can be estimated that about 863000 tonnes of seeds were discarded and disposed of as a waste product in this year. Utilization of such waste could be very important to date agriculture and to increase the income to this sector (Al-Farsi & Lee, 2008). It is clear that there is a potential under usage of date palm seeds despite the fact that thousands of tons of seeds are discarded annually.Utilization of such wastes could be very important to the date industry.

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CHAPTER TWO

Materials and Methods

This chapter presents the analytical methods used in this study. Most of the analytical methods used were carried out to official methods. The chemical compositions were determined according to Association of Official Analytical Chemists (AOAC, 1995). All chemicals and solvents used in this study were obtained from Sigma-Aldrich Ltd., UK and Fisher Scientific Ltd., UK with high purity grade. All analytical determinations were carried out in triplicate.

2.1 Materials

Date palm fruit (*Phoenix dactylifera* L.) at Tamar stage (complete maturity) of the commercially available date palm Deglet Nour variety were purchased from a local supermarket in Edinburgh, United Kingdom. The seeds of 40 kg of whole dates were manually isolated, soaked in water, washed to remove any remaining date flesh and then air dried for a week. They were then further dried overnight at 40˚C in an oven. Date palm seeds were milled using a hammer mill to pass 1–2 mm screens and then preserved at – 40˚C until analysis. The powder obtained was identified as date palm seed powder (DPSP). Date flesh was chopped into small slices approximately 8–10 mm thick using a kitchen knife, put into small polyethylene bags and stored at – 40˚C until required for further analysis.

2.2 Preparation of Defatted Seed Powder

Oil was extracted from DPSP using a Soxhlet apparatus (Figure 2.1).

Fifteen g of dried DPSP was weighed into an extraction thimble and sealed with a piece of cotton wool. The thimble was inserted in a Soxhlet extraction flask. A few anti-bumping agent granules were added before adding 300 ml of hexane to a cleaned, dried and weighed round bottom flask. The extraction unit was placed over an electric heating mantle. The temperature of the heater was adjusted to boil the hexane. When the solvent started to boil some hexane dripped from the condenser into the sample chamber where it extracts the oil from the DPSP. The extraction was continued for 10 hours or until the solvent in the sample chamber was colourless indicating that it was free from oil and that all the oil had been extracted. At this point the heat source was switched off and the sample was removed from the thimble and left overnight

to dry at room temperature. The defatted date palm seed powder (DDPSP) was kept in plastic container at –20˚C until further use.

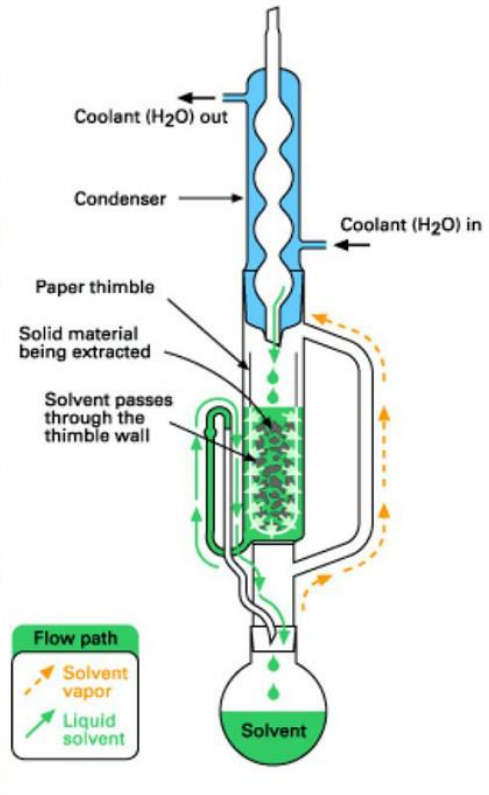

Fig 2.1: Soxhlet apparatus for extraction of crude fat (www.cremonaviolintools.com).

2.3 Physical Properties of Date Fruit and Seeds

The following is a brief description of each method used.

2.3.1 Physical Characteristics

Physical measurements of seeds are useful when developing handling and sieving devices for date fruits or seeds (Arafa, 2007). The weight of one hundred fruits and seeds was measured, and then the average for a single fruit and seed was calculated and recorded. The ratio of date palm seed (DPS) or date palm flesh (DPF) to the whole fruit was calculated as following:

$$\text{Ratio of DPS or DPF to whole fruit} = \frac{\text{Weight of DPS or DPF}}{\text{Weight of whole fruit}} \times 100$$

The length and width of fruits and seeds were measured using a micrometer (Moore & Wright Ltd., Sheffield, UK).

2.3.2 Bulk Density

Bulk density was determined after measuring the weight and volume of a sample of powder according to the method of Ocloo et al. (2010). A calibrated plastic micro centrifuge tube was weighed (W1) and filled to 2 ml with powder. Constant tapping was used to pack the powder into the tube until there was no change in volume. The tube was weighed again (W2). The bulk density is calculated from the difference in weights and expressed as grams per millilitre (g/ml) as follows:

$$\text{Bulk density (g/ml)} = \frac{\text{W2} - \text{W1}}{\text{ml}}$$

2.3.3 Swelling Power of Powder

Swelling power was determined according to the method described by Oladele and Aina, (2007). One gramme of each sample was weighed into a centrifuge tube that had been previously weighed (W1). Ten ml of distilled water was added to each centrifuge tube and vortex mixed before heating and shaking the samples at 80˚C for 30 min in shaking water bath. The mixture was centrifuged at 1000 xg for 15 min, the supernatant discarded and the centrifuge tube including the pellet was weighed (W2). The swelling power is then expressed by dividing the weight of pellet by weight of dry sample as follows:

$$\text{Swelling power (g/g)} = \frac{\text{W2} - \text{W1}}{\text{Dry sample (g)}}$$

2.3.4 Differential Scanning Calorimetry (DSC)

The thermal properties of crude date seed and flesh samples and extracted date palm proteins were measured by differential scanning calorimetry using a TA Instruments DSC 2010, differential scanning calorimeter (TA Instruments, New Castle, USA) according to the procedure of Sorgentini et al. (1995) and Renkema, (2001), with some modifications. Approximately 5 mg of each sample was sealed in an aluminium DSC pan. A sealed empty pan was used as a reference. The analysis was carried out using a temperature gradient of 20–140˚C at a scan rate of 10˚C per minute.

The denaturation temperature and enthalpy value of denaturation were determined from each thermogram using the TA analysis software supplied with the machine.

2.4 Chemical Properties

2.4.1 Moisture and Total Solids Content

Moisture and total solids in date palm seed was measured using AOAC method No. 925.10 (AOAC, 1995). Briefly, an aluminium dish previously cleaned and dried at 105ºC was weighed (A) and cooled in a desiccator to room temperature. Two gramme of seed powder was weighed (B) into the dishes and then heated at 105˚C in air oven until constant weight was achieved (approximately 5 hours). The dish was transferred to a desiccator, cooled and weighed (C). The moisture was calculated from the loss of weight as follows:

$$\% \text{ Moisture} = \frac{(C)-(A)}{(B)} \times 100$$

The total solids were calculated by subtracting the moisture from 100 i.e: Total solids = 100 – (% moisture).

2.4.2 Ash Content

The ash content was measured according to AOAC method No. 923.03 (AOAC, 1995). Five gramme of sample powder was weighed into a crucible that had previously been cleaned and burned in a muffle furnace at 550˚C before cooling in a desiccator to room temperature. A crucible containing a powder sample was ignited in the furnace at 550˚C until the powder was reduced to a light grey or white ash or until a constant weight was achieved. The crucible was transferred into a desiccator and the weight of ash was recorded soon after reaching room temperature. The total ash as a percentage was calculated as follows:

$$\% \text{ Total ash} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

2.4.3 Fat Content

A Soxhlet apparatus was used to extract the fat using the AOAC method No. 963.15 (AOAC, 1995) as previously used in Section 2.2. Fat was extracted from a known mass of date seed or flesh using the method given in Section 2.2 with the following modifications. First the round bottom flask was dried

in an oven, allowed to cool and then weighed. The fat was then extracted from the sample. After extraction was complete, the solvent was evaporated from the round bottomed flask using a rotavapor (Buchi, Laboratoriums-Technik AG, Switzerland) operating at 60˚C. After evaporation of the hexane the flask containing the residual crude fat was dried to constant weight in a fume hood to ensure no solvent remains along with the sample. The flask was then weighed and the mass of crude fat determined by difference. The results were expressed as percentage of crude fat as follows:

$$\% \text{ Crude fat } = \frac{\text{Weight of the fat}}{\text{Weight of sample}} \times 100$$

2.4.4 Protein Content

Crude protein content was determined according to the Kjeldahl method (AOAC, 1995, method No. 988.05; Lynch et al., 1998). Samples of 0.25–1.00 g were weighed into a digestion flask and two tablets of Antifoam S (each one containing 0.97 g sodium sulphate & 0.03 g silicone antifoam) and one tablet of KJELTAB S catalyst (containing 5g potassium sulphate and 5 mg selenium (Se)) were added.

Sulphuric acid (12 ml) was carefully added to each digestion flask. The sulphuric acid functions to digest the protein, thus releasing nitrogen from the sample which is converted into ammonium ions. The flasks were then transferred to a digestion unit (Tecator™ digester system 20, Foss-UK) and heated gradually up to 420˚C or until the samples become clear (approximately one hour). The flasks were heated for a further 10 min before the digestion flasks were cooled to room temperature and transferred to a distillation unit (Kjeltec™ 8100 distillation system, Foss-UK). Water (70 ml) and 40% (w/v) sodium hydroxide (50 ml) were added to each flask and the samples distilled for 3 min for each sample to release ammonia from the sample. The ammonia was received into a cleaned and dried beaker containing 25 ml of boric acid. The ammonia reacts with the boric acid to form ammonium borate.

Dilute hydrochloric acid (0.1N) was used to back-titrate the ammonium and the titre used to calculate the total nitrogen content. Protein content was calculated as percent of dry weight of sample by multiplying the total

nitrogen content by a conversion factor of 6.25. This value of 6.25 represents the average nitrogen content of a of a typical protein sample. The percent yield of protein from date palm seed or flesh is calculated using the following formula according to Onsaard et al. (2010) and Selling et al. (2013):

$$\text{Percent yield} = \frac{\text{mass extracted(g)} \times \text{protein content (\%)in sample}}{\text{starting mass(g)} \times \text{protein (\%)in date seed powder}} \times 100$$

2.4.5 Amino Acid Determination

The amino acid composition of date protein samples was determined using high performance liquid chromatography (HPLC). Dried sample (10 mg) was weighed into a screw capped glass hydrolysis tube and placed in ice before adding 0.2 ml of cold performic acid. This was mixed by placing the tube in an ultrasonic bath for 10 min, after which the tubes were capped and left to stand overnight in a refrigerator at 4˚C.

Sodium metabisulphite (50 mg) was added carefully to each tube and mixed using a vortex mixer. Hydrochloric acid (0.8 ml of 7.5 N) was added to the tube and this was mixed again by placing in an ultrasonic bath for 15 min. The tubes were placed unsealed onto a hot plate previously heated to 110˚C. After an hour, the tubes were sealed and hydrolysed for a further 24 hours on the hot plate.

After the hydrolysis was complete, the tubes were removed from the hot plate and cooled to room temperature. The contents were transferred to a 5 ml volumetric flask, diluted to volume with distilled water and filtered through filter paper before placing into a rotary evaporator (Buchi, Laboratoriums-Technik AG, Switzerland) to dry partial under vacuum at 40˚C. The residue left after evaporation was dissolved in 0.8 ml of 0.2M sodium carbonate buffer, pH 9.7 and stored frozen prior to dansylation and analysis.

Sodium carbonate (0.2 ml, 0.2 M, pH 9.7), 20 µl of internal standard and 20 µl of sample were added to a 1.5 ml screw capped reaction vial. Finally, 0.2 ml of dansyl chloride solution (5 mg/ml in acetone) was added before capping and vortexing the tubes. These were incubated overnight in the dark at room temperature. The contents of the reaction vial were transferred to a one ml volumetric tube and diluted to volume with water. This one ml of the dansylated product was used to run in HPLC and the results were expressed as mg amino acid/g dry matter.

2.4.6 Crude Fibre Content

Crude fibre was determined in samples as the loss on ignition of dried residue remaining after digestion in sulphuric acid and sodium hydroxide according to the AOAC method (AOAC, 1995, method No. 962.09). The sample (2 g) was weighed into a 500 ml conical flask. Sulphuric acid solution (200 ml, 1.25% (v/v)) was added to the flask, was then connected to a condenser and digested by boiling for 30 min with shaking of the flask every 5 min to ensure no adhesion of the sample on the flask walls. The boiled solution was vacuum filtered through a filter covered by filter cloth. The residue on the filter cloth was washed three times with hot water and transferred to a new conical flask which had been previously cleaned and dried.

The digestion was repeated but with 1.25% (w/v) sodium hydroxide added to the flask instead of sulphuric acid. After filtration of the samples the residue was washed three times with 1% (v/v) hydrochloric acid to remove any alkali and three more times with hot water to remove any trace of acid. The residue was transferred to a clean, dried and weighed crucible. The crucible was dried in an air oven at 105˚C until constant weight was achieved (approximately 5 hours) before being transferred to a muffle furnace and burned at 550˚C until any organic matter (black colour) has disappeared and the weight is constant. The percentage of crude fibre was expressed as the % loss of mass compared to the original mass of 2 g.

2.4.7 Total Carbohydrates

Total carbohydrates in DPSP were calculated by difference rather than direct analysis according to the FAO method (FAO, 2003). All components in the powder other than carbohydrate (moisture, ash, protein, fat and crude fibre) were individually determined, summed and subtracted from 100 (total percentage of powder components) using the following formula:

Total carbohydrates = 100 – (%moisture + % ash + % protein + % fat + % crude fibre).

2.5 Extraction of Date Palm Seed Protein

A range of methods were used to recover soluble protein from date palm seed and flesh, including methodologies that are standard in the protein industry for manufacture of protein powders from other seed products (e.g. soya bean protein).

2.5.1 Some Factors Affecting the Extraction of Protein from Date Palm Flesh & Seed

To study the effect of extraction time on water soluble protein yield, one gram of the defatted date palm seed powder (DDPSP) or dried powdered date flesh was added to 50 ml of distilled water. The mixture was stirred for 30, 60, 90 or 120 min using a magnetic stirrer (Stuart® stirrer SB 162, Bibby Scientific Ltd, UK). The suspension was centrifuged (Beckman Coulter, Avanti J-26 XP centrifuge with a JA 25.50 rotor, Beckman Coulter, USA), at 1000 xg for 10 min to separate the insoluble materials. Ten ml samples of the clear supernatant solution were taken for protein determination by the Kjeldahl method as described above (section 2.4.4).

To study the influence the pH on the extractability of the protein, the procedure above was repeated but this time the pH was adjusted using 0.1 M NaoH or 0.1 M HCl to make a set of protein powders solutions with pH in the range 1–12 with one pH unit intervals.

2.6 Preparation of Date Palm (Seed & Flesh) Protein Concentrates

Protein concentrates were prepared from defatted powder by removing soluble materials such as sugars, oligosaccharides and other minor components using dilute acid solution in the pH ranges from 4.0–4.8 according to the method proposed by Wang et al. (2004) with some modifications. In the general method (Figure 2.2), dried defatted powder is weighed and suspended in distilled water with ratio of 1:10 of sample: water. The pH of the mixture was adjusted to a specific acid pH in the range 4.0–4.8 using 0.1 M HCl and extracted by stirring the mixture for a specific time using a magnetic stirrer (Model CB162, Bibby Scientific Ltd, UK). Then, the mixture was centrifuged for a specific time using a Beckman-Coulter, Avanti J-26 XP centrifuge (Beckman-Coulter, USA).

The pellet containing the protein was neutralised to pH 7 with 0.1 M NaoH, whilst the supernatant was discarded. Finally, the neutral mixture was freeze dried to form the protein powder and kept in sealed container at –20˚C for later analysis.

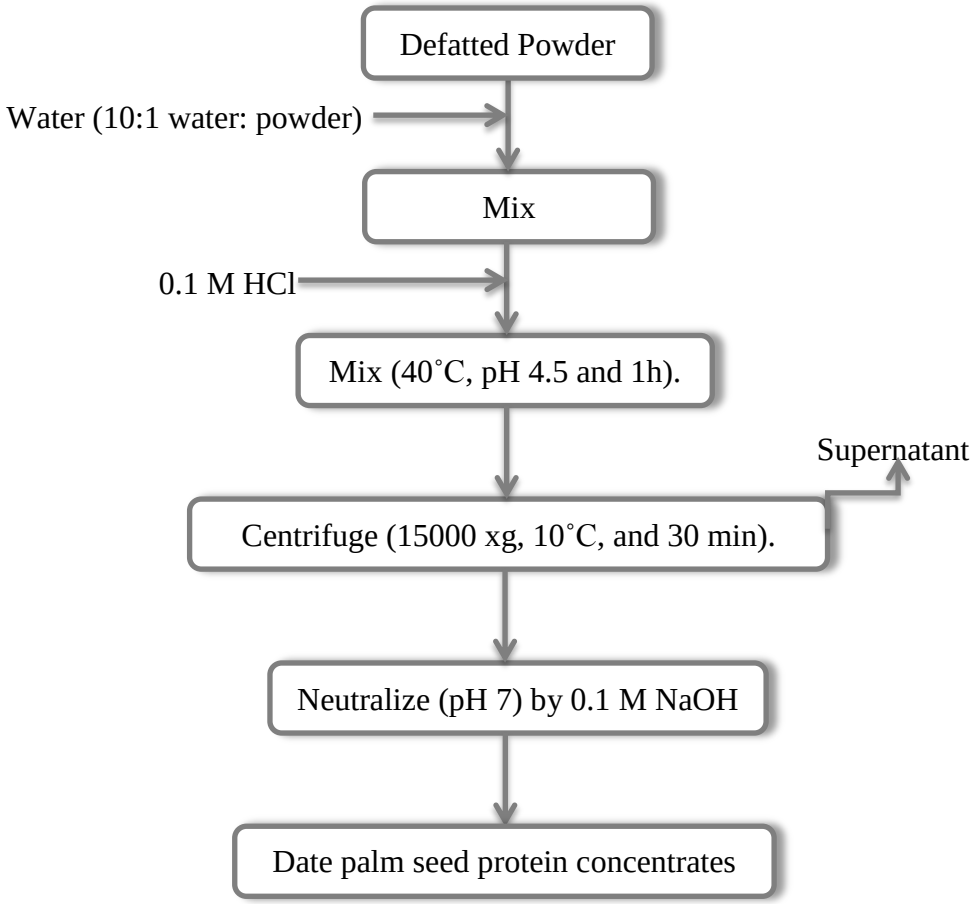

Fig 2.2: Production of Date Palm seed Protein Concentrates.

2.7 Preparation of Date Palm (Seed & Flesh) Protein Isolates

Protein isolate preparation was performed according to the method of L'hocine et al. (2006), with some modifications (Figure 2.3). Alkali (0.1 M NaOH) was used to adjust the pH of a defatted powder solution to between pH 9–10 to extract the soluble protein. The supernatant was decanted from the tube and the pelleted solid material discarded. The protein in the supernatant was precipitated at pH 4.5 using 0.1M HCl The mixture was centrifuged, the supernatant discarded and the pellet was redispersed and washed with water, centrifuged and then the washing and centrifugation repeated. The pellet was separated from the supernatant. The washed precipitate was resuspended in the minimum amount of water needed and neutralized to pH7 and freeze dried to produce a protein isolate.

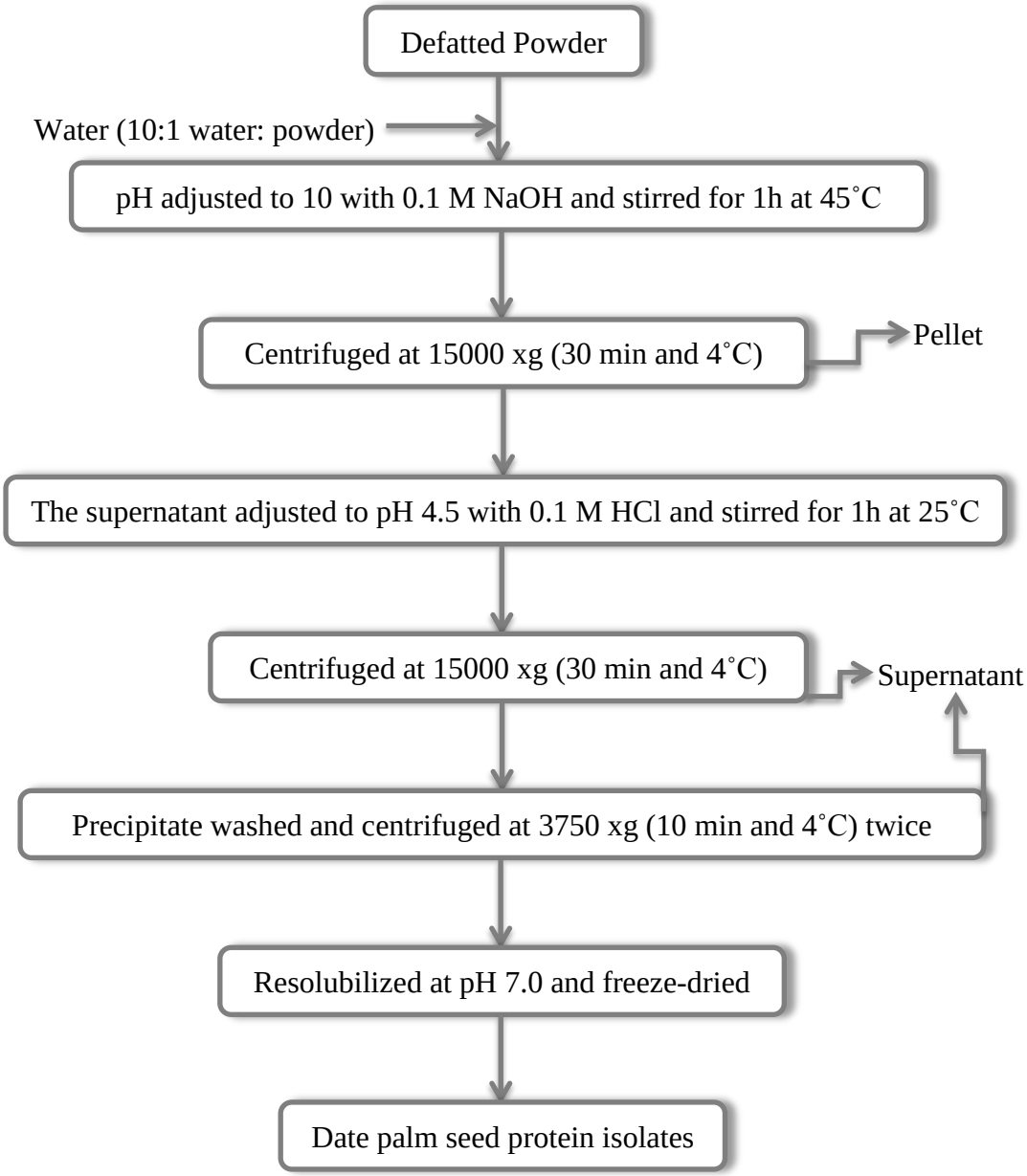

Fig 2.3: Production of Date Palm Seed Protein Isolates.

2.8 Improvement of Protein Extraction from DPSP by Enzymatic Hydrolysis

To improve purity of protein extracted from date seed powder various enzyme treatments were used to hydrolyse carbohydrate and protein components of the powder to facilitate protein isolation. The enzymes and methods used are outlined below.

2.8.1 Enzymes Used

The following enzymes were utilized in an attempt to improve protein isolation from DPSP:

- Mannaway 4.0 L batch KHN02011 was provided by Novozyme (Bagsvaerd, Denmark). This preparation contains the enzyme mannanase which hydrolyses mannans. The manufacturers give no information on the activity of the enzyme, but Cervero et al. (2010) have measured the mannanse activity of Mannaway as being more than 80000 nkat/ml which corresponds to > 4000 IU/ml.

- Viscozyme batch KTN02186 was provided by Novozyme (Bagsvaerd, Denmark). This product contains an endo-Beta-glucanase that hydrolyzes (1,3) or (1,4) linkages in Beta-D-glucans. This includes xylanase, cellulose and hemicellulase activity. The manufacturers report an enzyme activity of no less than 100 IU/mL.

- Cellulase (from *Trichoderma reesei* ATCC 26921), purchased from Sigma–Aldrich, UK. Cellulase catalyzes the endohydrolysis of 1,4-ß-D–glucosidic bonds in cellulose, lichenin and cereal ß-D-glucans. Enzyme activity reported by supplier, > 700 IU/mL.

- Pectinase (from *Aspergillus aculeatus*) purchased from Sigma–Aldrich, UK. Pectinase catalyzes the random hydrolysis of 1,4–α–D–galactosiduronic bonds in pectin and other galacturonans. Enzyme activity reported by supplier, > 3800 IU/ml.

- Protease (from *Bacillus licheniformis*) purchased from Sigma-Aldrich, UK. This is a protease that has an optimum pH in the range 8–10. Enzyme activity reported by supplier, > 2.4 IU/mL.

2.8.2 Enzymatic Extraction of DPSP Protein

DDPSP (20 g, three replicates) was mixed with 200 ml of distilled water at a ratio of 1:10 powder: water (w/v). The pH was adjusted depending on the optimum pH for each enzyme used (Table 2.1). Acetate buffer solution was used to obtain pH values of 5, 5.4 and 6 (Cevero et al., 2010), whilst pH 8 was achieved using phosphate buffer solution. The enzyme was added to give an enzyme loading (ml enzyme per 100 g dry weight sample) corresponding

to the optimum in Table 2.1. The mixture was incubated in a water bath with gently shaking for 24h using a shaking water bath (GLS Aqua18 plus, Grant Instruments Ltd, UK) at the recommended optimum temperature of each enzyme as shown in Table 2.1. The sample was then placed in hot water (95–100˚C) for 10 min to inactivate the enzyme and cooled to room temperature to finish the reaction. Immediately, the mixture was adjusted to pH 10 with 1M NaOH solution and stirred for 1 h at room temperature. Protein was then extracted from each enzyme treated sample using the procedure given in Section 2.7.

Table 2.1: Enzymatic treatment conditions, Protein content of each enzyme used

Enzyme	pH	Temperature (˚C)	Protein content (%)	Loading concentration (%)
Pectinase	6.0	50	1.97	5
Cellulase	6.0	45	3.45	5
Viscozyme	5.0	50	7.18	5
Protease	8.0	55	4.62	10
Mannaway	5.4	50	4.21	10

2.9 Date Palm Seed Protein Extraction by Osborne (OSB) Method

Protein was extracted using the procedure previously described by Osborne and Mendel (1914) with some modifications. DDPSP (30 g) was extracted with 300 ml of 2% (w/v) NaCl solution for one hour before centrifugation at 3000 xg for 15 min using a Beckman Coulter, Avanti J-26 XP centrifuge with a JLA 16.250 rotor, (Beckman Coulter, USA). The supernatant obtained contains albumin and globulin fractions. The extraction procedure was repeated by adding a further 30 mL of 2% (w/v) NaCl to the centrifuged pellet, and centrifuging under the same conditions. The supernatant from both centrifugation steps was combined and the salt in the supernatant was removed by dialysis for 72 hour against water using a 12 kDa cut off cellulose membrane. The centrifugal pellet was then extracted again with 70% ethanol and 0.1 M acetic acid to obtain prolamin and glutelin fractions respectively. The residue of the ethanol in the extracts was evaporated at

40˚C using a rotavapor (Buchi, Laboratoriums-Technik AG, Switzerland). The remaining powder residue was then extracted with 0.1M sodium hydroxide as described by Hamad et al. (2002) to remove any remaining protein. All extracted protein fractions were kept and analysed for the protein content of each fraction by micro Kjeldahl method as described in (Section 2.4.4).

2.10 Laboratory Preparation of Date Palm Seed Protein Extract

In an attempt to optimise the extraction of protein from DDPSP several different extraction methods were used, although some were relatively minor modifications of other methods. These methods are detailed below.

2.10.1 Method 1

Method 1 was based on the procedure proposed by Wang et al. (2004; with some modifications) for preparation of soybean protein concentrate. DDPSP was mixed with water at a ratio of 1:10 (w/v powder:water). The mixture was adjusted to pH 4.5 with 0.1 M HCl and stirred for 60 min at 45˚C. The mixture was centrifuged at 15000 xg for 30 min at 10˚C using a Beckman Coulter, Avanti J-26 XP centrifuge with a JLA 16.250 rotor, (Beckman Coulter, USA). The supernatant was discarded and the protein containing sediment was neutralised with 0.1M NaOH and freeze dried at – 60˚C for 72 h to form a powdered protein concentrate.

2.10.2 Method 2

This method was based on the procedure (with some modifications) proposed by L'hocine et al, (2006) for preparation of soy protein concentrate. DDPSP (50 g) was mixed with 500 ml distilled water. The mixture was adjusted to pH 10.0 with 0.1 M NaOH and stirred for 60 min at 55˚C. The mixture was centrifuged at 15000 xg for 30 min at 4˚C. The supernatant was adjusted to pH 4.5 with 0.1M HCl and stirred for 60 min at 25˚C and then centrifuged at 3750 xg for 15 min at 4˚C. The supernatant was discarded and the precipitate washed with distilled water and centrifuged at 3750 xg for 10 min at 4˚C. The washing of the precipitate and centrifugation was repeated. The pellet was resolubilised at pH 7 with 0.1 M NaOH and freeze dried.

2.10.3 Method 3

Protein was isolated from date palm seed according to the method of Bouaziz et al. (2008) with some modifications. DDPSP was mixed with water at a ratio of 1:10 (w/v powder:water). The mixture was adjusted to pH 10.0 using 0.1 M NaOH and stirred for 60 min at room temperature. The mixture was then centrifuged at 3750 xg for 15 min at 25˚C. The supernatant was collected and kept for further treatment whilst the pellet was mixed with 100 ml distilled water, the pH readjusted to 10.0 and the mixture centrifuged again under the same conditions as above. The supernatant was combined with the previous one and concentrated by tangential crossflow filtration using a 10000 Da cut off membrane (model Vivaflow 200, Sartorius Stedim Biotech, UK, Figure 2.4). The retentate from the filtration was freeze dried to a powder concentrate.

Fig 2.4: Vivaflow 200 Tangential Crossflow Filtration Cartridge Units, http://www.sartorius.co.uk.

2.10.4 Method 4

This was the same as method 2 except that after the pellet was solubilised the slurry was then concentrated by tangential crossflow filtration with a 10 kDa cut off membrane and the retentate freeze dried to a protein concentrate.

2.10.5 Method 5

Method 5 was a Ph/TCA extraction procedure based on methods (with some modifications) proposed by Wang et al. (2003) and Gomez-Vidal et al. (2008) for olive and *Phoenix dactylifera* L. leaves respectively. DDPSP (10 g) was mixed with 30 ml of ice-cold acetone, vortex mixed and then centrifuged at 12000 xg for 10 min at 4˚C using a Beckman Coulter, Avanti J-26 XP centrifuge with a JA 25.50 rotor, Beckman Coulter, USA.

The supernatant was decanted and discarded and the residual pellet washed with ice-cold acetone twice and allowed to dry at room temperature. After the pellet had dried it was ground to a fine powder and rinsed with 15% (w/v) TCA in acetone, vortex mixed and then centrifuged at 12000 xg for 10 min at 4˚C). The rinsing with TCA/acetone and centrifugation was repeated three times. The pellet was then rinsed with cold 15% (w/v) TCA in water and centrifuged. The rinsing with cold TCA and centruifugation was repeated three times. The pellet was then rinsed with cold 80% (v/v) acetone followed by centrifugation, and this was also repeated three times.

The pellet was then air dried. To extract the protein two g of the dry pellet was suspended in 10 ml of Ph/Tris-buffer, pH 8.0 and 10 ml of dense SDS buffer (2%(w/v) SDS, 5%(w/v) sucrose, 0.1 M Tris-HCl, pH 8.0, 5% (v/v) β-mercaptoethanol). The mixture was vortex mixed and the pellet was obtained by centrifugation at 12000 xg for 10 min at 4˚C. The pellet was resuspended in Ph/Tris-buffer and dense SDS solution, and centrifuged again under the same conditions.

The pellets from both centrifugations were mixed and precipitated with five volumes of cold 0.1 M ammonium acetate in methanol, refrigerated at 4˚C overnight and then centrifuged at 12000 xg for 10 min at 4˚C. The pellet from this centrifugation was then washed three times with cold methanol plus 0.1 M ammonium acetate and centrifuged as above followed by the same process with cold 80% (v/v) acetone. The dried pellet (0.5 g) was then mixed with 5 ml of cold aqueous 24% (w/v) TCA, vortex mixed and left to precipitate on ice for 30 min, followed by centrifugation at 20500 xg for 15 min at 4˚C. The pellet was washed with 2 ml ice cold acetone, incubated for 15 min on ice and then centrifuged at 20500 xg for 15 min at 4˚C. The final pellet (DPS protein extract) was air dried and stored at –20˚C for further analysis.

2.11 Laboratory Preparation of Date Palm Flesh Protein Extract

For preparation of protein from date palm flesh three methods were used which are detailed in the following sections.

2.11.1 Method 6

This is based on the method described by Ahmed et al. (1995) with some modifications. Date flesh (50 g) was suspended in 100ml of phosphate

buffered saline (PBS, pH 7.5, containing 0.01% (w/v) sodium azide) and crushed in a kitchen blender (BL370 series, Kenwood Ltd, UK). The blend was stirred for 24 h at 4˚C and then centrifuged at 48500 xg for 10 min at 4˚C. The supernatant was ultrafiltered using a 10kDa cut-off membrane and the retentate freeze dried. The final powder was stored at –20˚C for later analysis.

2.11.2 Method 7

This method was based on that proposed by Elleuch et al. (2008) with some modifications. Date palm flesh (50 g) was mixed with 600 ml of hot water and stirred for 10 min to solubilise the sugars. The dietary fibre and protein were separated by centrifugation at 7500 xg for 10min. The solid pellet was then washed with 300 ml of water at 40˚C and centrifuged as above. This operation was repeated five times to ensure that all sugars were removed. After the final washing with water the solution was concentrated using tangential crossflow filtration with a 10 kDa cut-off membrane. The retentate was freeze dried to obtain a protein concentrate and the final powder was kept refrigerated until analysis.

2.11.3 Method 8

In this method, pectinase and cellulase enzymes (in liquid state) were used to remove carbohydrate from the sample prior to protein extraction (El-Sharnouby, 2009). Date flesh (20 g) was mixed with 60 ml of distilled water before the pH was adjusted to 6.0 ± 0.2. Then the enzyme preparations (1:1) pectinase to cellulase was added to a concentration of 1.0 % (v/w). The mixture was placed in water bath (GLS Aqua18 plus, Grant instruments Ltd, UK) at the recommended optimum temperature of 45˚C (Table 2.1) for 24 h. After the incubation, the samples were mixed in a kitchen blender (BL370 series, Kenwood Ltd., UK) before the slurry was filtered through a cheese cloth with a hand press. The solid filtrate residue was then placed in hot water for 10 min and then cooled to room temperature to finish the reaction. The mixture was adjusted immediately to pH 9 with 0.1 M NaOH solution and stirred for 1 h at room temperature. Then, the protein was extracted from the enzyme hydrolysed powder using the method given in Section 2.7.

2.11.4 Method 9

This was the same as method 4 (described above) for preparation of DPS protein extract using a 10 kDa tangential crossflow filtration membrane.

2.12 Separation and Identification of Date Palm Proteins

A combination of electrophoresis and mass spectrometry methods were used to separate and identify the proteins of date palm seed and flesh. These methods are detailed in the following sections.

2.12.1 Electrophoresis (SDS-PAGE) of DPS and DPF proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on DPS and DPF protein on a 12% polyacrylamide gel to determine molecular weights for proteins according to the method of Laemmili (1970).

2.12.1.1 Preparation of Samples for 1D SDS-PAGE

Protein was extracted according to the method proposed by Sekhar and Demason (1988) in 0.06 M Tris-HCl, 2.3% SDS buffer at pH 6.8 for 5h at 4˚C. The suspension was centrifuged for 10 min at 3750 xg, and then the supernatant precipitated by adding four volumes of cold acetone. A protein pellet was separated by centrifuging at 18500 xg for 10 min after which the pellet was air dried.

2.12.1.2 Separation of Proteins by 1D SDS-PAGE

All parts of the gel casting unit (glass plates, combs, spacers, and assembled gel cassette) were washed thoroughly with light detergent to remove any residues of previous gels, before rinsing well with distilled water and drying on paper towel. The gel casting tray was assembled by placing the glass plates together with a spacer between them. A rubber strip was placed at the base of the casting unit and the glass set on top of it. The screws on the casting unit were tightened to hold the glass plates in place. The gap between the glass plates was filled with water to ensure there is no leakage from the base or sides of the casting unit. After this, a comb was inserted between the glass plates and a mark was made on the plate to ensure filling of the gel to a sufficient height.

The gel was cast by pouring the 12% separating gel buffer (1.5M Tris-HCl buffer pH 8.7, acrylamide/bis-acrylamide 40% solution, 10% SDS buffer, 10% APS buffer, 20 µl TEMED solution) between the glass plates up to the mark. n-butanol was spread over the surface of the gel to ensure a flat surface and left for an hour to allow the gel to solidify.

Once the gel had polymerized n-butanol was removed carefully with filter paper before pouring the 4% stacking gel (0.5M Tris-HCl pH 6.8, acrylamide/bis-acrylamide 40% solution, 10% SDS, 10% APS and 20 µl TEMED solution) over the separating gel and immediately inserting a comb into the stacking gel to form the wells in the gel. The gels were allowed to polymerize for a further one hour. The glass plates were removed from the casting unit before clamping them onto the gel running unit. The assembly was inserted into an electrophoresis tank and both the chambers were filled with 1x SDS-PAGE running buffer (25mM Tris-HCl (pH 8.3), 192 mM Glycine and 0.1% SDS) before the combs were carefully removed. The air dried pellet of protein (7 mg) obtained from the extraction step was dissolved distilled water (1 ml). This protein solution (100 µl) was added to an equal volume of SDS sample buffer (0.5M Tris-HCl pH 6.8, SDS, glycerol, 2-mercaptoethanol and 0.1% bromophenol blue solution). This was mixed well before 10–20 µl were loaded into the wells in the gel using a pipette ensuring no dispersal in the running buffer in the tank. The tank was covered with a lid and connected to the power pack.

The gel was run at constant voltage of 160 V for approximately one hour or until the dye reached the bottom of the gel. After completion the gel was gently removed from the glass plates and placed in a tray before staining with colloidal Coomassie brilliant blue solution (5% (w/v)aluminum sulfate hydrate, 10% (v/v) ethanol, 0.02% (w/v) Coomassie brilliant blue-G250, 2% (v/v) orthophosphric acid) and left overnight on a shaker. The stain solution was removed and replaced with destain solution (10% (v/v) ethanol and 2% (v/v) orthophosphoric acid) until the background become clear and protein bands were visible. Gels were scanned using a *BIO-RAD* Molecular imager® ChemiDoc™ XRS+) and analysed with gel analyzer software using GelAnalyzer 2010a software to estimate the molecular weight of protein bands.

2.12.2 Two dimensional (2D) Electrophoresis Separation of Date Palm Proteins

Two-dimensional gel electrophoresis involves separation of proteins based on isoelectric pH (isoelectric focusing) followed by a second SDS-PAGE separation based on molecular weight. The method allows separation of proteins with similar molecular weights and variants of the same protein that may differ in amino acid sequence or degree of post-translational modification (glycosylation, phosphorylation etc.). It is used as a separation step prior to protein identification using peptide mass fingerprinting via MALDI-TOF mass spectrometry.

2.12.2.1 Preparation of DPSP samples for 2D Electrophoresis

DDPSP (10 g) was subjected to protein extraction method 5 (section 2.10.5). The final dried pellet was dissolved in solubilisation buffer (7 M urea, 2 M thiourea, 4% (w/v) 3-3-cholamidopropyl dimethylammonio-1-propanesulfonate (CHAPS) and 100 mM dithiothreitol). The mixture was incubated for one hour at room temperature to achieve protein solubilisation. The insoluble material was removed by centrifugation at 20500 xg for 20 min. The pellet was discarding whilst the supernatant containing soluble protein was kept at –20˚C until used for electrophoresis.

2.12.2.2 Casting the Gel for 2D Electrophoresis

Glass plates (26 cm width, 20 cm length and 1mm thick, GE Healthcare, UK) were soaked overnight in light detergent and thoroughly washed to remove any previous gels before rinsing in distilled water and air-drying for at least 2 hours. Four ml of bind-silane solution (80% (v/v) ethanol, 0.2% (v/v) glacial acetic acid and 0.01% (v/v) bind-silane) was applied over the plates and again allowed to air-dry before washing with a small amount of 70% (v/v) ethanol. Large 12% SDS-PAGE resolving gels (30% T, 2.6% C monomer solution (30% acrylamide and 0.8% N,N-methylenebisacrylamide), 4% resolving buffer (1.5 M Tris Base HCl pH 8.8), 10% SDS buffer, 10% APS buffer and TEMED) were cast in the Ettan Dalt*six* apparatus system (GE Healthcare, UK). The solution was filtered using a 0.22 µm filter prior to addition of APS and TEMED.

The solution was gently mixed well and then poured between the glass plates. n-butanol was applied over the surface of gels. The gels were allowed to polymerize overnight at room temperature. The n-butanol was then removed and the gels stored at 4˚C until required for use.

2.12.2.3 First Dimension (isoelectric focusing (IEF)) of Date Palm Proteins

After extraction and solubilisation of date palm protein (section 2.12.2.1), sufficient protein powder was mixed with rehydration buffer (7M urea, 2M thiourea, 2% (w/v) CHAPS, 2% (v/v) IPG Buffer pH 3-11 NL, 40 mM DTT and 0.002% (w/v) bromophenol blue) to make a final volume of 450 µl containing at least 500 µg of protein. The mixture was then distributed evenly over a 24 cm ceramic strip holder previously cleaned and air-dried. A 24 cm immobiline Drystrip (pH 3-11 NL) was lowered over the sample with the anodic (+) region facing the pointed end of the holder. Care was taken to ensure no air bubbles formed under the plastic strip. Drystrip cover fluid (GE Healthcare) was applied over the strip before covering the lid to prevent sample evaporation. IEF was achieved using an IPGphor II manifold following settings suggested by the manufacture (Table 2.2). Strips were immediately equilibrated (as shown below) or stored at –70˚C before running the second dimension.

Table 2.2: IEF Conditions and Settings for 24cm Drystrip (pH 3-11 NL)

Voltage (V)	IPGphor ^{TM} II setting	Time (h)
30	Step and hold	12 (active rehydration)
300	Step and hold	2
1000	Gradient	1
1000	Gradient	2
8000	Step and hold	8

2.12.2.4 Equilibration of Proteins (Reduction and Alkylation)

The Immobiline strips were removed with care from their ceramic strip holder and placed in a plastic cylinder before reduction was performed for 15

min at room temperature with gentle shaking using 10ml of equilibration buffer (6 M urea, 75 mM Tris-HCl (pH 8.8), 20% (v/v) glycerol, 2% (w/v) SDS and 0.002% (w/v) bromophenol blue) containing 1.5% DTT, followed by another 15 min to alkylate the strip in a new cylinder containing 10ml equilibration buffer with 2.5% iodoacetamide. The equilibrated strips were then used immediately to run the second dimension.

2.12.2.5 Second Dimension Separation of Date Palm Seed Proteins

Following equilibration (above) the strips were placed on top of a laboratory prepared SDS gel (12% (w/v) polyacrylamide resolving gel) with the anodic (+) end of the strip facing the left hand side and the plastic backing touching the larger glass plate. The strip should be completely touching the gel surface before sealing by applying an agarose sealing solution (0.01% (w/v) bromophenol blue and 0.5% (w/v) agarose) dissolved in 1x SDS running buffer (section 2.12.1.2) and heated to 100˚C before applying on top of the gel. The remaining empty gel holder slots were filled with blanks before lowering into the Ettan^{TM} DALT*six* (GE Healthcare) electrophoresis apparatus unit. Approximately 4.5 L of 1x SDS running buffer (section 2.12.1.2) was used to fill the lower (anodic) chamber while the cathode chamber was filled with 3x SDS running buffer before sealing the system with the lid. This solution was prepared by diluting three volumes of 10x SDS running buffer (250mM Tris-HCl (pH 8.3), 1.92M Glycine and 1% SDS) with seven volumes of double distilled water. The electrophoresis unit was equilibrated to 10˚C using a MultiTemp^{TM} III temperature controller unit (GE Healthcare) before running the gels at 25˚C at 2W per gel for the first 45min and then 17W/gel until the dye front had reached the end of the gels (approximately 5 hours). The gels were removed from the electrophoresis unit and stained overnight with colloidal Coomassie brilliant blue, and then destained as described in Section 2.12.1.2.

2.12.3 Peptide Mass Fingerprinting (PMF)

Protein spots were excised from colloidal Coomassie brilliant blue stained 2D SDS-PAGE maps using a manual spot picker and digested with Trypsin Gold (Promega, UK) following the Trypsin protocol (in-gel protein digestion). The gel slices (head diameter:1.5 mm) were placed into 0.5 ml microcentrifuage tubes previously washed twice with 50% (v/v) acetonitrile

(ACN), 0.1% trifluoroacetic acid (TFA). The gel slices were then destained with 200µl of destain buffer (100mM ammonium bicarbonate (NH_4HCO_3), 50% (v/v) ACN) for 45 min at 37˚C. The destaining was repeated with fresh destain solution. The destain buffer was removed and the gel slices were dehydrated in 100 µl of 100% ACN for 5 min at room temperature. At this stage, the gel slices are reduced in size and had become opaque in appearance. The gel slices were dried in a SavantAES2010 Speed Vac® (Thermo Fisher Scientific, USA) for 10–15 min at room temperature to remove the ACN.

Trypsin Gold (Promega) was suspended at 1µg/µl in 50 mM acetic acid before dilution to 20µg/ml with 40mM NH_4HCO_3, 10% (v/v) ACN. The dried pellets were then rehydrated in trypsin solution with minimal volume (10–20 µl) at room temperature for 1 hour. After 1 hour if the gel slices appeared white or opaque, an additional 10–20 µl of trypsin solution was added and incubated for another 1 hour at room temperature. Digestion buffer (40 mM NH_4HCO_3, 10% ACN) was added to completely cover the gel slices before capping tightly to avoid evaporation and incubation overnight at 37˚C. After overnight incubation, Milli-Q water (150µl) was added to the gel slice digests and incubated at 37˚C for 10 min with frequent mixing. The liquid was removed and transferred to a new microcentrifuge tube. The gel slices were extracted (twice) with 50 µl of 50% (v/v) ACN, 5% (v/v) TFA for 1 hour at room temperature. All liquid extracts were pooled and dried in a Speed Vac® for 2–4 hours.

The concentration of extracted peptides were performed using Zip Tip® C18 pipette tips (Millipore Corporation, Billerica, USA) as per the manufacturer's instructions. To purify, peptides were then eluted in 2.5 µl of 70% (v/v) ACN, 0.1% (v/v) TFA, 10mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) and finally 1–2µl was spotted directly onto the MALDI-TOF using the Ettan MALDI-TOF Pro system (Amersham Biosciences, UK) for mass spectrometry protein identification. Unfortunately, the MALDI-TOF mass spectrometer in our laboratory developed a fault during our attempts at analysing the spots from the 2-D gel. Due to time constraints on the project, it was decided to procure the services of the mass spectrometry facility in the School of Chemistry at the University of Edinburgh where the results for

liquid-chromatography coupled tandem mass spectrometry (LC-MSMS) were obtained.

2.12.4 Date Palm Seed Protein Identification by Liquid-Chromatography Coupled Tandem Mass Spectrometry (LC-MSMS)

The sample preparation below in section 2.12.4 was kindly carried out at the University of Edinburgh by Prof. Cait MacPhee, of the School of Physics & Astronomy prior to LC-MSMS analysis in the School of Chemistry, UoE.

2.12.4.1 Preparation of Sample

Date palm seed protein (10 mg) was resuspended in 50 µl of distilled water (dH_2O), followed by denaturation with 250 µl of 8M Urea and dilution with 950 µl dH_2O prior to trichloroacetic acid (TCA) precipitation with 310 µl of 100% TCA, 1250 µl methanol and 625 µl chloroform. Samples were vortex mixed and incubated (4˚C, 10 min) before centrifugation (4500xg, 4˚C, 10 min). The top phase was removed before adding 1 ml methanol.

The sample was vortex mixed before centrifugation (as above), the supernatants were removed and the solid sample washed twice with 1 ml acetone, centrifuged at 10000 xg, at 4˚C for 5 min and dried under vacuum. Then, the sample was resuspended in 100 µl dH_2O. Protein concentrations were estimated by Bradford assay of 10 µl of the protein extract diluted with 30 µl of 2.7M urea solution and quantified using a Bovine serum albumin (BSA) as standard in 2M Urea. Protein digestion was carried out using the method proposed by Le Bihan et al. (2011) on 20 µl of protein extract. Briefly, samples were denatured in 8M urea, reduced by incubating with dithiothreithol (DDT) prior to cysteine alkylation with iodoacetamide and overnight digestion with 60 µg trypsin at room temperature.

Four µg of peptide samples were acidified (1% formic acid) before centrifugation and cleaning using Stage tips (Rappsilber, 2007). Finally, the peptide samples were vacuum-dried and stored at –20˚C until further analysis.

2.12.4.2 Liquid-Chromatography Coupled Mass Spectrometry (LC-MSMS)

Peptide samples (2 µg) were analysed in a randomised sequence by capillary HPLC-MSMS, using 140 minute gradients as described by Martin

et al. (2012), on an on-line system consisting of a micro-pump (1200 binary HPLC system, Agilent, UK) coupled to a hybrid LTQ-Orbitrap XL instrument (Thermo-Fisher, UK). ACN and water were HPLC quality (Fisher, UK). Formic acid was Suprapure 98–100% (Merck, Darmstadt, Germany) and trifluoroacetic acid was 99% purity sequencing grade.

2.12.4.3 Identification and Quantification of the Peptides

Multicharged (2+, 3+ and 4+) ion intensities were extracted from LC-MS files and MSMS data were searched using Mascot Version 2.4 (Matrix Science Ltd, UK) against the NCBI protein database (02/10/2010; 11,961,441 sequences) using a maximum missed-cut value of 2, variable oxidation (M), N-terminal protein acetylation and fixed carbamidomethylation (C); precursor mass tolerance was 7 ppm and MSMS tolerance 0.4 Da. The significance threshold (p) was <0.05 (MudPIT scoring) and a minimum peptide cut off score of 20 was set. Proteins identified and quantified with 2 or more peptide sequences were retained.

2.13 Functional Properties of Protein

The following functional properties of date palm protein were characterised using a number of standard tests: solubility, emulsifying properties, water and oil adsorption properties. Details of these methods are given below.

2.13.1 Solubility of Date Palm Seed Protein

Each protein sample (200 mg) was suspended in 20 ml of Milli-Q water. The pH of the solution was adjusted using 0.1M NaOH or 0.1M HCl to a pH of 2, 4, 6, 8, 10 or 12. The mixture was then stirred using a magnetic stirrer for 30 min at room temperature. The samples were heated at either room temperature, 55˚C or 85˚C in shaking water bath (GLS Aqua18 Plus, Grant instruments Ltd, UK) for 10 min, then cooled immediately on ice to 15˚C. Each treated sample (10 ml) were centrifuged at 1000 xg, 20˚C for 10 min using a Beckman Coulter, Avanti J-26 XP centrifuge with a JA 25.50 rotor, Beckman Coulter, USA. The protein in the supernatant was determined using the micro-Kjeldahl method (AOAC, 1995) (section 2.4.4) and the protein solubility was then calculated as follows:

$$\% \text{ Solubility} = \frac{\text{Protein content of the supernatant}}{\text{Total protein content of the sample}} \times 100$$

2.13.2 Emulsifying Properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) were determined by a turbidimetric method according to Kinsella (1979), Liu et al. (2008) & Ogunwolu et al. (2009) with some modifications. Four hundred and fifty milligrams of protein sample was dispersed in 45 ml of Mill-Q water. The protein solution was then mixed with 15 ml of vegetable oil and the pH was adjusted to 2, 4, 6, 8, 10 or 12 using 0.1 M HCl or 0.1M NaOH.

The mixture was homogenised using an Ultra-turrax high speed homogenizer (IKA-Werke GmbH, Germany) for 1min to make a protein-stabilised oil-in-water emulsion. The emulsion (50 µl) was pipetted from the bottom of the container into 5 ml of 0.1% (w/v) SDS solution. This was carried out immediately at 0 min and 10 min after the homogenisation. Absorbance of the diluted emulsions was measured at 500 nm using a spectrophotometer (Model Genesys 6, Thermo Electron Corporation, USA). The ability of the protein to form an emulsion (emulsifying activity index, EAI) and the stability of the formed emulsion (emulsion stability index, ESI) were calculated using the following formulae:

$$\text{EAI } (m^2/g) = \frac{2 \times \text{T} \times \text{dilution factor}}{\text{C} \times \emptyset \times 1000}$$

$$\text{ESI (min)} = \frac{\text{A0}}{\text{A0} - \text{A10}} \times \Delta\text{t}$$

Where, T = 2.303, A_0 = the absorbance immediately after the homogenisation, dilution factor = 100, C = the weight of protein per unit volume (g/ml), Ø = the oil volumetric fraction (0.25), A_{10} = the absorbance after 10 min of the homogenisation, Δt = 10 min.

2.13.3 Water Absorption Capacity (WAC)

The sample (100 mg) was mixed with 1 ml of distilled water and vortex mixed for 1 min. The sample suspension was then centrifuged at 1000 xg for

15 min at room temperature using a Beckman Coulter, Avanti J-26 XP centrifuge with a JA 25.50 rotor (Beckman Coulter, USA). The supernatant was removed and discarded, and the tubes were drained at 45° angles for 10 min. The water absorption capacity was calculated by dividing the volume of water absorbed by the weight of the sample as follows:

$$\text{WAC (g/ml)} = \frac{\text{Wet mass of sample after centrifugation (g)}}{\text{Dry mass of the sample before centrifugation (g)}}$$

2.13.4 Oil Absorption Capacity (OAC)

Oil absorption capacity was measured by vortex mixing 0.1 g of protein powder sample with 1 ml of vegetable oil in an Eppendorf tube. The mixture was left at room temperature for 30 min before centrifugation at 11500 xg using microcentrifuge (VWR micro Star 12) for 10 min at room temperature. The supernatant was decanted and drained at a 45° angle for 20 min. To obtain the oil absorption capacity, the volume of oil absorbed was divided by the protein sample mass as follows:

$$\text{OAC (g/ml)} = \frac{\text{Wet mass of sample after centrifugation (g)}}{\text{Dry mass of the sample before centrifugation (g)}}$$

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CHAPTER THREE

Physicochemical Properties of Date Palm Fruit (Flesh Seed)

3.1 Introduction

The fruit of date palm (*Phoenix dactylifera* L.), has been consumed by both humans and livestock for millennia, and is one of the oldest plants cultivated by humans. Dates could be used for generations to come due to their superb nutritional and health properties and high economic value. There are nearly 2000 varieties of dates distributed around the world. Many of these varieties are available during an eight month growing period each year. Dates can be packed without any preservatives due to their high sugar content which acts as an effective preservative (Sulieman et al., 2012). It has been reported that date palm seed contains sufficient amounts of fat, protein and total carbohydrate to be used to meet part of the nutritional needs of animal feed. In addition waste seeds from the date processing industry could be an excellent source of food ingredients as it contains dietary fibre with interesting functionality (Besbes, 2004).

Date palm seed is a rich source of mineral ions. Ali-Mohamed & Khamis (2004) have compared date palm seed with barley and coffee and they found date seeds have the lowest concentrations of heavy metals such as lead (Pb) and cadmium (Cd) compared to coffee and barley. In general, daily intakes for the minerals of date palm seeds are within the dietary intake level required by the human body (Ali-Mohamed & Khamis, 2004). Dates have been evaluated for their fruit quality since ancient times (FAO, 1999; Al-Hooti et al., 2002).

The physical and chemical properties are essential for the quality control in the date industry through grading, sorting and handling of dates. Physical measurements of date palm fruit are good indicators of growth and maturity as well as predictive of shelf life. The chemical characteristics of dates relate to the health benefits and nutritional values of date palm fruit (Khan et al., 2008; Manickavasagan et al., 2012). Study of the physical characteristics of date fruit and their seeds like weight, length, width, flesh percentage, seed percentage are of importance in differentiation between the cultivars.

In this chapter the physical properties and chemical composition of date palm fruit (flesh and seed) at the Tamar stage, which are used in this study, are assessed. Dates are mostly consumed and traded in this stage. It is important for us to understand the composition of our raw materials prior to their use for the extraction of functional proteins for study in this project.

3.2 Results and Discussion

3.2.1 Chemical Compositions of Crude Date Palm Seed and Date Palm Fruit

The optimization of the DPF or DPS as a functional food requires understanding of the physicochemical properties of both flesh & seed.

Most previous studies focused on the chemical characteristics and nutritional value of date palm flesh rather than seed, and information on the composition of seeds are limited. Figure 3.1 presents the proximate composition of date palm flesh (DPF) and date palm seed (DPS). Not unexpectedly, the major component of both the date flesh and date seed used in this work are carbohydrates. The total carbohydrate content of the flesh used in this work was higher than that in the seed, although as noted in Chapter 1 Section 1.8.4 the carbohydrate content of the flesh changes with maturation stage (here we have used Tamar stage where the carbohydrate content is at its highest).

It is also reported that the form of the carbohydrate differs between date flesh and seed. In the flesh the carbohydrate consists mainly of mono- and disaccharides such as sucrose, fructose and glucose which make up 85% of the total carbohydrate in some varieties (Manickavasagan et al., 2012).

In date seed it is reported that only a small part of the total carbohydrate consists of simple sugars with the rest of the carbohydrates being found as more complex polysaccharides such as cellulose, hemicelluloses and lignin. The carbohydrate content of date seed has been characterised due to the use of the product as an animal feed. Table (3.1); (FAO, 1993) shows data reported by the FAO on the composition of date seed carbohydrates. The polysaccharide content of animal feed is often expressed in terms of the fibre content, and is divided into crude fibre, neutral detergent fibre (NDF) and acid detergent fibre (ADF).

Crude fibre represents the indigestible carbohydrate (hemicelluloses, cellulose, lignin etc.). It is determined using an acid treatment followed by an alkaline treatment to simulate the effect of digestion in the stomach followed by the intestine. The remaining material is termed crude fibre, although it only consists of a fraction of the total fibre content. Crude fibre has little nutritional value, but plays an important physiological role by adding bulk to stools which aid the normal peristaltic action in the digestive system and facilitate waste removal from the gut. In humans crude fibre intake has also

been linked to beneficial health effects. It is believed to remove cholesterol from the gut before it can be adsorbed, thus having positive effects for lowering obesity and cardiovascular disease (Buttriss & Stokes, 2008).

NDF is determined by boiling the feed in a neutral detergent solution that extracts protein, lipids and pectins but leaves lignin, hemicellulose and cellulose. Similarly, ADF is quantified by boiling in an acid detergent solution that solubilises everything apart from the least digestible polysaccharides (cellulose and lignin).

Table 3.1: Composition of date seed carbohydrates (% of dry weight) (FAO, 1993).

NDF	75.0 (neutral detergent fibre, total cell wall content)
ADF	57.5 (acid detergent fibre, NDF less hemicellulose)
Hemicellulose	17.5 (NDF minus ADG; hemicellulose is a long-chain carbohydrate composed of pentoses.)
Lignin	11.0 (determined by potassium lignin procedure on ADF residue, oxidising the lignin)
Cellulose	42.5 (burning above residue)
Ash	4.0 (what remains upon burning)

Crude protein, crude fat are the other major fractions in both date fruit and seed. The seed contains lower levels of moisture, but higher levels of fat and protein compared to the flesh. This can be explained in terms of the differences in metabolic process that occur between the two parts of the date fruit. The seeds have a low moisture content to suppress metabolic activity during the dormancy periods before they germinate. The seeds also require stores of triglyceride as well as carbohydrate as an initial food source during germination, and the prerequisite enzymes (proteins) required for metabolic activity during germination. We will see later (Chapter 5) that many of the proteins associated with the date palm seed proteome are metabolic proteins.

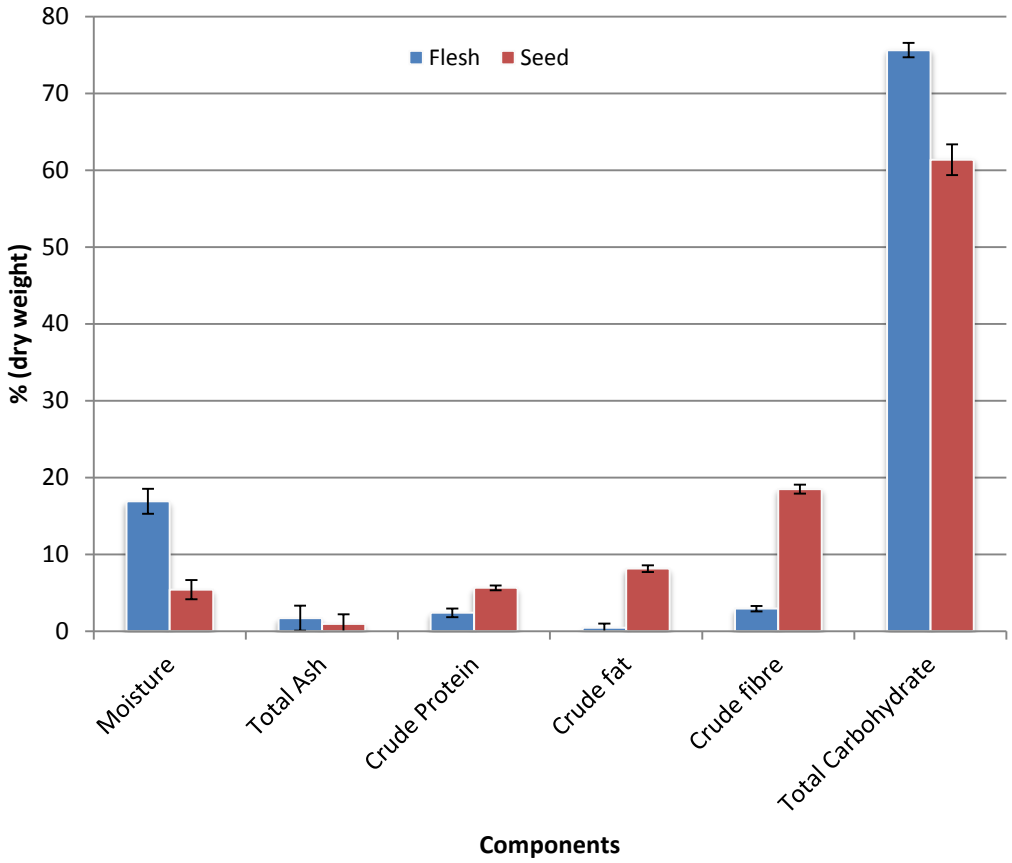

Fig. 3.1 Proximate Analysis of Date Palm Flesh and Seed

There is a significant variation in the proximate composition of date varieties. In this study results for DSP were in general agreement with those of Hamada et al. (2002) who measured the chemical composition of three date seed palm varieties (Fard, Khalas and Lulu) growing in UAE. These seeds contained 7.1–10.3% moisture, 9.9–13.2% crude fat, 5.2–6.0% crude protein and 1.0–1.8% ash. On the other hand the results in this study differ from those reported by Besbes et al. (2004) who studied the chemical composition of two date palm seed varieties growing in Tunisia (Deglet Nour and Allig) and found the moisture, fat, ash and total carbohydrate compositions were 8.6–9.4%, 10.19–12.67%, 1.12–1.15% and 81.0– 83.1% respectively, whilst the protein content was slightly lower than our results at 5.17–5.56%. The proximate composition of date flesh obtained in this study are comparable with those reported at Tamar stage by Ahmed et al. (1995), Elleuch et al. (2008) and Besbes et al. (2009) for twelve varieties of date palm fruit produced from the four major regions in UAE, and two of the most popular

Tunisian varieties of dates. The composition of dates is known to vary at the different stages in the growth cycle. Al-Hooti (1997) studied the chemical composition of five cultivars of dates from the UAE during the different stages of growth and maturity. All components except water were highest at the first stage (Kimri stage) and gradually decreased as the fruit matures to the final stage (Tamar stage). The exception to this was the sugar content which increased with maturity. There were differences observed between cultivars, but in general, trends were the same for all cultivars.

3.2.2 Amino Acid Composition of Full Fat Date Palm Seed Powder (FFDPSP)

The amino acid composition of a protein is important in terms of the nutritional value of the protein source, and in particular the content of the essential amino acid which cannot be synthesized by humans.

The amino acid composition of FFDPSP is shown in Table 3.2. Sixteen amino acids were found and identified by HPLC. All the essential amino acids such as isoleucine, leucine, lysine, S-containing (methionine and cysteine), phenylalanine, threonine and valine, were present but not tryptophan in both samples. In addition, a small amount of histidine was detected. Of the essential amino acids in FFDPSP, the most abundant was found to be leucine, and it is widely reported to be the most common amino acid in food proteins (WHO, 2007). The amount of threonine in FFDPSP is also relatively high. Threonine is considered particularly important in nutrition since the loss into the large bowel of this amino acid is high (Fuller et al., 1994). The concentration of phenylalanine in the FFDPSP is also relatively high. In humans phenylalanine is important because it is converted into tyrosine which is used to make catecholamine such as the neurotransmitter dopamine, and the hormones norepinephrine and epinephrine. Tryptophan is another essential amino acid that is a precursor for biologically important molecules such as the neurotransmitter serotonin and the B vitamin nicotinamide. The lack of tryptophan in the protein of FFDPSP has already been mentioned. Tryptophan is known to be present at lower concentrations than other amino acids in many proteins and in particular cereal proteins (WHO, 2007). However, the fruit of date palm has

been shown to contain tryptophan with the concentration ranging from 0.12–0.66 mg/g depending on variety for Hallawi, Sayer, Deglet Nour and Algerian varieties (Manickavasagan et al., 2012). We would expect to have found some tryptophan in the FFDPSP. The disappearance of tryptophan from date seed might be attributed to loss during hydrolysis by concentrated hydrochloric acid as reported by Al-Showiman & Baosman (1992). It is has also been reported by Suryaprakash et al. (2000) and Yada (2004) that the amino acid composition of oil seed proteins can be influenced by the presence of some phenolic acids such as quinic and caffeic acid. Quinic acid has been shown to interact with amino acids such as tryptophan, arginine and lysine while caffeic acid interacts with tyrosine, lysine and tryptophan.

Table 3.2 also include a column listing the recommended daily amounts (RDA) of the essential amino acids as recommended by the World Health Organisation in 2007 (WHO, 2007). The essential amino acid profile of full fat date seed powder is compared to the RDA in Figure 3.2.

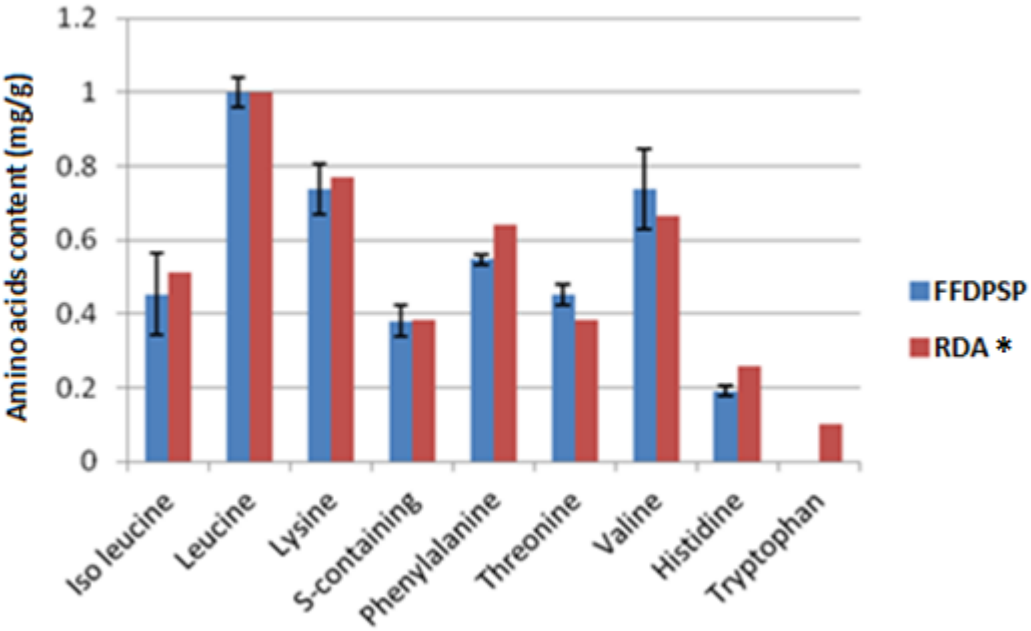

Fig 3.2: Essential amino acid profile (relative to leucine content) for (FFDPSP) compared to the recommended daily amount (WHO, 2007).

* Mean nitrogen requirement of 105 mg nitrogen/kg per day (0.66 g protein/kg per day)

With the exception of tryptophan the amino acid profile of the date seed protein is very similar to that of the RDA for humans, which would suggest that it could be a suitable protein additive in human foods, subject to supplementation of the tryptophan content with other proteins rich in this amino acid.

Table 3.2: Amino Acid Composition (mg/g of protein) in FFDPSP

Amino acids	**FFDPSP**	***RDA(WHO, 2007)**
Essential amino acids:		
Iso leucine	33.5±4.1	30
Leucine	74.1±5.3	59
Lysine	54.7±8.8	45
S-containing	28.2±5.3	22
Phenylalanine	40.6±1.8	38
Threonine	33.5±3.5	23
Valine	54.7±4.1	39
Histidine	14.1±1.8	15
Non-essential amino acids:		
Aspartic acid	82.9±17.0	–
Glutamic acid	183.4±15.3	–
Serine	60.0±7.1	–
Arginine	128.7±13.5	–
Glycine	68.8±14.1	–
Alanine	61.7±7.1	–
Proline	40.6±1.8	–
Tyrosine	40.6±10.6	–

* Mean nitrogen requirement of 105 mg nitrogen/kg per day (0.66 g protein/kg per day)

The amino acid composition of FFDPSP from this study can be compared with the amino acid composition found in other studies. Most of the amino acid composition of our sample was in line with those reported by Al-Showiman & Baosman (1992) who examined the amino acid contents of five Saudi date seed cultivars namely; Berhey, Sekkeri, Berni, Maktomey and Shaqra. Sulphur-containing amino acids, glycine and tyrosine were higher in our sample than the five cultivars studied by Al-Showiman & Baosman (1992) as shown in Table 3.3.

Table 3.3: Amino Acid Composition (mg/g) in FFDPSP Compared to other Studies

Amino acids	FFDPSP	Behey(*)	Sekkeri(*)	Berni(*)	Maktomey(*)	Shaqra(*)
Essential amino acids:						
Iso leucine	0.19±0.08	0.26	0.18	0.19	0.20	0.20
Leucine	0.42±0.03	0.52	0.40	0.39	0.44	0.44
Lysine	0.31±0.05	0.57	0.35	0.71	0.36	0.35
Methionine	0.16±0.03	0.04	0.02	0.05	0.06	0.07
Phenylalanine	0.23±0.01	0.26	0.24	0.24	0.25	0.19
Threonine	0.19±0.02	0.23	0.20	0.20	0.19	0.20
Valine	0.31±0.08	0.24	0.30	0.30	0.33	0.32
Histidine	0.08±0.01	0.15	0.12	0.12	0.13	0.05
Non-essential amino acids:						
Aspartic acid	0.47±0.21	0.77	0.71	0.60	0.73	0.64
Glutamic acid	1.04±0.20	1.39	1.22	1.10	1.30	1.09
Serine	0.34±0.04	0.35	0.28	0.27	0.38	0.24
Arginine	0.73±0.19	1.08	1.01	0.88	1.09	1.13
Glycine	0.39±0.08	0.29	0.28	0.28	0.26	0.29
Alanine	0.35±0.04	0.34	0.32	0.32	0.37	0.36
Proline	0.23±0.01	0.45	0.41	0.26	0.38	0.39
Tyrosine	0.23±0.06	0.09	0.09	0.09	0.09	0.80

(*): Al-Showiman and Baosman, (1992)

On the other hand aspartic acid, arginine and proline were lower in our sample than in the five cultivars. This could be due to differences in composition of amino acid between varieties. Glutamic acid was the most abundant amino acid in full fat date seed, followed by arginine, aspartic acid, leucine, glycine, alanine, lysine, valine and phenylalanine. These were in agreement with the results reported by Bouaziz et al. (2008) in their study of the amino acid profiles of two Tunisian date palm seed (Deglet Nour and Allig), They also found that most of the amino acid in the seed of Deglet Nour variety were present at higher concentrations than those in Allig variety.

3.2.3 Physical Characteristics

3.2.3.1 Some Physical Measurements of DPS and DPF

Physical characteristics of date palm fruit are considered important information in the date industry that helps trading, processing and storage of dates. In Table 3.4 some physical measurements are shown for the dates used in this study (Deglet Nour variety). The date palm fruits and their seeds had an average weight of 9.5 g and 0.98 g respectively, which meant that the seed and flesh comprised 10% and 90% respectively, of the whole fruit. The average fruit weight was in agreement with the study by Sulieman et al. (2012) who reported that the weight of five Sudanese date fruits were 9.41, 6.57, 12.78, 7.90 and 9.14 g for White Gau, Red Gau, Black Gau, Barakawi and Gondeila variety respectively. Some date varieties have smaller fruits, such as Bushibal, Gaafar, Habash, Lulu and Shahla with flesh weights of 4.9, 5.0, 4.9, 4.9 and 4.9 g respectively (Al-Hooti et al., 1997).

Obviously there is variation in fruit weight between the different varieties, but the fruit weight could also have been affected by different season, agriculture treatments or environmental conditions during growing of the dates. The average seed weight was in the range of those reported by El-Alwani & El-Ammari (1999) who examined thirteen date palm cultivars grown in Libya which showed that the highest value of seed weight was 1.43 g for the Tediss variety while the lowest of 0.79 g was for the Deglet variety.

Table 3.4: Some Physical Measurements of DPS and DPF.

	Weight (g)	Length (mm)	Width (mm)
Whole fruit	9.51±1.36	37.59±0.73	16.45±0.62
Flesh	8.53±0.93	37.59±0.90	16.45±0.75
Seed	0.98±0.14	29.81±0.81	08.06±0.59
Ratio of flesh to whole fruit		89.70%	
Ratio of seed to whole fruit		10.30%	
Number of fruit per kg		106.0±1.22	

Our results show that the average weight of date seeds is 10.30% of the whole fruit weight which is within the range of pit percentage reported by Sulieman et al (2012) who found the highest value of seed percentage of 15% for Barakawi variety, whereas the Black Gau variety has the lowest pit percentage of 9%. Both fruit and seeds are ellipsoidal in shape with an average aspect ratio (length: width) of 2.16:1 for the whole fruit/flesh and 3.7:1 for the seed. The length and width of fruit were 37.59 and 16.45 mm respectively. There is considerable variation in the dimensions of the date fruit between varieties. The average length of the fruit used in this study was lower than those found by Sulieman et al. (2012) of five date varieties of 47.4, 45.4, 51.0, 44.2 and 43.0 mm for White Gau, Red Gau, Black Gau, Barakawi and Gondeila varieties respectively. A similar variation in average fruit widths between varieties was also reported by Sulieman et al., (2012) of 16.8, 14.0, 18.2, 13.8, and 18.0 mm respectively.

3.2.3.2 Bulk Density of Full Fat Date Palm Seed Powder (FFDPSP), Defatted Date Palm Seed Powder (DDPSP), Compared with Soybean Protein Isolates (SPI) and Whey Protein Isolates (WPI)

Bulk density depends on the particle size, polydispersity of particle size and the particle packing of the powder. The bulk density is important for determining material handling, applications in the food industry and for packaging requirements (Ocloo et al., 2010).

Ortega-Rivas, Juliano & Hong Yan (2005) report that the bulk density of most food powders lies in the range 200–800 kg/m^3 with few exceptions. Figure 3.3 shows the bulk density of the powders used in this study with data for whey protein concentrate and soy protein isolate powders for comparison. As expected we do see that all values lie in the range 300–800 kg/m^3.

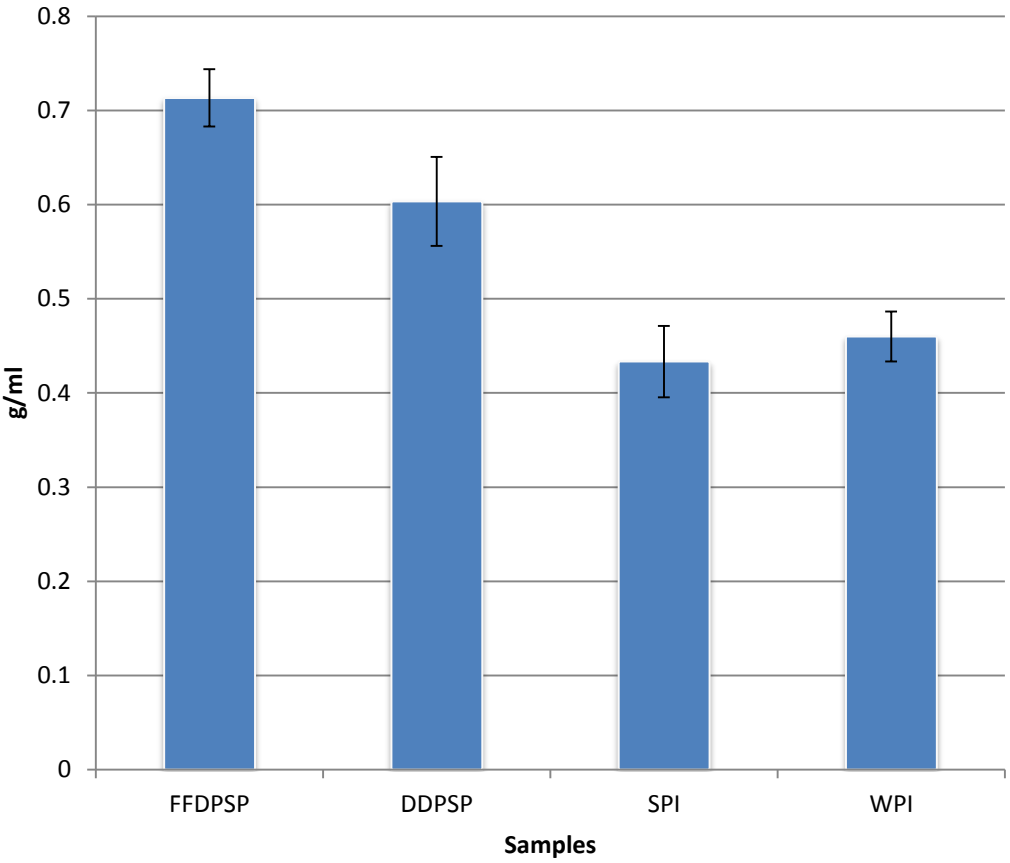

Fig 3.3: Bulk densities of FFDPSP and DDPSP, compared with SPI and WPI.

FFSP is 0.71g/ml and was found to be the highest of the samples under investigation with the bulk density of DDPSP at (0.60 g/ml) being less than that of FFDPSP. This observation agreed with that of Fagbemi et al. (2005) who noted that removing fat reduced the bulk density of fluted pumpkin seed flour regardless of the process applied. The bulk density depends on a number of factors both compositional and structural. It is believed that the low values of bulk density for powders cannot be explained by geometric packing considerations alone, and must reflect a porous inner structure for the powder particles. If this is the case then it might explain why the date seed powders in this study have a higher bulk density than the WPC and SPI samples. The date seed powders have not been spray dried, but have simply

been milled from the seed and are unlikely to have a highly porous structure in the same way that a spray dried powder (WPC or SPI) might which have very low bulk density (Figure 3.3) and presumably highly porous powder particle structure. The bulk density values for our date seed powders can be compared to other plant protein powders. The bulk density values for seed powders reported by Oladele & Aina, (2007) for tiger nut flour (0.55–0.62 g/ml), by Akubor and Badifu (2004) for African bread fruit seed flour (0.54 g/ml) and wheat flour (0.70 g/ml) and by Ocloo et al. (2010) for jackfruit seed (0.80g/ml) all fall within the expected range for bulk density. High bulk density is a requirement of powders used in food products as a thickener (Ocloo et al., 2010); hence, the date palm seed powder might find application as a thickener.

3.2.3.3 Swelling Powers of Date Seed Samples Compares with Soybean and Whey Powder

The swelling power is a measure of hydration capacity of a powder. In flours it can be related to the swelling of starch granules, gums or proteins in the powder (Ocloo et al., 2010). The quality of carbohydrate rich food is often related to the retention of water in the swollen starch granules (Rickard et al, 1992), which in turn is related to the interaction forces between water and starch molecules. The measurement of the swelling power of the flour granules can be used as an indicator of the extent of associative forces within the granule (Moorthy & Ramanujam, 1986; Olu et al., 2012). The results for swelling power are presented in figure 3.4.

The highest swelling power was obtained for soybean powder (7.72 g water/g powder) whilst the lowest was for the FFDPSP (3.36 ml water/g powder). Date seed has swelling power values of 3.74 and 3.36 ml water/g powder for DDPSP and FFDPSP samples respectively. The reason for the swelling power of FFDPSP being lower than the DDPSP could be due to can inhibition of swelling power by the lipid which has been reported by Morrison et al. (1993) and Sasaki & Matsuki (1998).

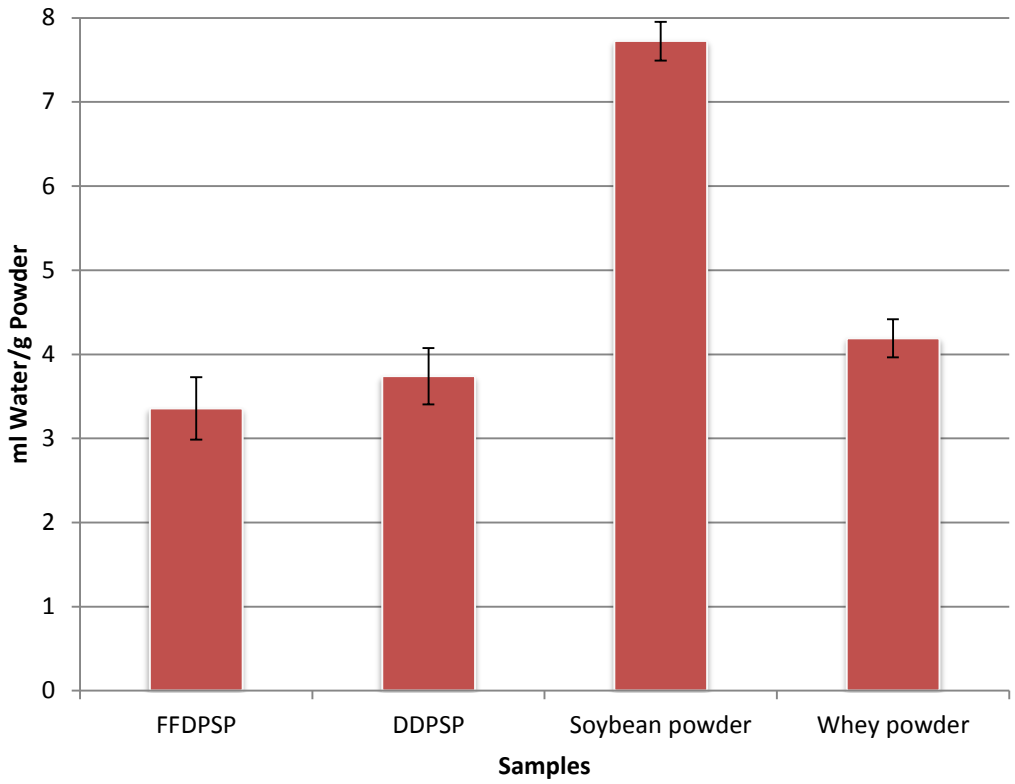

Fig 3.4: Swelling Powers of Date Seed samples Compares with Soybean and Whey Powder.

It is not surprising that the soybean and whey protein have the highest swelling powers since these contain high levels of protein (93% and 87% w/w protein respectively). The lower swelling powers of the date seed proteins can be explained by their low protein contents (5–6% w/w) combined with a relatively high proportion of non-swelling polysaccharides and fibre.

3.3 References

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CHAPTER FOUR

Extraction of Date Palm Seed Proteins

4.1 Introduction

The increasing cost of proteins from animal sources such as meats, eggs and dairy products has encouraged the food industry to find other sources of proteins for use as functional ingredients in formulated foods. In addition it is becoming evident that protein sources such as fish meal or soy protein that are often used in animal feed are unsustainable or economically not viable. The edible part of the date palm (*Phoenix dactylifera* L.) is called the fruit, flesh or simply the date. Date fruit can be eaten raw (fresh), soft or dry, has a high nutritional value and is considered by many to be one of the most delicious of fruits (Ramawat, 2010). It is also one of the richest of fruits in protein (Ahmed et al., 1995).

The seeds of the date fruit, which are a waste product from date processing, also contain protein. If it is possible to extract the protein from the seeds it might be used as a source of protein for human or animal nutrition. Robust methods for the extraction of proteins from date seeds could facilitate the utilization of date palm wastes such as seeds in the human and animal diet. In addition, the extraction of proteins can be effective at decreasing anti-nutritional components of the seeds as well as producing high protein products with good nutritional value and functional properties (Yada, 2004).

There is a lack of information in the literature on the extraction of proteins from date palm fruits (flesh or seed).

For this reason we have initiated a study to investigate the extraction of protein from dates (flesh & seed). The main target of this study is to provide the best method to recover a high proportion of the soluble protein from date seed and flesh. Previous studies by Hamada et al. (2002) suggest that at least 53% of the total protein from date seed can be recovered using simple aqueous extraction techniques. We attempted to improve on this by using methodologies that are standard in the protein industry, and are commonly used for protein powders derived from other seed products (e.g. soybean) and/or other protein sources.

This chapter describes using different purification methodologies to prepare protein powder of protein content up to 70%. This will include methodologies such as solvent extraction, dialysis and/or tangential crossflow filtration and enzyme assisted extraction to remove non-protein material from the extracted powder.

4.2 Results and Discussion

4.2.1 Some Factors Effecting Protein Extraction from Date Palm Seeds (DPS)

Extraction using alkaline solution has been used extensively for protein extraction from seeds, including from date palm seeds (Hamada et al., 2002; Bouaziz et al., 2008) but its effectiveness depends heavily on the extraction conditions used. These include extraction time, extraction temperature, solvent concentration and the ratio of sample to extraction solvent (Sun & Tian, 2003; Guo et al., 2005; Shen et al., 2008). However, no systematic study of the effect of pH on extraction of protein from date seed has been presented in the literature, and so as a first step in optimising protein extraction from the date seed experiments were carried out to confirm that high pH gave the best extraction conditions.

4.2.1.1 Effect of pH on Extraction of DPS Protein

The effect of different pH values on extractability of date palm seed protein is shown in Figure 4.1.

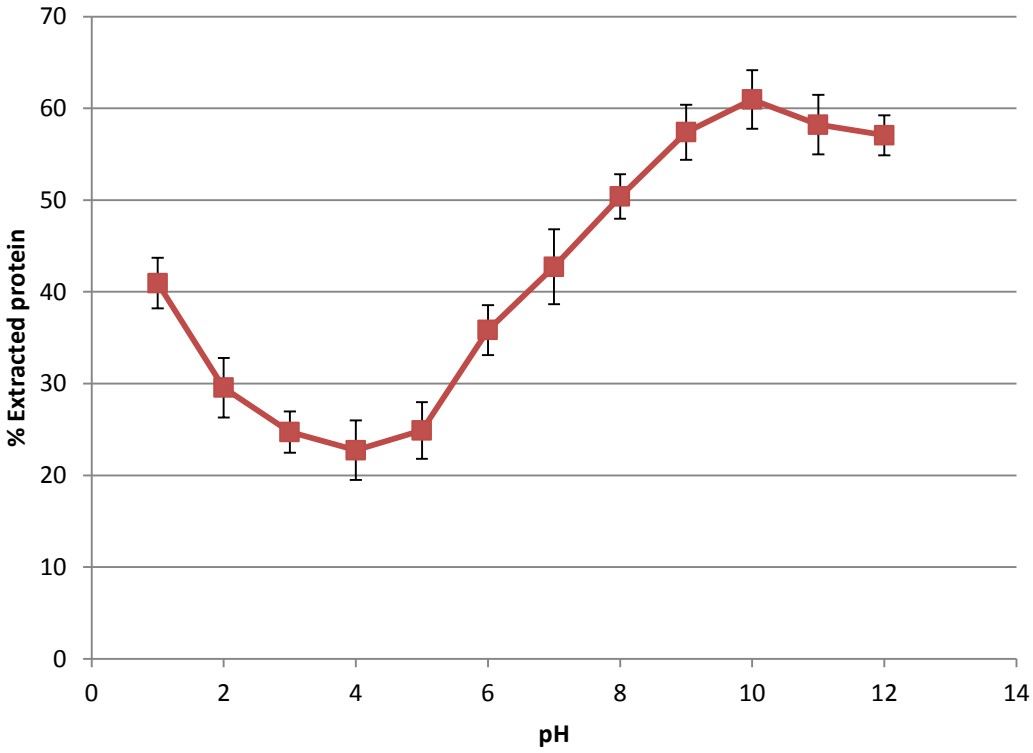

Fig 4.1: Effect of the pH on Extraction of DPS Protein.

High extractable protein (defined as the percentage of the total protein present that can be extracted) was obtained in both alkaline and acidic pH. 40% of the total protein in DPS was extracted at pH 1.0 which decreased to 22% at pH 4.0, but increased again to 61% at pH 10.0. The minimum extractable protein which occurs at the isoelectric point of DPS protein was observed at pH 4.0. This can be interpreted as when the net charge between the particles tends to zero at the isoelectric point, the protein aggregates into an insoluble mass due to a decrease in electrostatic charge repulsion between the protein molecules which promotes protein monomer aggregation (Eromosele et al., 2008).

Other researchers have recorded a similar isoelectric point of pH 4.0 for different plant proteins such as mung bean, red kidney and pea bean (Hang et al., 1970), conopher seeds (Ekpenyong, 1986), defatted soy flour and broad bean protein (Arogundade et al., 2006). The acid iso-electric point (IEP) of the mixed protein powder is not surprising. Analysis of the proteome of many organisms suggests that there is a universal biomodal distribution of IEP's with clusters of acidic and basic proteins but very few having an IEP point close to pH 7 (Weiller et al., 2004). The reason for this is that proteins have lowest solubility and therefore reduced biological activity at the IEP. Since the pH of the interior of most cells is close to neutral, evolutionary pressures to maximise protein stability have led to the preference for acid and basic proteins. For the DPS sample, which is a mixture of many proteins, the low solubility at acid pH suggests that the acidic proteins are more abundant in the sample than basic proteins.

Padilla et al. (1996), Queiroga et al. (2001) and Eromosele et al. (2008) reported a slightly higher IEP at 4.5, 5.0 and 5.0 for barinas nut flour, cashew nut protein and African yam bean respectively. Others authors observed a range of pH in maximum protein precipitation. Madhusudhan & Singh (1983), Dev et al. (1986), Lazos (1992), Mahajan & Jha (1995) and Jyothirmayi et al. (2006) reported that IEP ranged from 3.0 to pH 7.0 for a range of protein powders as shown in Table 4.1 below.

It was observed that the extractable protein increased as the pH increased from 5.0 to 10.0. In solution there are a number of opposing forces that either act to prevent precipitation of the protein (electrostatic repulsion) or promote protein precipitation (van der Waals, hydrophobic, hydrogen bonding).

Table 4.1: Iso-electric point (IEP) of some Plant Proteins and Seeds

Sample type	Isoelectric point	Reference
Linseed protein	3.0–6.0	(Madhusudhan & Singh, 1983)
Defatted linseed meal	3.8–4.6	(Dev et al., 1986)
Pumpkin seed flour	3.0–7.0	(Lazos, 1992)
Rape seed protein isolates	5.0–6.0	(Mahajan & Jha, 1995)
Barinas nut flour	4.5	(Padilla et al., 1996)
Cashew nut protein	5.0	(Queiroga et al., 2001)
Defatted *Erythrina variegate* flour	3.0–4.0	(Jyothirmayi et al., 2006)
African yam bean (*S. stenocarpa*)	5.0	(Eromosele et al., 2008)

At the isoelectric point the net charge on the protein is zero and attractive forces will dominate over repulsive forces and lead to a reduced solubility. At pH values below the isoelectric point the net proteins charge is positive and like charged proteins will repel each other thus improving solubility, When the pH increases, the net charge (negative charges) increases and the solubility will gradually increase (Boulet et al., 2000; Eromosele et al., 2008). At pH 11.0 and pH 12.0 the extractable proteins were 3.49 g/100g protein and 3.43 g/100 g protein (58% and 57% of total DPS protein) respectively which was slightly lower than the extractable protein at the optimum pH of 10.0 of 3.66 g/100 g protein (61% of total DPS protein). Optimum extraction of seed proteins at alkaline pH has been observed before. Abu-Tarboush et al. (1995) found that the highest protein extractability for Karkade seed flour (*Hibiscus sabdariffa*) was at pH 11.0 with the difference between this and our DPS seeds most likely due to composition differences between the samples. The relatively low solubility of DPS proteins in aqueous solution is may due to insoluble proteins or perhaps there was loss of

these proteins during precipitation of extracted protein. One conclusion is that the most of DPS proteins are not water soluble.

4.2.1.2 Effect of Extraction Time on DPS Protein Extracts

The effect of extraction time on extractable protein is shown in Figure 4.2. The percentage of extractable protein (as a percentage of total protein in DPS) increased from 46% at an extraction time of fifteen minutes to a maximum of 57% for an extraction time of sixty minutes. Above an extraction time of 60 minutes the percentage extraction of protein becomes constant within statistical limits. It is clear that a long extraction time of more than 60 min had very little influence on the extraction of DPS protein. The slight decrease in average extracted protein for extraction time above 60 min could be due to the possible aggregation of soluble protein with other food components such as phytate (Alli & Baker, 1981) which can be solubilised after a long extraction time.

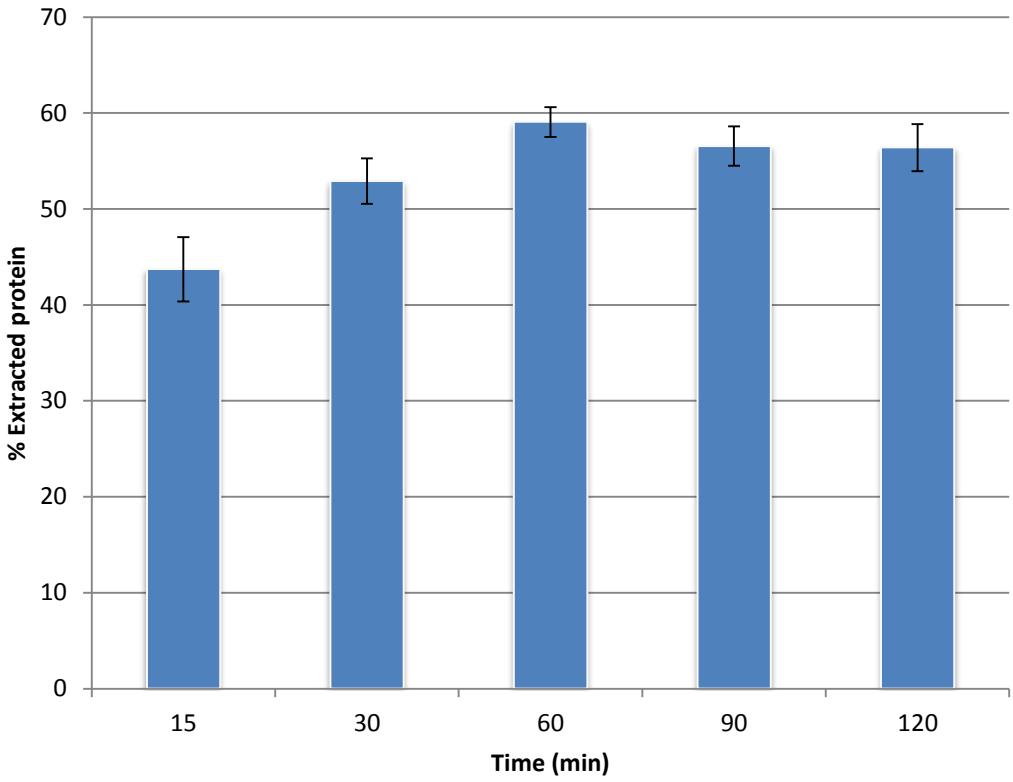

Fig 4.2: Effect of the Extraction Time on DPS Protein Content in DPS Powder.

Extraction under basic conditions with sodium hydroxide is a common extraction method for plant proteins, suggesting a relatively high proportion of acidic proteins in plants. The results from thus study are comparable to

those reported by Narsing Rao et al. (2011) for extraction of a wood apple (*Feronia limonia* L.*)* seed protein concentrate. They concluded that the optimum protein extraction time using NaOH was 60 minutes. The optimum extraction time is not, however, the same for all seed protein containing samples. For example, studies by Dev et al. (1986) and Jyothirmayi et al. (2006) for the extraction of protein from linseed flour and defatted *Erythrina variegate* have observed a maximum extraction at a time of 35 and 45 minutes respectively.

4.2.1.3 Effect of Sample to Solvent ratio on DPS Protein Extracts

The mass: volume ratio between the extraction solvent and the mass of DPS plays a significant role in DPS protein extraction as shown in Figure 4.3.

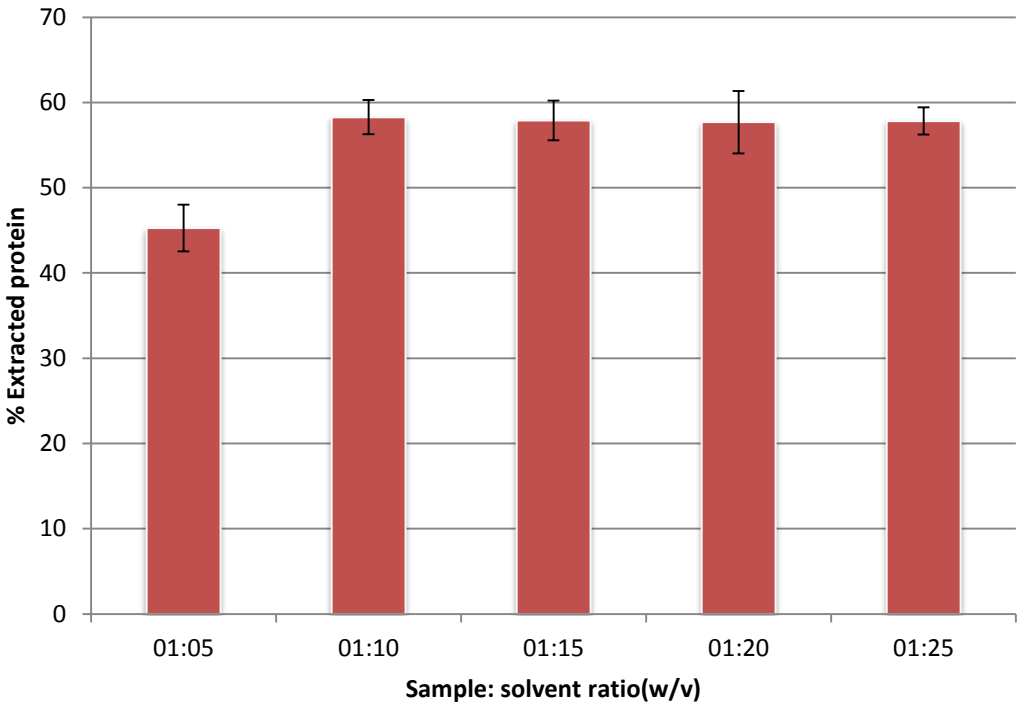

Fig 4.3: Effect of sample to Solvent ratio on DPS Protein Extracts.

The extractable protein increased from 47.2% of total protein to 58.3% of total DPS protein when the DPS: solvent ratio was increased from 1:5 up to a ratio of 1:10 i.e. an increase in extraction of 11%. At the highest mass-volume ratios (1:15 and above) there are no further changes observed in extractable protein. The small decrease in extractable protein observed in Figure 4.3 at the DPS: solvent ratios above 1:10 may be connected with the co-extraction of other ingredients from the powder, which form insoluble complex aggregate with the soluble protein as earlier stated (Eromosele et al., 2008).

For other studies using NaOH extraction, some researchers have reported optimal protein extractability at powder: solvent ratios higher than 1:10. Oomah et al. (1994), observed an optimum powder:solvent ratio of 1:40 for defatted flax seed, Jyothirmayi et al. (2006) an optimum ratio of 1:30 for defatted *Erythrina variegate* flour and Eromosele et al. (2008) an optimum ratio of 1:20 for yam bean flour. Clearly the optimum solvent to powder ratio is specific to the particular powder from which the protein is being extracted.

4.2.1.4 Effect of Solvent Concentration on DPS Protein Extracts

The effect of different alkali concentration (0.01, 0.05, 0.1, 1.0 and 2.0 M) on DPS protein extraction is presented in Figure 4.4.

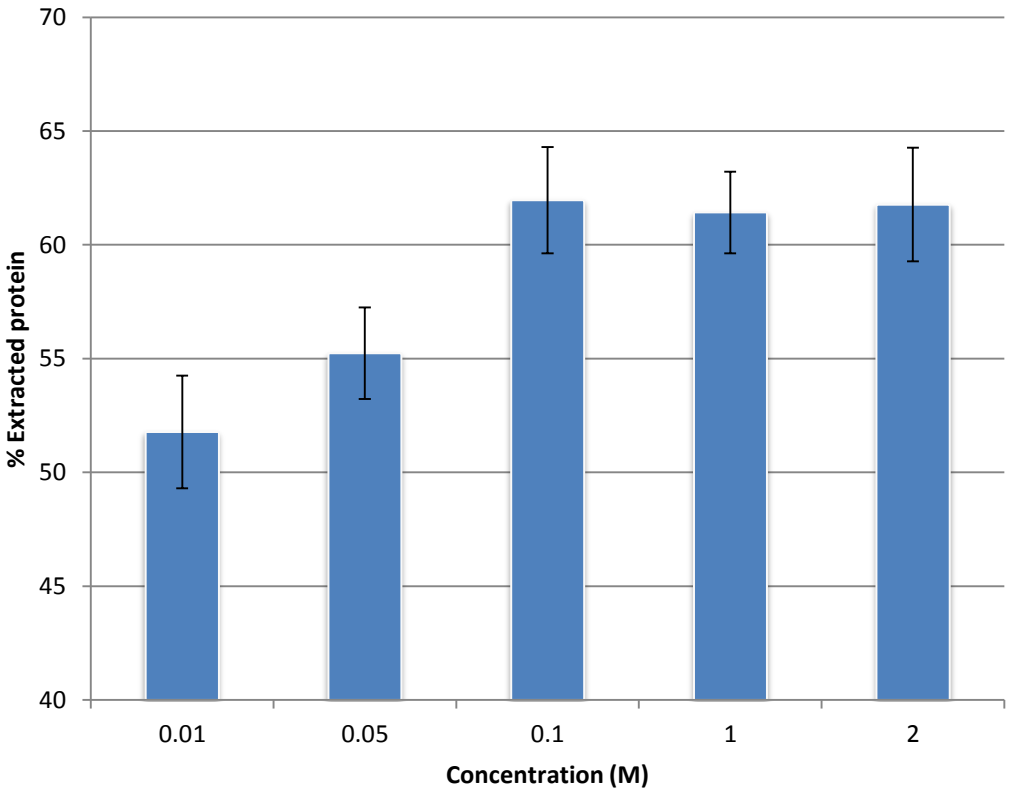

Fig 4.4: Effect of Solvent Concentration on DPS Protein Extracts.

The proportion of protein extracted increases from 57.2 % to 61.5% as the alkali concentration increases from 0.01 M up to 0.1 M. Above a NaOH concentration of 0.1 M the percentage of extracted protein remains constant. However, even with 0.1 M NaOH there are 38.6% of the DPS proteins that remain unextractable. Extraction efficiencies, using 0.1 M NaOH, as high as 81% of the total protein content have been reported by Borght et al. (2006)

for rice endosperm protein. This suggests that DPS proteins are less soluble than rice proteins.

It is possible that higher concentrations of NaOH than 0.1 M could cause protein modification, and that this is contributing to a relatively low yield of protein from the DPS. Shen et al. (2008) report that a higher alkaline concentration is believed to help break hydrogen bonds and to dissociate hydrogen from acidic amino acid side chains and sulphate groups. However, high concentration may also lead to protein modification (Hamad, 1997) and cause disaggregation of the proteins as well as reduction of the molecular size making it less soluble (Borght et al., 2006).

Other researchers have also found that NaOH concentrations around 0.1 M give optimum extraction conditions for some protein powders. For example, Shen et al. (2008) observed that the optimum concentration of alkali for extraction of tea protein was 0.14 M.

4.2.1.5 Effect of Temperature on DPS Protein Extracts

Temperature is one of the extraction conditions that could affect the extractability of DPS protein and therefore it can be used as a variable parameter to optimize the yield of protein extracted from DPS. For most molecules the solubility increases with increasing temperature. For proteins, however, we must be aware that overheating could cause protein denaturation and precipitation which will reduce overall protein recovery in the alkali phase. Thus there is a limitation on the range of temperatures that we would want to use. Figure 4.5 shows that the percentage extractable protein increases from 59% to 63% of total DPS protein as the temperature increases from 25 to 45˚C, and then remained constant above this.

This is a relatively small change in the extracted protein (4%) for a relatively big temperature change (20˚C). This would suggest that it is not worth the extra cost of heating to 45˚C for such a modest change in extracted protein. The optimum extraction temperature for seed proteins differs greatly from seed to seed (Moure et al., 2001).

Shen et al. (2008) reported that 40˚C is a suitable temperature for tea protein extraction; whereas Moure et al. (2001) observed that the highest extraction yield of rose rubiginosa seed protein was obtained at 60˚C. Long periods using high temperature of 60˚C or more could denature the protein

(Kwon et al., 1996 and Massoura et al., 1998). So, for technical reasons, 45˚C would be a suitable choice for DPS protein extraction, although for economic reasons the extra cost of heating may not give a big enough change in protein extraction for it to be justified.

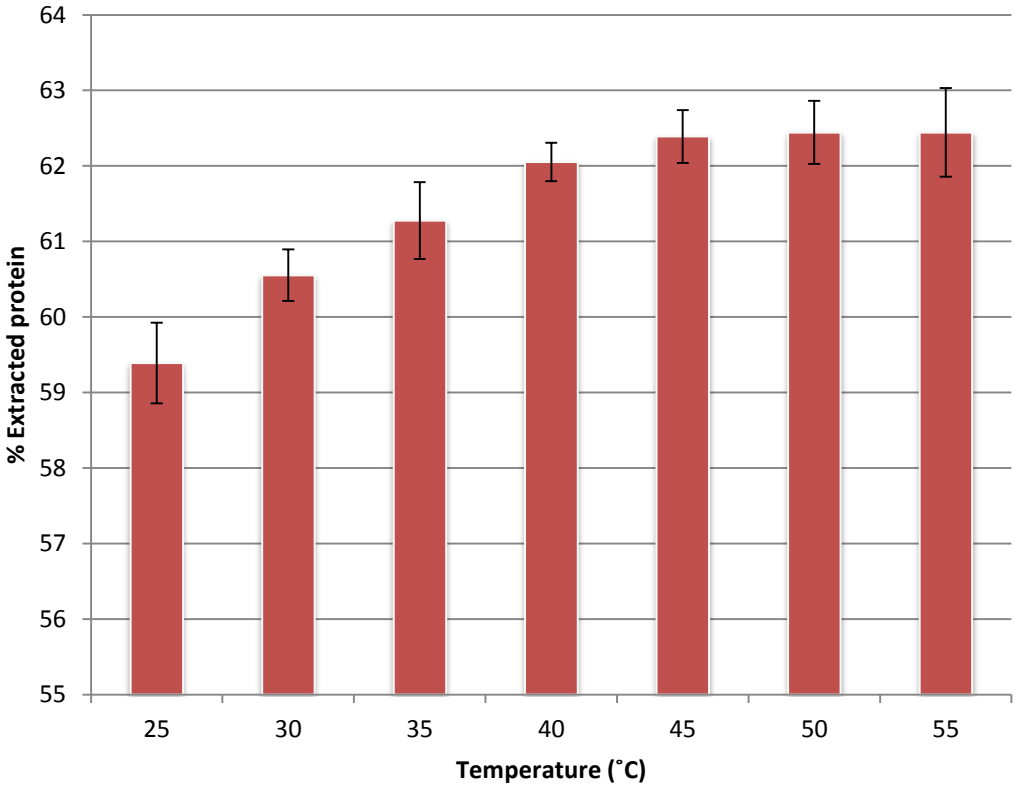

Fig 4.5: Effect of Temperature on DPS Protein Extracts

4.2.2 Improvement of Protein Extraction from DPS by Different Enzymes

Enzymes have been widely used to extract both oil and protein from fruits and seeds (Dominguez et al., 1994 & Rosenthal et al., 1996). The main function of enzymes is to degrade the cell walls of plants (Hanmoungjai et al., 2002) and to achieve this enzymes should associate with components of the cell wall of plant matrix material such as pectin, cellulose, hemicellulose and protein (Christensen, 1991) and hydrolyse them.

Most of the previous studies on protein extraction by enzymatic hydrolysis have been with plant protein sources. These found that cell wall components could be hydrolyzed by enzymes which increased extractable protein (Grossman et al., 1980 & Tang et al., 2003). It is reported that one other efficient means to improve the protein solubility and functionality of oilseed

protein is to subject them to enzymatic hydrolysis with proteolytic enzymes (Delvalle, 1981; Shen, 1992 & Radha et al., 2007). In addition it has been known for some time that hydrolysis of protein improves emulsifying and foaming ability with studies having been carried out on whey protein (Brittenet al., 1994 and Lieske & Konrad, 1996), casein (Slattery & Fitzgerald, 1998), soy protein (Qi et al., 1997; Wu et al., 1998) and gluten (Linares et al., 2000).

Studies on whey protein hydrolysate (WPH) with a degree of hydrolysis (DH) in the range 8−45% found that emulsifying ability was greatest between 10 and 20% DH, and decreased sharply above DH 27% (Agboola, et al., 1998 & Euston et al., 2001). These changes in emulsifying ability as a function of degree of hydrolysis seem to be related to an optimum peptide molecular weight for emulsifying properties (Adler-Nissen, 1978; Lee et al., 1987; Chaplin & Andrews, 1989; Jeon, Byun, Kim, 1999), with peptides of molecular weight greater than 10kDa (Jeon et al., 1999) and more than 20 amino acids in length (Lee et al., 1987; Chaplin & Andrews, 1989) being required for good emulsifying properties.

Most of the seed proteins are stored in protein bodies (Kozlowski & Pallardy, 1997) which are also called protein storage vacuoles (PSV) and range from 0.1 to 25 μm in diameter. These storage proteins can be divided to three types, albumins, globulins, and prolamins, and are sequestered in protein storage vacuoles (Bewley et al., 2013).

The aim of this part of the project is to examine different enzymes for their ability to improve the extraction of protein and to obtain high yield of DPS protein. This includes enzymes that break down complex polysaccharides such as pectinase, cellulase, and mixtures such as Viscozyme and Mannaway, but also protease.

4.2.2.1 Effect of Enzyme Concentration on DPS Protein Extractability

Enzymatic hydrolysis of DDPSP was carried out using the different enzyme preparations detailed in Chapter 2, section 2.8.2 at three different enzyme concentrations of 0.02, 0.05 and 0.1 ml enzyme per g sample of DDPSP. The percentage protein content of DPS obtained using enzyme assisted extraction showed an increasing level of protein extraction as the enzyme concentration was increased at all of the enzyme concentrations (Figure 4.6).

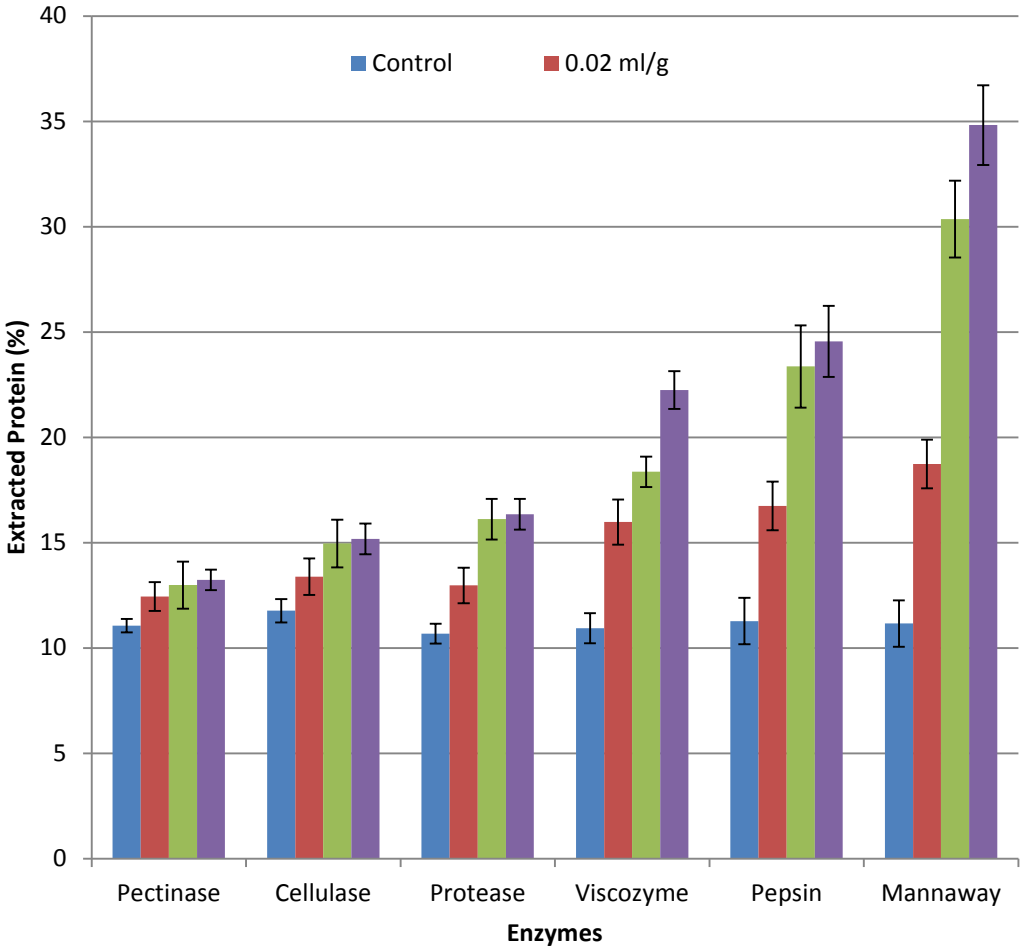

Fig 4.6: Effect of Enzymes Concentration on DPS Protein Extractability.

The highest extracted DPS protein content was observed when the sample was digested with Mannaway which gave protein percentage extractions of 18.74%, 30.36% and 34.82% protein with enzyme concentrations of 0.02, 0.05 and 0.1 ml of enzyme/g sample respectively. The lowest percentage of extracted protein was obtained after using pectinase digestion. At 0.1 ml of enzyme/g sample, the percentage protein extractions were 13.23%, 15.18%, 16.35%, 22.24% and 34.82% for pectinase, cellulase, protease, Viscozyme and Mannaway respectively, compared with 12.44%, 13.37%, 12.96%, 15.97% and 18.74% protein for the same enzymes at an enzyme concentration of 0.02 ml/g. Shen et al. (2008) noted that when using enzymes to extract tea proteins there was an optimum enzyme concentration above which protein extraction yield decreased. They explained this as being due to a possible competitive inhibition mechanism where the products from the hydrolysis reaction act as inhibitors of the enzyme.

It is worth noting that the results in Figure 4.6 do not suggest that inhibition of the enzyme is occurring at high enzyme concentrations, at least for Viscozyme and Mannaway. This suggests that further increases in extracted protein content might be achieved at even higher enzyme concentrations. James et al. (2005) reported that increasing the enzyme concentration generally had a greater effect on reducing hydrolysis time than increasing the temperature. These results were in agreement with Shen et al. (2008) who found that the maximum protein content of powder extracted from tea was obtained at high concentration of enzyme and the protein content increased with increasing enzyme concentration. The results were also in agreement with those of Wang et al., (2008) who reported that peanut protein yield increased as enzyme concentration was increased from 0.5% to 3% (v/w). In addition, Guan & Yao (2008) reported that there was a linear relationship between Viscozyme enzyme concentration and the protein content of extracted oat bran protein.

4.2.2.2 Effect of Hydrolysis Time on Extractability of DPS Protein by Different Enzymes

Figure 4.7 shows the percentage of protein recovered from DPS powder after hydrolysis times of 0 h, 6 h, 12 h, 24 h and 48 h, the temperature and enzymes concentration used here were as listed in Table 2.1 (Section 2.8.2).

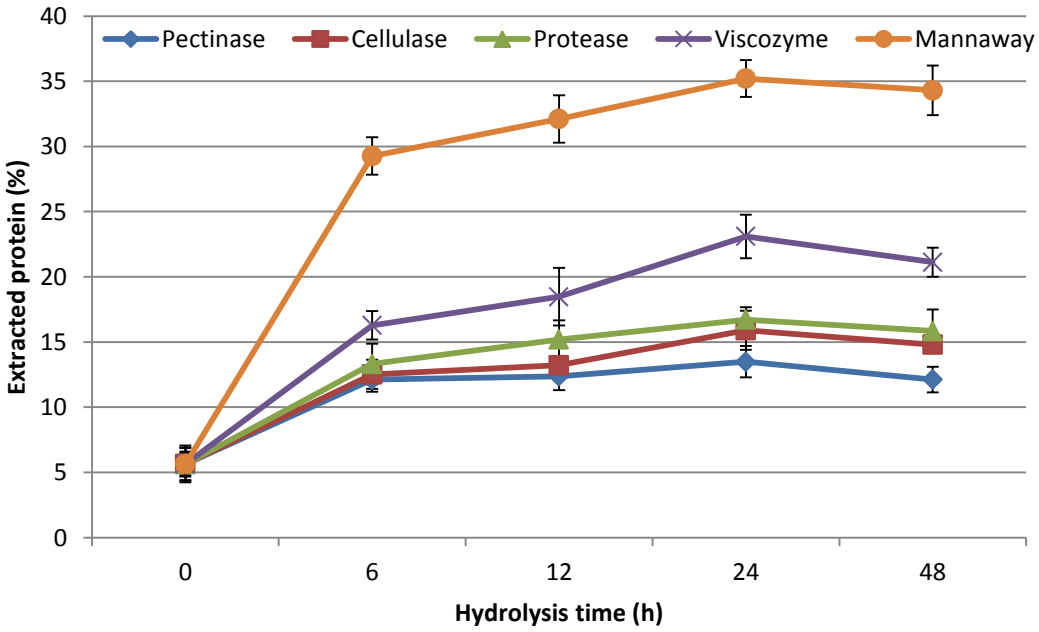

Fig 4.7: Effect of Hydrolysis Time on Extractability of DPS Protein by Different Enzymes.

For all enzyme samples the extractable protein was observed to increase up to a hydrolysis time of 24 hours after which it decreased slightly at 48 hours. During the first 6h, rapid hydrolysis of the DPS was found for all enzymes used. The highest extractable DPS protein was obtained by Mannaway throughout the period of hydrolysis from 0h to 24 h. Hydrolysis using Mannaway gave a protein content of 35.21% at 24 h of hydrolysis which was three times the protein content obtained for the control (11.16%) with no enzyme added. The order of increasing protein content was the same at all times, i.e. Mannaway > Viscozyme > Protease > Cellulase > Pectinase.

The results for protein yield must be interpreted bearing in mind the relative activities of each of the enzymes, although the fact that the protein content levels off at 48 hours suggests that the reaction has reached completion by this stage. Both Mannaway and Pectinase have similar activity (>4000 and >3800 IU/mL respectively). Therefore the results clearly indicate that the DPS sample must contain more mannans than pectin. Buckeridge (2010) has reported that the most abundant polysaccharides present in date palm seed (*Phoenix dactylifera* L.) are mannans.

Previous studies have indicated that the cell wall of DPS is composed mainly of a linear 1,4 β-D-mannan as a storage carbohydrate (Meier, 1958; Meier & Reid, 1982; Aspinall, 1983; Sekhar & Demason, 1988). Viscozyme and cellulase both hydrolyse cellulose and β-glucans, with Viscozyme also active with xylans. The lower activity (>100 IU/mL) but higher extracted protein for Viscozyme compared to the celllulase (>700 IU/mL) may indicate that the xylan content of the DPS powder is relatively high. Protease enzymes are often used to aid extraction of protein from plant materials through partial hydrolysis and solubilisation of the protein. This method was only able to give a modest increase in extracted protein yield, although it was higher than the yield increase achieved with pectinase and cellulose.

4.2.2.3 Enzymes Hydrolysis with Individual Enzyme: Extracted Protein Content and Protein Yield

The effect of the different enzymes on the protein content of the freeze dried powder after extraction, and the yield of protein (protein obtained as a percentage of the total protein in the DPS powder) is presented in Figure 4.8.

For each of these experiments the enzymes loading was 0.1 ml enzyme/g sample and the samples were held at a temperature in the range 45˚C to 50˚C depending in the optimum of the particular enzyme type as shown in Table 2.1 (Section 2.8.2) for 24 hours before the soluble protein was separated and freeze dried. Results in Figure 4.8 indicate that the protein powder with the highest protein content was obtained after extraction with Mannaway (protein content of 31.46% compared to the control with 11.35% protein).

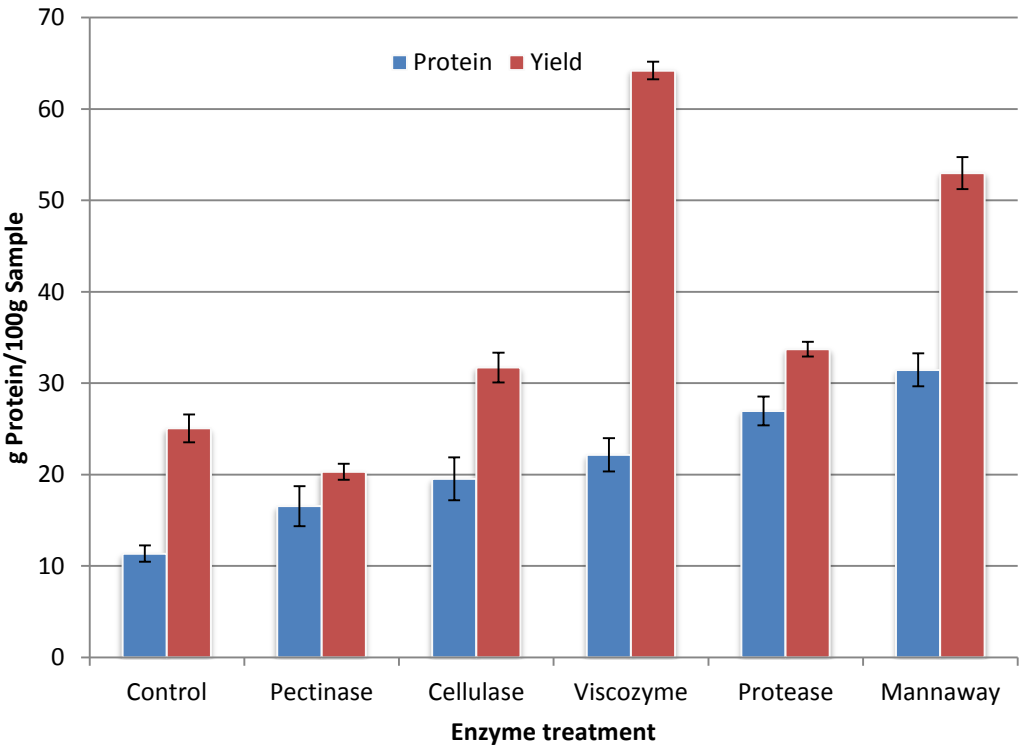

Fig 4.8: Improvement of Protein Extraction from DPS by Enzymes.

The yield of the protein obtained after Mannaway extraction is above 50% (52.99%), but is not as high as that obtained after hydrolysis with Viscozyme which allowed extraction of 64.21% of the total proteins. The higher yield of protein after treatment with Viscozyme compared to Mannaway could suggest that the protein in date seed is more closely associated with cellulose, β-glucans and xylans rather than the mannans. Sekhar & Demason (1988) have found that 75% of the protein in date palm seeds is found in the cotyledon parenchyma cells (part of the embryo), whilst only 17% is found in the endosperm. Since mannans are found mainly in the endosperm, this may explain why there are differences in the quality and yield of proteins

extracted with the Viscozyme and Mannaway enzymes. Hydrolysis of the mannans in the endosperm with Mannaway will expose protein containing material from the cotyledon, but will not breakdown the polysaccharides in the cotyledon other than the relatively low levels of mannans.

The lower yield of protein when extracted with Mannaway may be because some of the protein is associated with cotyledon polysaccharides, which tend to be the less soluble cell wall material, and this protein will not be partitioned into the aqueous extraction phase. When hydrolysed with Viscozyme any protein associated with the cotyledon polysaccharides will become more soluble and will be more readily extracted. However, the mannans which will not be hydrolysed by Viscozyme and some will be extracted into the protein containing aqueous phase leading to a higher yield of protein, but at a lower overall protein concentration in the final dried powder. The results of this study clearly indicate that pretreatment of DPS with certain enzymes greatly enhances protein extractability.

Our results are comparable with previous studies of enhancing the protein extraction from rice bran using Viscozyme where more than 50% of the protein was extracted under optimum conditions of pH, incubation time, temperature and enzyme concentration (Ansharullah et al., 1997). Grossman et al. (1980) found that pectinase and hemicellulase improved the protein extraction from buckwheat bran whilst Guan and Yao, (2008) found that Viscozyme enhanced protein extraction from oat bran and that conditions of pH 4.6 and temperature 44˚C gave the highest protein content in the powder of 55.7%.

4.2.2.4 Enzymes Hydrolysis of Protein with Enzymes Mixtures

Since individual enzyme preparations were able to enhance the extraction of protein from DPS, mixtures of two or more of the enzymes were tested to see if this gave further enhancement of protein recovery. Figure 4.9 shows the results for the binary mixtures Mannaway + Viscozyme, pectinase + cellulose, and the quaternary mixture Mannaway + Viscozyme + pectinase + cellulase.

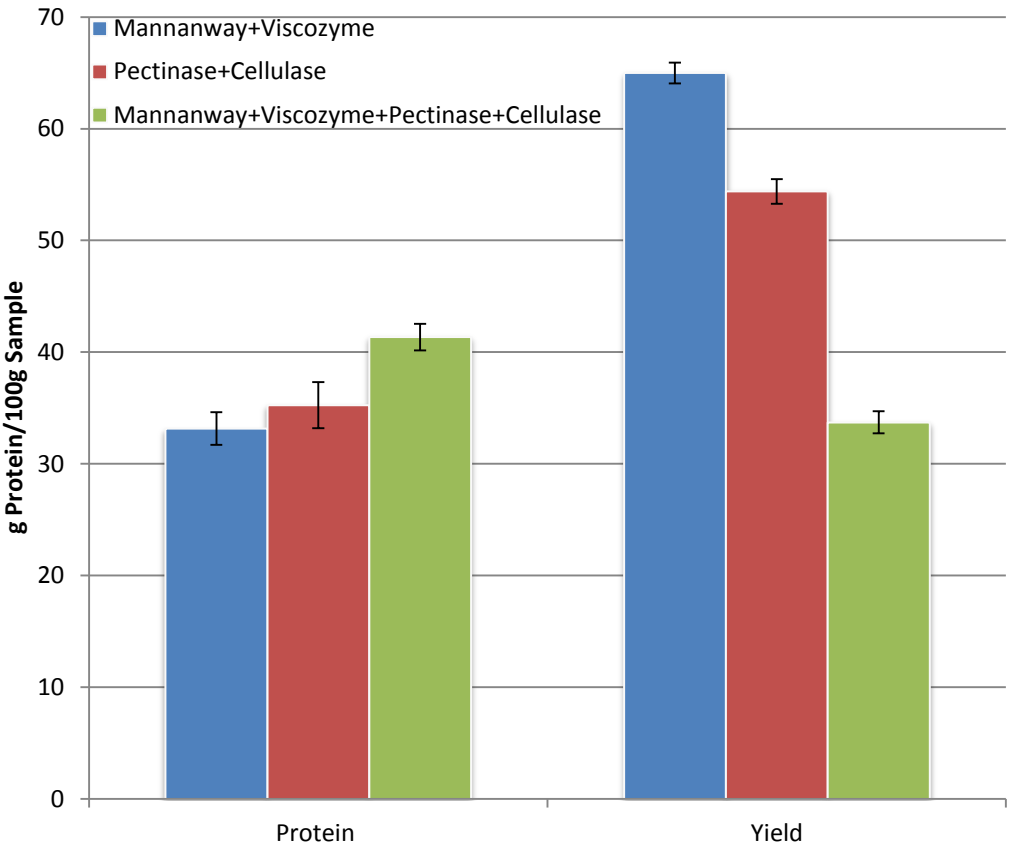

Fig 4.9: Effect of Mixtures Enzymes Hydrolysis on DPS Protein Yield and Protein Content of the Powder.

When Mannaway and Viscozyme are combined to extract protein from DPS the yield of protein was the same as when only Viscozyme was used and the protein content of the final powder was the same as when Mannaway alone is used. Combined pectinase and cellulase gave a large increase in both the protein yield and the protein content of the dried powder compared with using the individual enzymes on their own. When all four enzymes were used together, however, the protein yield was low compared with the binary combinations of the enzymes, although the protein content of the powder was the highest of all the enzyme extraction techniques.

Our findings indicate that extraction rate of DPS using combined enzymes is often lower than when individual enzymes are used. It is likely that when the enzymes are combined competitive inhibition leads to a loss of activity of one or other or both of the enzymes and consequently decreases the hydrolysis rate and protein yield. Previous work by Hanmoungiai et al.

(2002) who found that the combined of Alcalase enzyme with carbohydrase (Celluclast, Hemicellulase and Viscozyme) to extract rice bran protein gave lower protein yield than using Alcalase alone. Norsker et al. (1999) indicated that a combination of different enzymes to isolate the insoluble potato dietary fibre by enzymatic degradation shows an inhibitory effect on each other and consequently decrease the hydrolysis rate and thereby the yield.

4.2.3 Laboratory Preparation of Date Palm Seed Protein Extract

In Figure 4.10 the results for percentage protein and yield of protein from DPS for different non-enzymic extraction methods are shown.

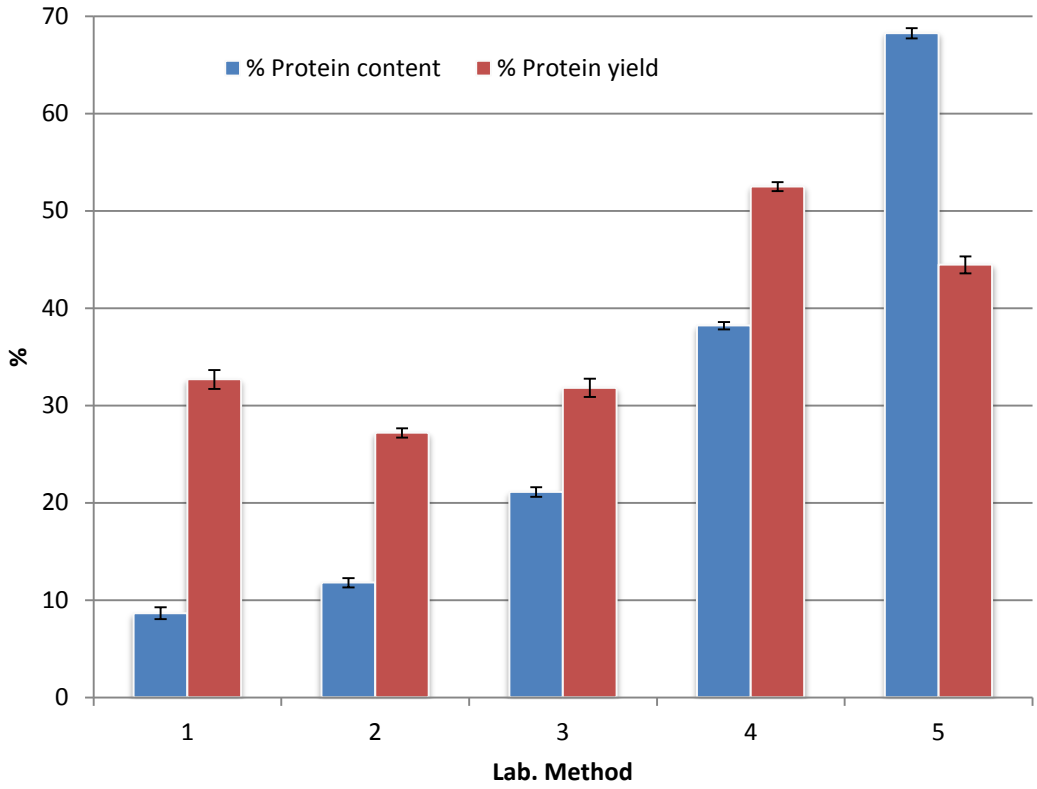

Fig 4.10: Protein content and protein yield obtained with different extraction methods.

The protein content of the dried powder prepared using these methods increased from Method 1 to Method 5 (8.65% to 68.24%). The highest protein content of the prepared powder was obtained using Ph/TCA with acetone precipitation (method 5) while the lowest protein extract was obtained by acid extraction (method 1). The yield of protein does not follow the same order as the protein content of the final powder. In particular, although the protein content of the powder is low after extraction with HCl

alone (method 1) the yield is higher than that for methods 2 and 3 which give higher protein contents in the powder. One possible explanation is that method 1 also extracts non-protein components as well as protein and this reduces the overall powder protein content. It was also notable that the highest yield of protein was not obtained with the phenol/TCA (Method 5) extraction method which gave the highest powder protein content.

The highest protein yield was obtained by using tangential crossflow filtration after alkaline extraction (method 4) whereas using just the alkaline extraction (method 2) gave the lowest protein yield. These differences are likely to be due to extraction of polysaccharides or other sugars along with the protein. It cannot be ruled out that proteins are conjugated with DPS cell wall polysaccharides, and that this leads to co-extraction of the protein and polysaccharide.

It is clear that using tangential crossflow filtration in method 4 increases the protein content and yield markedly compared with method 2 which uses the same extraction procedure except ultrfiltration is not carried out.

Tangential crossflow filtration in this case was through a 10 kDa membrane which is too small to allow polysaccharides to be removed in the filtrate but will remove monosaccharaides and small oligosaccharides.

The increase in protein content from method 2 to method 4 can be explained by the removal of contaminating sugar molecules during filtration, but this does not explain why the yield increases as well. Tangential crossflow filtration is clearly of use in preparing a date seed protein powder of high protein quality and it has the potential to be used as part of a method for protein recovery from date palm seeds which does not rely on non-food grade chemical extraction such as with phenol/TCA. However, because the Ph/TCA method gave the highest quality of protein in the final powder (68.24% w/w) this powder was used for the proteomic analysis of date seed protein in Chapter 5 and the functional property testing in Chapter 6.

4.2.4 Laboratory Preparation of Date Palm Flesh (DPF) Protein Extract

Protein extracted from DPF was presented in Figure 4.11 as obtained by four different lab methods (6, 7, 8 and 9, Chapter 2, Sections 2.11.1−2.11.4).

The highest protein content in the dried powder was obtained using method 9 which involved alkaline extraction, adjustment of the pH to 4.5 followed by tangential crossflow filtration.

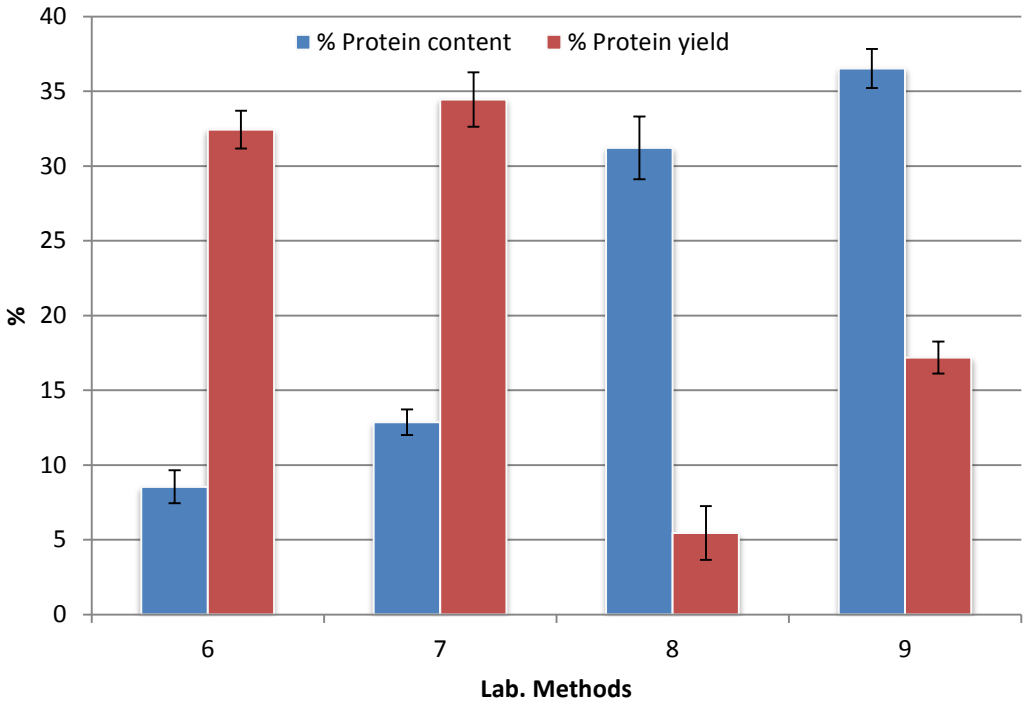

Fig 4.11: Extraction methods of date palm flesh protein.

The lowest protein content was obtained using method 6 where the date fruit was extracted with PBS buffer alone. Method 9 gave relatively high protein content in the powder but a low yield of protein, whereas the yield from method 9 was high, but the protein content of the powder low. This suggests that method 9 selectively solubilises protein, but not all of it, whilst method 6 solubilises more of the protein but also a high proportion of non-protein components of the flesh. The protein content of the final powder was increased when hot water was used to extract soluble sugars before extraction of the protein with PBS (method 7). This method gave the highest protein yield. The lowest protein yield was obtained with method 8 where hydrolysis of the flesh polysaccharides was carried out with cellulase and pectinase prior to alkaline extraction in 0.1 M NaOH. It is clear that use of pectinase and cellulose enzymes to hydrolyse polysaccharides is efficient to extract the protein from DPF but with low protein yield. This could be due to the low levels of pectin and cellulose in date palm flesh which is considered the substrate of both enzymes. This agrees with previous reports which indicate that the flesh of date palm fruit at Tamar stage consists of about 60% sugar, 25% water with the remainder pectin, cellulose, ash and vitamins (FAO, 1992 & El-Sharnouby et al., 2009).

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CHAPTER FIVE

Separation and Identification of Date Palm Seed Proteins

5.1 Introduction

Date palm (*Phoenix dactylifera* L.) is one of the major fruit crops produced in dry and semidry regions. It is an important commercial crop in different regions of the world (Manickavasagan et al., 2012) and is considered the third most important palm species in the global agricultural industry after coconut and oil palms (Gomez-Vidal et al., 2009). Date by product in the form of seeds is available in large quantities as a waste after processing of the fruit. Date palm seed contains 5−7% protein (Aldhaheri et al., 2004), but very little is known about the composition, or the functional properties of the seed protein. In this chapter we have analysed the proteome of the date seed using various techniques. Analysis of proteins using proteomic tools is a multistep process that usually includes protein extraction, separation, fractionation and mass spectrometry analysis (Thiellement et al., 2007). SDS-PAGE of proteins is a powerful tool for identification of genetic diversity in plant species (Javid et al., 2004; Iqbal et al., 2005 & Ehsanpour et al., 2010). The seed protein composition can be used for distinguishing cultivars of a particular crop species (Jha & Ohri, 1996; Seferoglua et al., 2006) and the identification of species can be done by SDS-PAGE as a reliable method (Gepts, 1989).

The seed storage protein diversity of wheat has been reported by (Khan et al., 2002) as well as the identification of three wheat genotypes achieved by protein markers (Zeb et al., 2006). There are many proteomic tools that can be applied for accurate detection and identification of proteins such as liquid chromatography mass spectrometry, two dimensional polyacrylamide gel electrophoresis, and Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometry. Limited information is available in the literature on date palm seed protein composition using these tools. The most critical step for 2D analysis studies of date palm seed protein is the preparation of samples. This is due to the presence of interfering compounds (non-protein materials) such as polysaccharides, salts, nucleic acids, polyphenols and pigments. Removal of those compounds that can interfere in electrophoretic separation becomes a crucial matter for sample preparation.

To avoid interference from other components of the seed, denaturation, reduction and rehydration/solublization of the proteins should be achieved in order to ensure that each spot in the gel represents an individual polypeptide

(Gomez-Vidal, 2008). In this work, we have used a combination of wet chemical methods, gel electrophoresis (SDS-PAGE electrophoresis and 2D gel electrophoresis) and proteomics techniques (LCMSMS) to characterize the proteins in date palm seed samples.

Originally soluble proteins were classified into groups according to their solubility in water (albumins), dilute salt solutions (globulins), aqueous alcohols (prolamins) and dilute acid or alkali (glutelins) (Osborne, 1924; Gottsehalk, 1983). More recently, after analysis of plant storage protein genes and the structure of the proteins themselves these groups have been reclassified to include two major groups the globulins and prolamins, where the albumins have been included in the globulins and the glutelins in the prolamin fraction (Shewry & Tatham, 1990).

The albumins are a group of proteins found mainly in animals and in some plant seeds. They are water soluble, have low molecular weight, contain a high proportion of acidic amino acids, are globular and denature upon heating. Globulins are soluble in salt solution or water and generally of higher molecular weight than the albumins. Examples of plant globulins are vicillin and legumin from pea (Sanchez-Monge et al., 2004), and the 7S and 11S storage proteins of legumes (Tandang et al., 2010). The aqueous solubility of the albumins and globulins has led to their being classified in the same group (globulins).

Prolamins are a superfamily of plant proteins that are rich in proline and glycine. They contain a characteristic α-helical bundle and a conserved motif of three or four disulphide bonds (Mills et al., 2004).

Examples of prolamins are the gliadins and glutenins, indulines, non-specifc lipid transfer proteins and 2S albumin seed storage proteins.

Glutelins are a major storage protein in plants and have some similarity to the prolamins which has led to their reclassification into this group. One of the best known glutelins is glutenin which is responsible for some of the baking properties of wheat flour.

Separation and molecular weight profiling of the proteins can be carried out using SDS-PAGE electrophoresis (Figure 5.1). With 2D gel electrophoresis (Figure 5.2), isoelectric focusing (IEF) in which the protein is separated according to their isoelectric point (pI) is carried out in the first dimension followed by SDS-PAGE electrophoresis in order to separate the protein

according to their molecular weight in the second dimension (Shaw and Riederer, 2003). This gives extra information on the both the molecular weight profile and isoelectric profile of the samples.

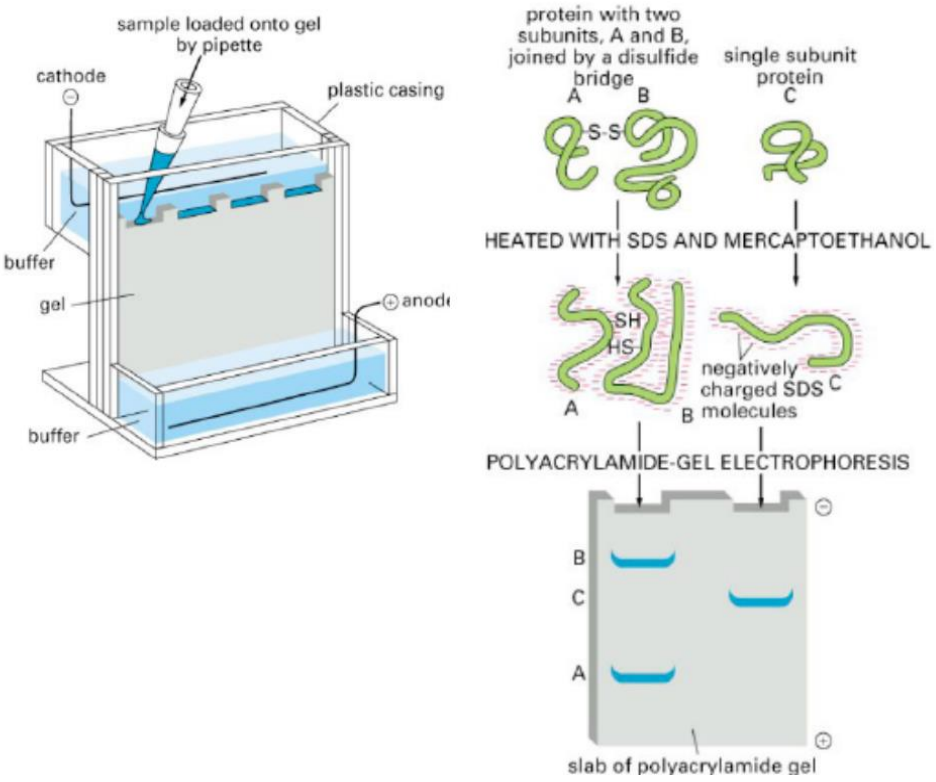

Fig 5.1: Scheme of 1D Electrophoresis Technique (Cavatorta, 2008)

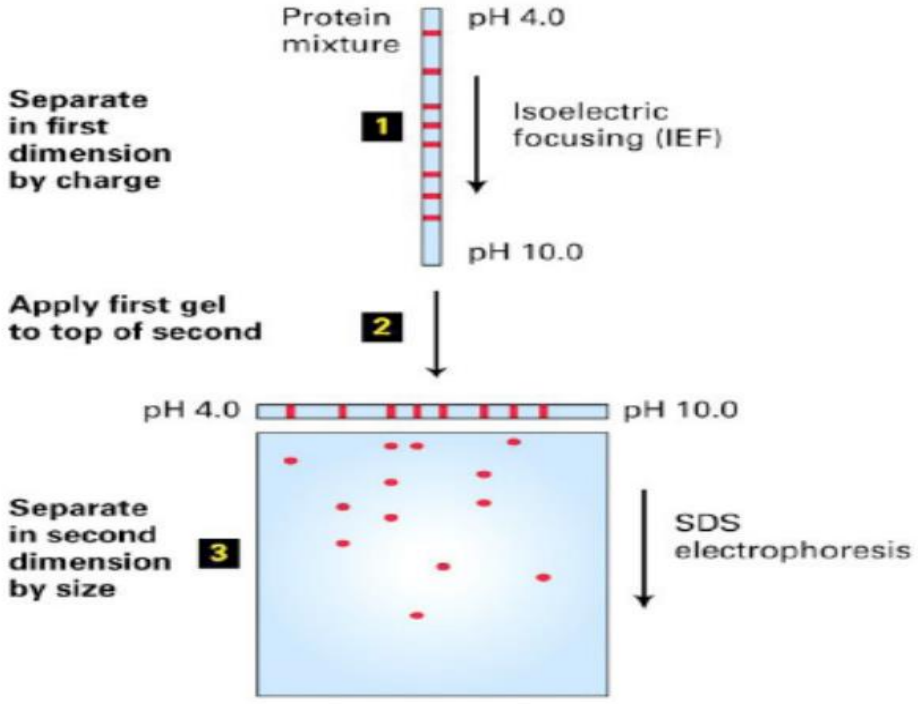

Fig 5.2: Scheme of 2D Electrophoresis Technique (Cavatorta, 2008)

More advanced analysis of the protein samples was carried out using either MALDI-TOF or liquid chromatography tandem mass spectrometry, although only the latter produced useful results. With MALDI-TOF MS initial separation is by 2-D gel electrophoresis and then selected protein spots are subjected to MALDI-TOF mass spectrometry, and characterised using peptide mass fingerprinting in order to identify the proteins in complex mixtures. Limited studies of date palm proteomics have been published to date for *Phoenix dactylifera* L. leaves (Gomez-Vidal et al., 2008), somatic and zygotic embryos (Sghaier-Hammami et al., 2009), date palm sap (Ben-Thabet et al., 2010), and date palm leaf proteome (Dakhlaoui-Dkhil et al., 2013). The 2D gel electrophoresis technique has never applied to date palm seed (*Phoenix dactylifera* L.) protein.

5.2 Results and Discussion

5.2.1 The Soluble Proteins Fractions Extracted from Defatted Date Palm Seed Powder (DDPSP)

In this study we have chosen to use the Osborne (1924) classification of protein type based on solubility in various solvents so that we can compare to other studies of the proteins of date seed (Hamada et al., 2002). Protein content and yield of albumin and globulin, prolamin and glutelin fractions obtained from DDPSP are presented in Table 5.1 along with the residue protein that could not solubilized in such solvents, but could be extracted and solubilized in 1 M NaOH.

Due to the scarcity of soluble proteins of date seeds, the albumins and globulins have been treated together as a salt soluble group. The protein content of albumin and globulin, prolamin and glutelin fractions extracted by Osborne method were 1.27%, 0.95%, and 0.29% respectively, and the residue protein soluble in NaOH was 3.43%. Protein yield of albumin and globulin, prolamin and glutelin fractions were 21.17, 15.82% and 4.83% respectively. The proportion of each of the protein groups varies between date varieties. The albumin and globulin yield in this work was higher than those found by Hamada et al., (2002) for the Lulu variety (19%) but lower than for the Fard and Khalas varieties (24% and 23% respectively). The prolamin and glutelin yields obtained in this work were slightly lower than for Lulu, Fard and Khalas varieties (16%, 16% and 29% for prolamin and 5%, 7% and 6% for

glutelin respectively). More than half (57.16%) of the protein in our date sample was soluble in NaOH which was lower than for the Lulu variety (60%) but higher than Khalas and Fard varieties (42% and 54% respectively) (Hamada et al., 2002).

Table 5.1: Protein Fractions of Date Palm Seed Proteins

protein fractions	Protein content (%)	Yield (%)
Albumin & Globulin	1.27±0.13	21.17±2.06
Prolamin	0.95±0.11	15.82±1.82
Glutelin	0.29±0.08	4.83±1.31
NaOH soluble protein	3.43±0.20	57.16±3.27
Total	5.94±0.52	–

5.2.2 Electrophoresis (SDS-PAGE)

5.2.2.1 Electrophoretic Profile of Date Palm Seed Protein samples

As an initial study of the protein composition of date palm seed using SDS-PAGE electrophoresis was carried out to estimate the molecular weight profile of the major proteins. SDS-PAGE of defatted date palm seed powder(DDPSP), date palm seed isolate (DPSPI), date palm flesh (DPF) and soy protein isolate (SPI) has been carried out under reducing and non-reducing conditions and at different protein loadings. The SDS-PAGE gels are shown in Figures 5.3, 5.5 and 5.7.

To facilitate analysis of the gels and to enable molecular weight determination of sample proteins, a protein molecular weight ladder was included with each gel. Results for the analysis of the molecular weight of the major bands on each gel imaged using either BIO-RAD Molecular imager® ChemiDocTM XRS+ and analysed with gel analyzer software using GelAnalyzer 2010a software (Figures 5.3 and 5.5) or using ImageJ (Figure 5.7) are shown in Figures 5.4, 5.6, 5.8 and 5.9 with the results summarised in Table 5.2. Major protein bands are observed at a number of molecular weights for the date seed proteins. The most abundant protein band occurs at 60 kDa, with minor bands identified at 75, 68−70, 19 and 14 kDa for

DDPSP, and 83, 72, 60−62, 32−34, 25−27 and 18−20 kDa for the DPSPI. Using non-reducing conditions did not alter the protein band profile significantly. This suggests that disulphide bonds are absent from these proteins. It has been reported previously that, in particular the albumins of oil palm seeds do not have disulphide bonds present (Morcillo et al., 1997). The similarity between the protein bands in the crude and purified date seed proteins suggests that all major proteins are extracted during the phenol/TCA extraction procedure used to purify the protein isolate.

Two high molecular weight proteins are observed in one of the gels for the DPSPI (Figure 5.7) which are not seen in the DDPSP. These two proteins will have a relatively low abundance and are only detected when their concentrations are increased by purification. Khoshroo et al. (2011) have reported similar results based on an analysis of seed protein from twelve varieties of date palm (Bazmani sefid (Bw.Ji), Mahminai, Gordial, Kharok, Almehtari, Mordar sang, Kaluteh, Halilehi, Bazmani sefid (Bw.Ba), Mazafati, Khorbak syah, Khosh kang) grown in different Iranian regions. They found one heavily stained band at around 65 kDa and minor bands ranging from 12 to 369 kDa. Bouaziz et al. (2008) found three similar prominent protein bands in date seeds of Allig and Deglet Nour varieties at 32, 60 and 70 kDa, whilst Ehsanpour et al. (2010) have reported slightly lower molecular weight profiles results for four date seed varieties namely Fandoghi, Akbari, Ahmad Aghaei, and Kaleghouchi with the most intensive bands at 45, 33, 32, 27 and 20 kDa.

The differences in protein profile between our results and the previous work (Bouaziz et al., 2008; Ehsanpour et al., 2010 & Koshroo et al., 2011) could be explained by a number of factors. The extraction process used by the other workers differs from our own and this may lead to differential extraction of proteins, Variation between the seed storage proteins is expected within different varieties of the same species. In particular, extensive genetic polymorphism of seed proteins is observed both within the same genotype and among genotypes of the same species. This genetic polymorphism may occur through the presence of multigene families within the same species, or through post-translational glycoslylation of proteins, or proteolytic action on the proteins (Miernyk & Hajduch, 2011; Shewry et al., 1995). Glycosylation, in particular, will lead to several proteins with the same amino acid sequence

but differing molecular weight due to the presence of one or more sugar chains of variable length and position.

Two-dimensional electrophoresis is better at identifying genetic polymorphism since glysoylation is likely to also lead to a change in the charge distribution on the protein and thence the isoelectric point. Finally, the protein composition of the seed varies during the embryo development process, with the major storage protein not appearing until three months after fertilization. Thus, the level of maturity of the date fruit will also influence the protein profile found in the seed. This may partly explain the differences in molecular weight profile for the seeds proteins found in our study and those of Bouaziz et al. (2008), Ehsanpour et al. (2010) and Koshroo et al. (2011).

Purification and characterization of storage proteins in oil palm embryo (the same family as the date palm) has been studied by Morcillo et al. (1997). They identified the major storage proteins as being 2S and 7S globulins. The 2S proteins were made up of two polypeptides (one acidic and one basic) of 22 kDa and 19 kDa molecular weight respectively. The 7S proteins were the major fraction identified using SDS-PAGE. These were shown to be a heterogeneous group of polypeptides of molecular weight between 45 and 65 kDa with no disulphide bonds. They were also found in the form of oligomers with molecular weights of 156 and 201 kDa.

The major protein band in date flesh is found at 30 kDa, with minor bands at 69, 45 and 14 kDa. Ahmed et al. (1995) reported that there are only two prominent bands around 72 kDa and 30 kDa detected when SDS-PAGE electrophoresis is carried out on different varieties of date flesh (Baeneh, Bunaringa, Faedh, Zabad and Khasab). Varieties such as Hilaly and Khalas had a prominent band at 72 kDa while bands around 30 kDa were prominent for the other varieties studied.

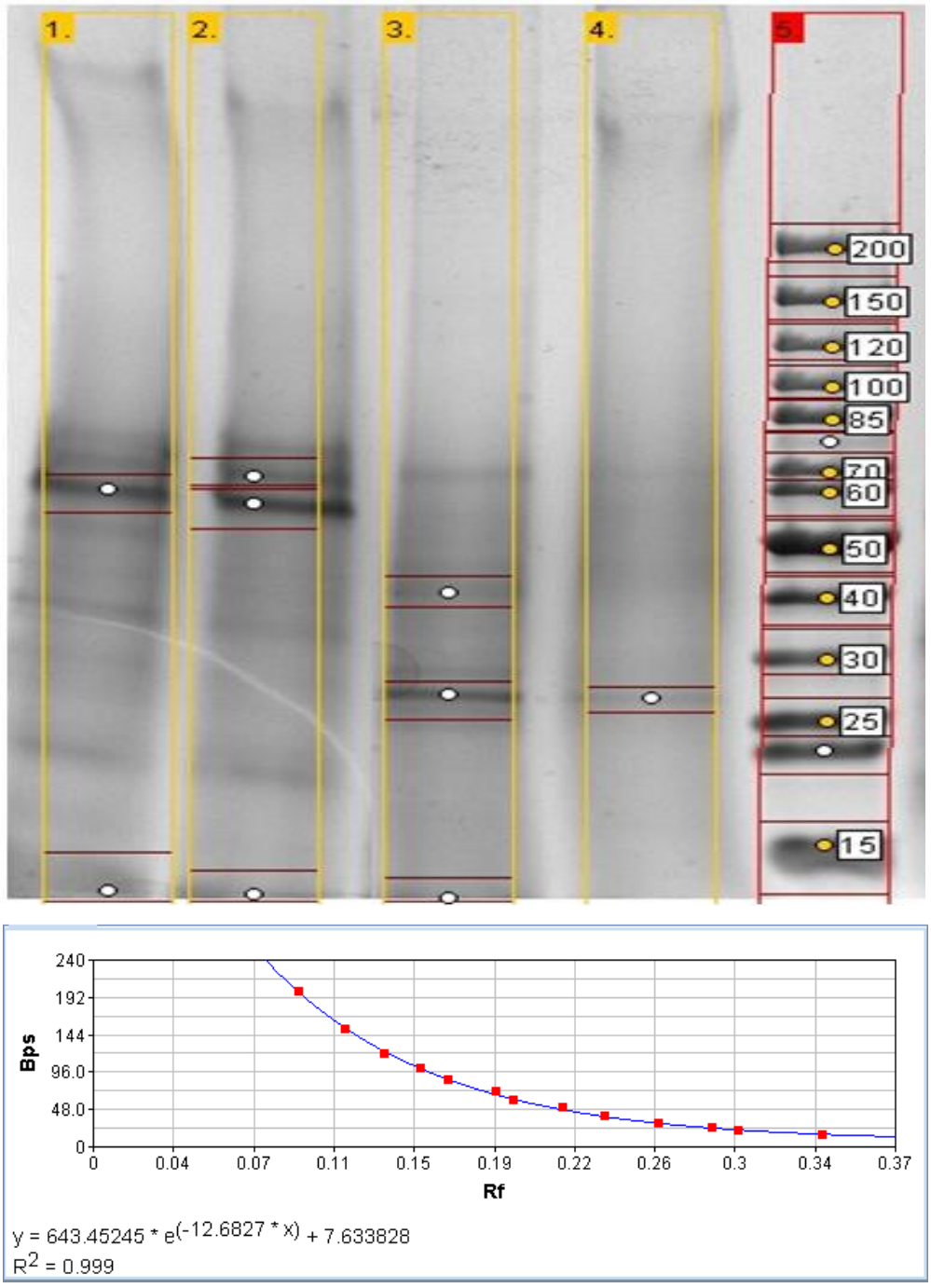

Fig 5.3: Reducing and non-reducing SDS-PAGE of DDPSP (lanes 1 and 2), DPF (lanes 3 and 4) compared with the standard protein (lane 5). Both reducing (lanes 2 and 3) and non-reducing (lanes 1 and 4) conditions were used to prepare the samples.

Table 5.2: Summary of the molecular weight (kDa) of the protein bands identified in reduced SDS-PAGE gels from Figures 5.3, 5.5 and 5.7. Date seed proteins marked with [a] superscript were found in the DDPSP, and those marked with a superscript [b] were found in the DPSPI.

Date Palm Seed	Date Palm Flesh	Soy Protein Isolate
621[b]	75	535
493[b]	68	113
150[b]	60	82
83[b]	45	72
72[b]	30	64
70[a]	19	54
69[a]	14	50
62[b]		41
60[a,b]		36
42[a]		35
34[b]		30
32[b]		22
30[a]		20
27[a,b]		16
25[b]		13
20[b]		
18[b]		
14[a]		

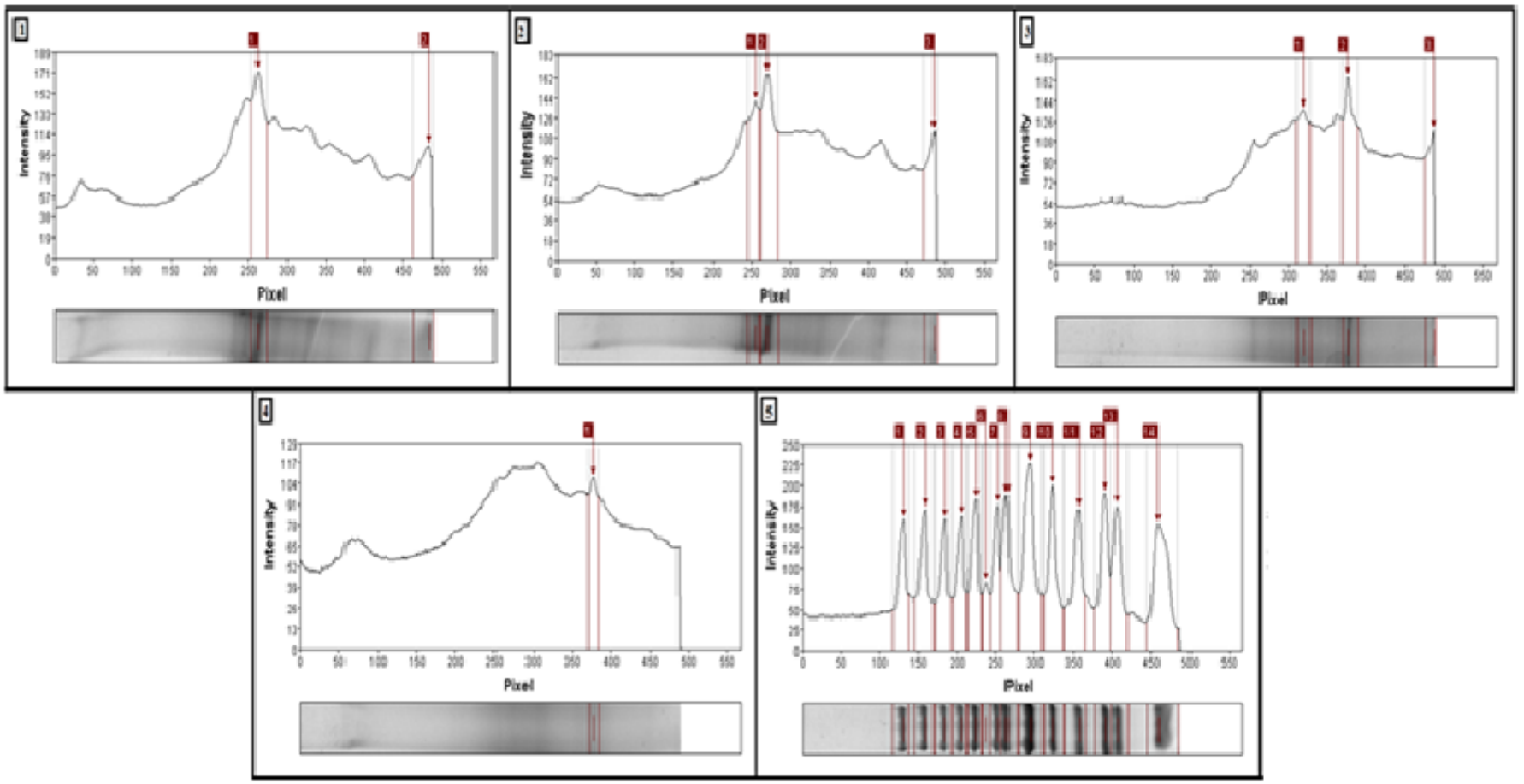

Fig 5.4: Bands intensities analysis of DDPSP, date palm flesh and date palm seed protein isolates compared with the standard protein under reducing and non-reducing SDS-PAGE.

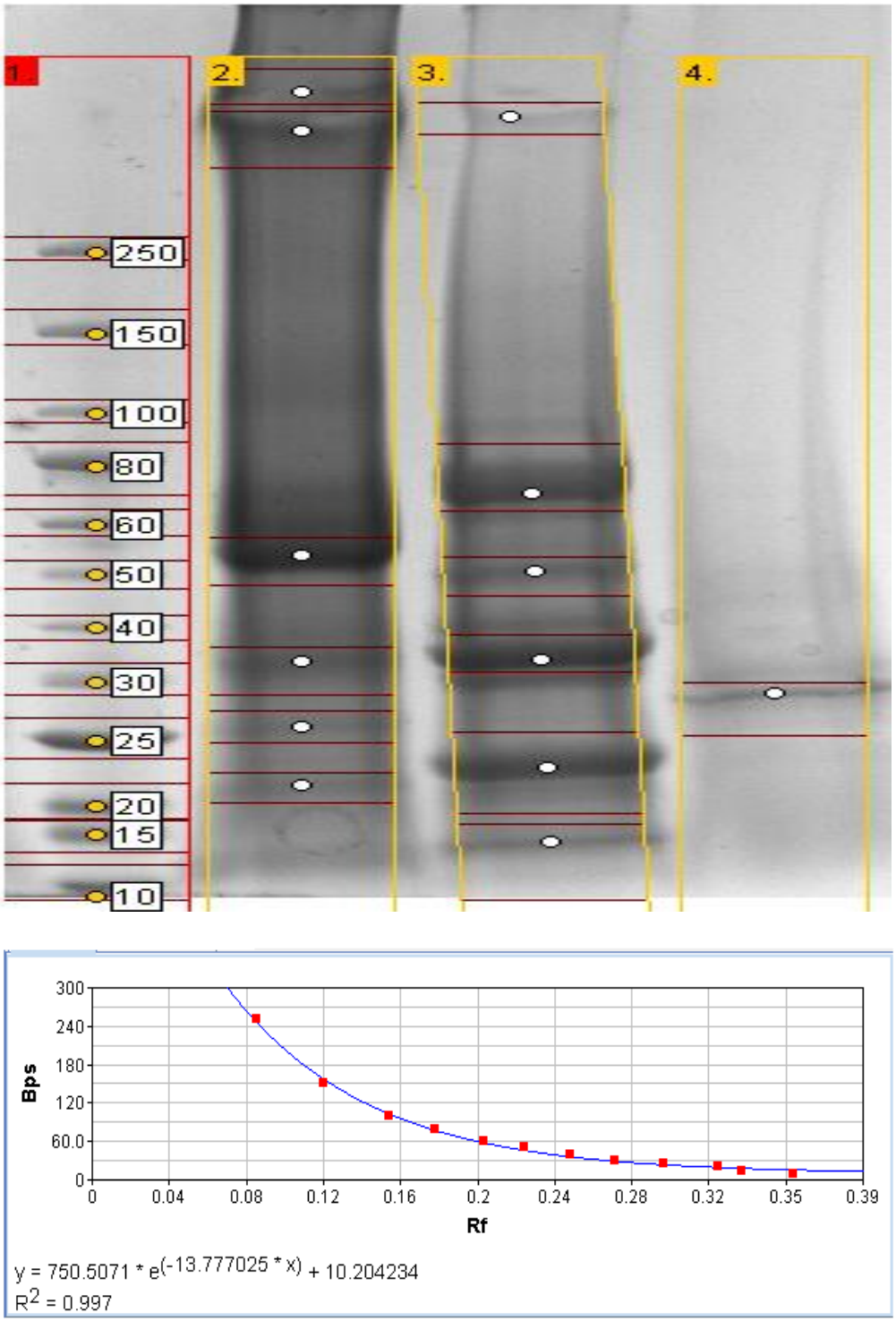

Fig 5.5: SDS-PAGE profiles of DPSPI (lane 2), SPI (lane 3), and DPF (lane 4) proteins compared with the standard protein (protein marker, lane 1).

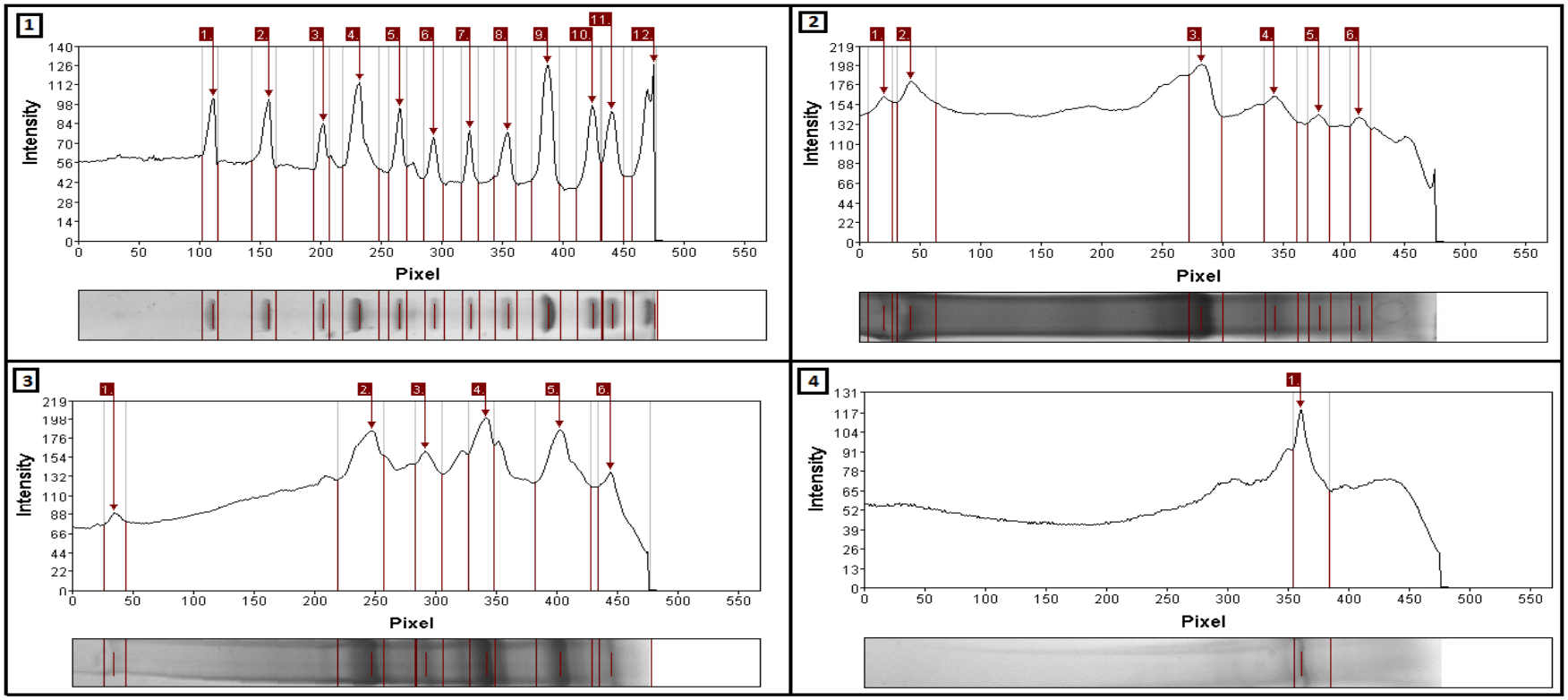

Fig 5.6: Bands intensities analysis of DPSPI, DPF and SPI compared with the standard protein.

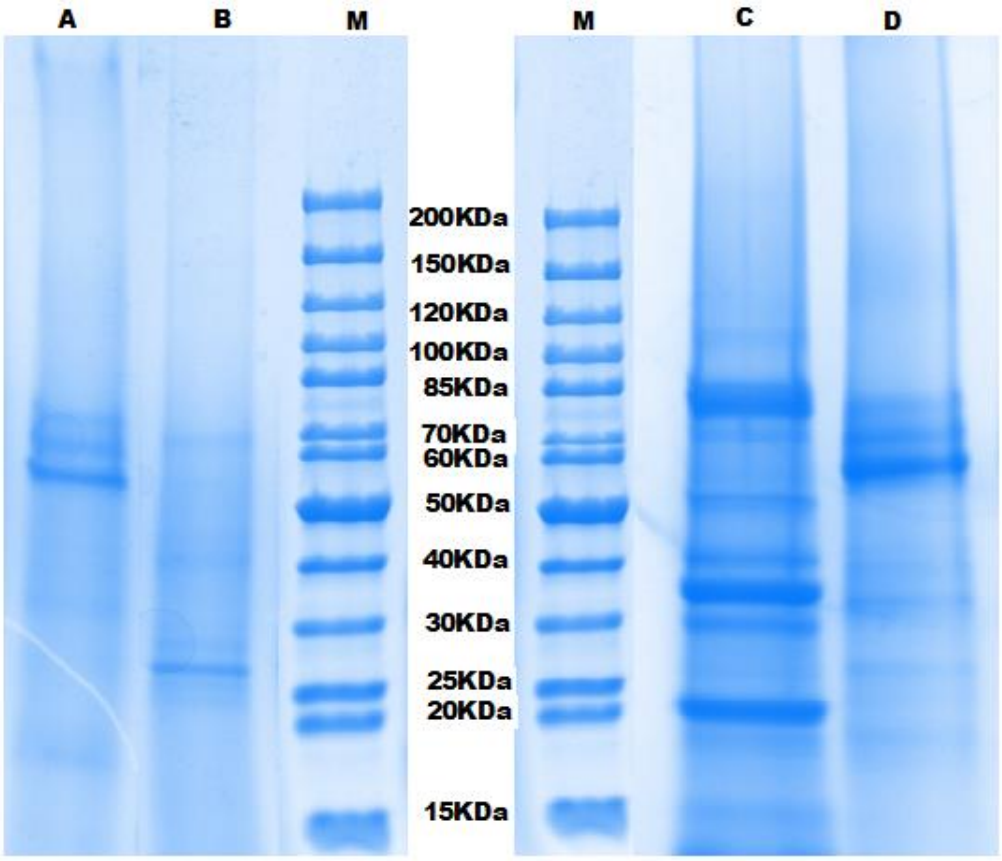

Fig 5.7: SDS-PAGE of DDPSP (A), DPF (B), SPI (C) and DPSPI (D) compared to protein markers (M).

For comparison purpose a soy protein isolate sample was also run on an SDS PAGE gel. There are six intense, detectable bands in lane 3 of Figure 5.5 corresponding to soy protein isolate. These six bands are located at approximately 535, 64, 50, 36, 22 and 16 kDa respectively. These bands might be identified with basic polypeptides of glycinin which have an accepted molecular weight range from 16–22 kDa, acidic polypeptides of glycinin with molecular weight range 34–36 kDa (Abtahi & Aminlari, 1997; Roesch & Corredig, 2005), β-subunit (40–50 kDa) and α-subunit (64 kDa) (Roesch et al, 2005&Chove et al., 2007). The high molecular weight band at 535 kDa could correspond to oligomers of glycinin.

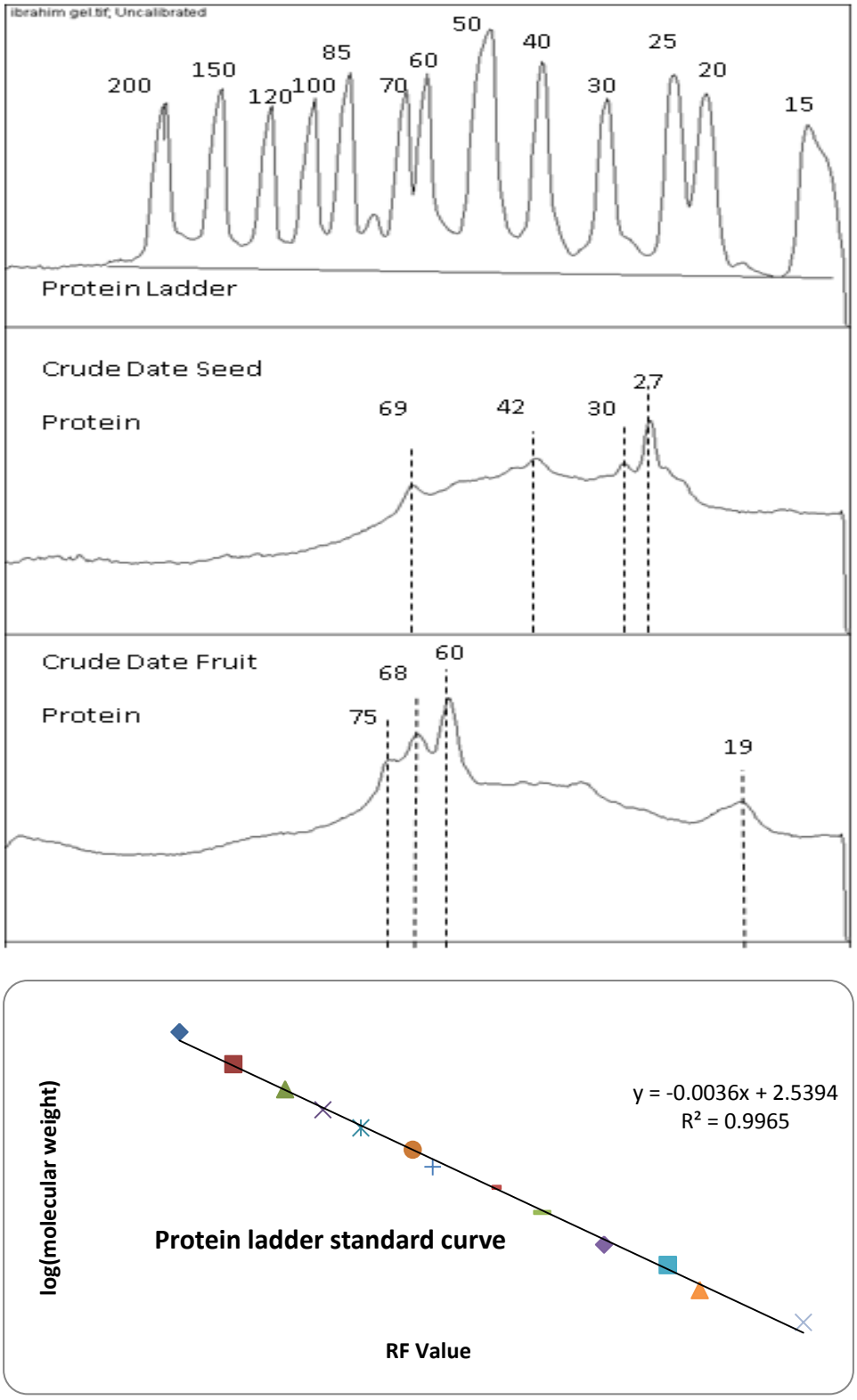

Fig 5.8: Bands Intensities Analysis of DDPSP, DPF compared to protein markers.

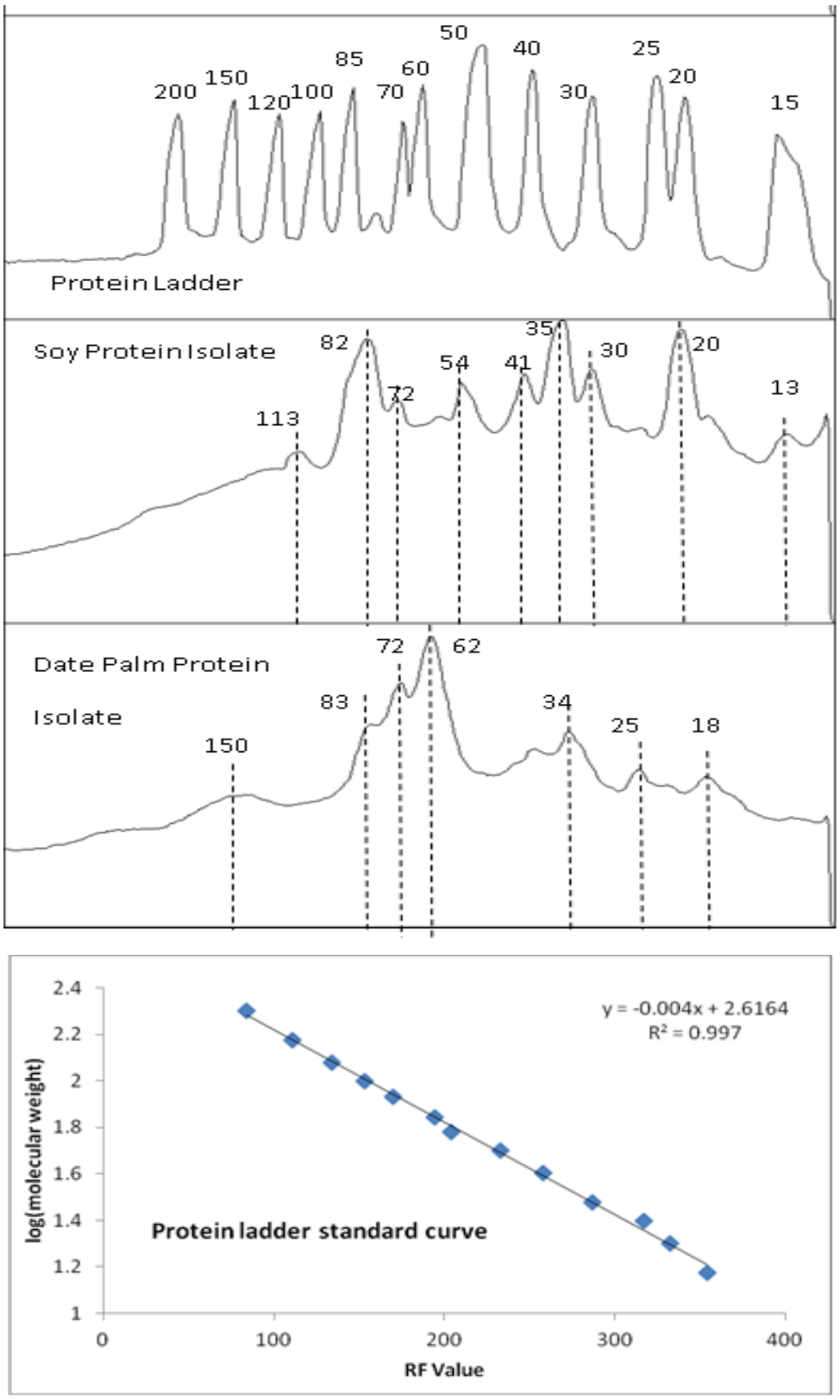

Fig 5.9: Bands Intensities Analysis of DPSPI and SPI compared to protein markers.

5.2.3 Two Dimensional Electrophoresis-Mass Spectrometry

5.2.3.1 Proteomic Map of the Date Palm Seed Proteins

The primary objective of this study was to identify date palm protein using mass spectrometry techniques. From the protein fractionation by SDS-PAGE it was very clear that the date palm seed protein isolates (68.24 %protein) were easier to analyse and gave clearer protein bands than the full fat (5.64% protein) and defatted seed powder (6.13% protein). In order to identify these protein bands, protein powder samples were separated using two dimensional SDS-PAGE as shown in the high resolution 2D gel image in Figures 5.10, 5.11 and 5.12.

It was clear that the date palm seed protein isolates gave a much better quality 2-D gel (Figure 5.12) with a large number of protein spots compared to the defatted (Figure 5.10) and full fat (Figure 5.11) date seed powders after staining with colloidal Coomassie brilliant blue. Under normal circumstances the gel in Figure 5.12 would be ideal for MALDI-TOF mass spectrometry, since the spots are well defined and well separated and could easily be excised from the gel for digestion for mass spectrometry. However, a lot of time and effort was spent in trying to do this with no results. Unfortunately, problems with the MALDI-TOF mass spectrometer in our lab meant that it did not prove possible to analyse a single spot for identification. Due to time constraints on the project a decision was made to stop attempts at MALDI-TOF mass spectrometry, and to send a sample of the date protein for LC-MSMS analysis at the University of Edinburgh, Chemistry Dept. mass spectrometry facility.

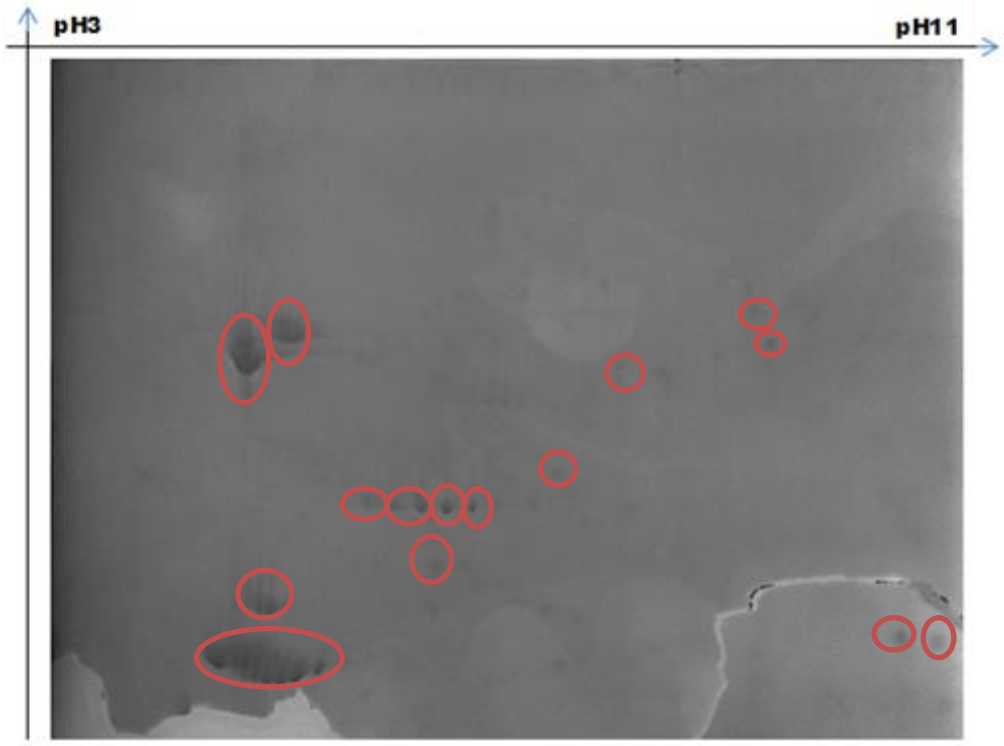

Fig 5.10: Separation of full fat date palm seed powder on a large 24 cm 2D (pH 3-11 NL, 12% (w/v) polyacrylamide gel).

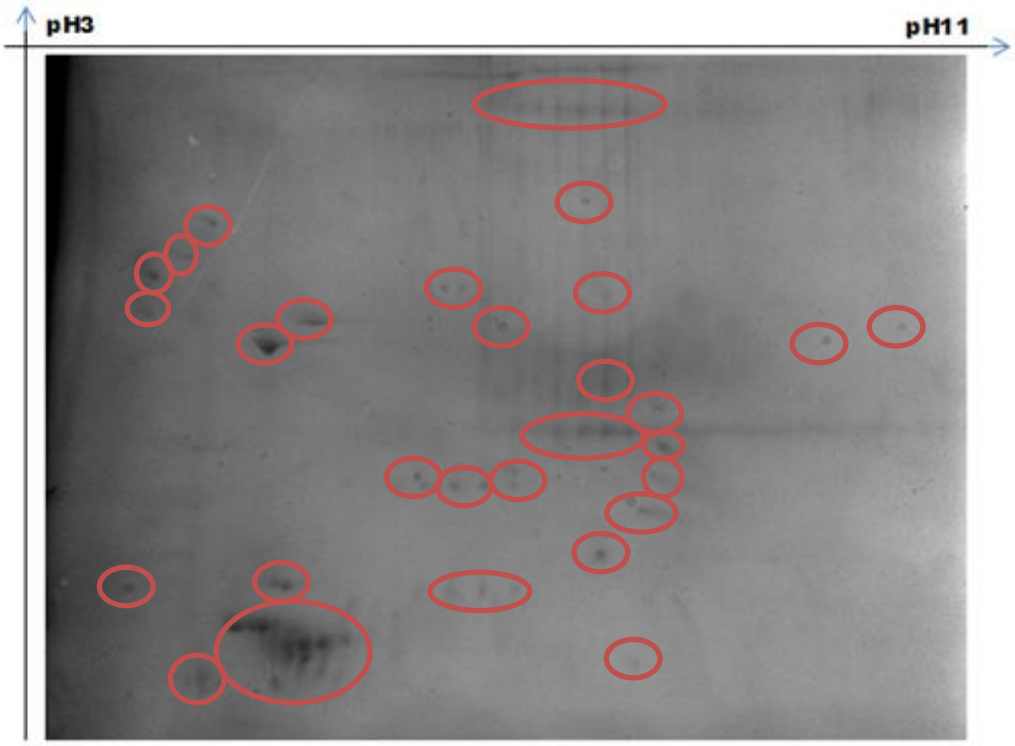

Fig 5.11: Separation of defatted date palm seed powder DDPSP on a large 24 cm 2D (pH 3-11 NL, 12% (w/v) polyacrylamide gel).

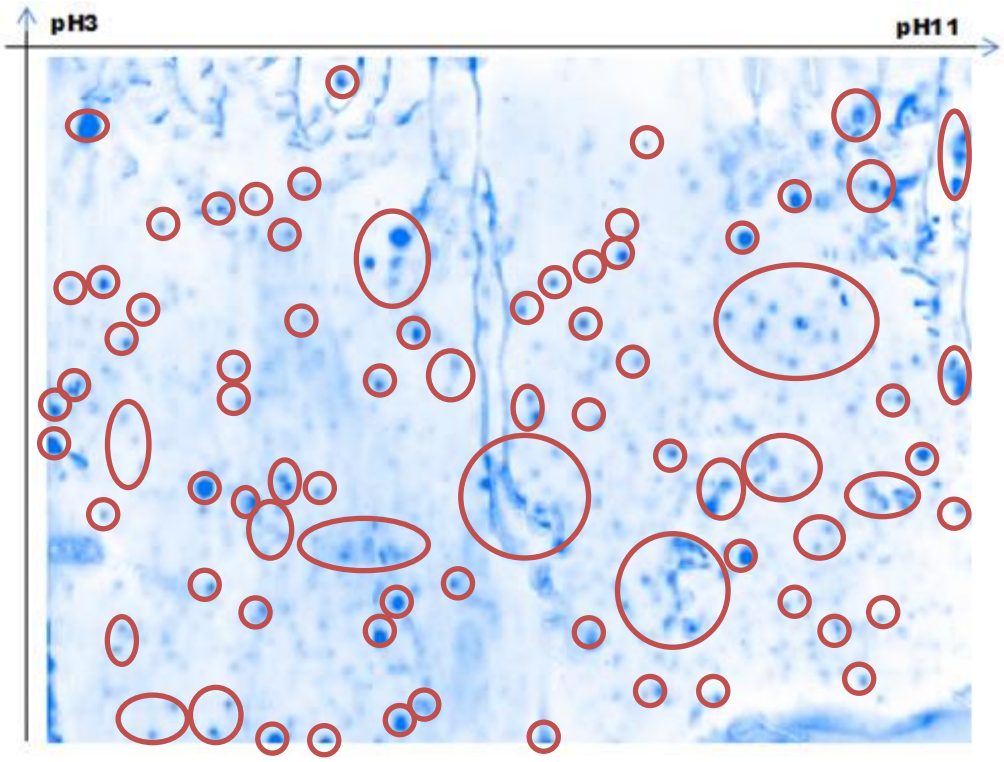

Fig 5.12: Separation of date palm seed protein isolates on a large 24 cm 2D (pH 3-11 NL, 12% (w/v) polyacrylamide gel).

5.2.3.2 Identification of the Date Palm Seed Protein Isolates by LC-MSMS

In this study, LC-MSMS was able to separate a large number of spots obtained from the date palm protein isolates sample. Protein identification was achieved after the MSMS data were compared to known sequences on the NCBI database using Mascot Version 2.4 (Matrix Science Ltd, UK). This search resulted in 318 hits, each of which corresponds to a unique protein. We screened these proteins to remove any hits that we considered probable contaminants (e.g. proteins that the database only identified as being found in humans or animals). For each of the protein hits that remained several candidate proteins were identified, most of which corresponded to the same protein but from different organisms. To determine how accurate the identification of the proteins is we used two criteria, the MOWSE score, and the condition that the identification be based on at least two peptides being matched to the predicted peptide map of the protein. MOWSE (Molecular Weight Search) is a method for identifying proteins based on molecular weight of the peptides formed from proteolytic digestion of the protein

sample. The method was first developed by Pappin et al. (1993). This method calculates the probability that the peptide has been mis-identified during database searching, i.e. the identification is a random event. A low probability of mis-identification is required for correct identification. Since it is more common to express a more accurate identification as a higher number, the probability of mis-identifcation is converted to a MOWSE score using the formula,

$$MOWSE\ Score = -10.\log_{10}(P) \qquad (1)$$

For example, using equation (1), protein identification with a probability of 10^{-10} that it is a misidentification will have a MOWSE score of 100. The probability is calculated based on the number of peptide matches identified for a particular protein match compared to the sequence database using an algorithm detailed by Pappin et al. (1993). To determine whether a particular MOWSE score is significant, a cut-off value is defined based on the assumption that a random event is acceptable if it occurs less than 5% of the time. To calculate the cut-off MOWSE score we need to calculate the probability of a random event across the whole of the protein database that is searched for matches. Currently the NCBI reference database contains 37,371,278 sequences (as of 21st January 2014, http://www.ncbi.nlm.nih.gov/refseq/, accessed 27/2/14). A 5% probability of a random identification is equivalent to 1 in 20 mismatches, so the MOWSE cut-off score will be:

$$MOWSE\ cut-off\ = -10.\log_{10}\left(\frac{1}{20\times 37371278}\right) = 88.7 \qquad (2)$$

Therefore any protein match with a MOWSE score of 89 or greater will have less than a 5% chance of being an incorrect identification. The first 100 hits were considered to have been successfully identified since they all have a MOWSE score of 90 or greater and therefore can be considered to be found in date palm (*Phoenix dactylifera* L.) seed. However, on closer inspection not all of these have been identified as a particular protein, with some being labelled unknown proteins, and some hypothetical but which are nonetheless

in the NCBI database. The results for the first 100 proteins are shown in Table 5.3; the rest of the hits which have a MOWSE score less than 90 have been discarded. The twenty most abundant proteins are listed in a separate table (Table 5.4).

Data listed in Table 5.3 and 5.4 include the hit number (HN), protein description, molecular weight search score (MOWSE score), protein molecular weight (MW) and number of peptide matches compared to total number of peptides produced.

The hit number is a rough indicator of protein abundance in the sample, with a higher hit number indicating a more abundant protein. Since the preparation method for the LC-MSMS requires digestion of the sample with trypsin, fragments of this will always be found in the sample and will always have the hit number 1 (i.e. the most abundant protein). Therefore, this protein is ignored and removed from the results table since it is an added protein. A second protein, keratin, with hit number 59 was also removed from the results list as this was considered to be a contaminant.

The LC-MSMS technique is more efficient than MALDI-TOF for the identification of the proteins although the LC-MSMS method is more complex than MALDI-TOF. Xu et al. (2006) pointed out that the identification of soybean leaf proteins by LC-MSMS produced sixty seven extra hits that were not identified when MALDI-TOF MS was used instead. This was in agreement with results obtained by Lin et al (2005) who indicated that using LC-MSMS technique was more efficient than MALDI-TOF in identification of rice protein where it was possible to identify fifty-four proteins with known function out of seventy seven proteins that were picked up by LC-MSMS.

Table 5.3: Date Palm Seed Proteins Identified by Liquid-chromatography Coupled Mass Spectrometry (LC−MSMS)

HN	Proteins description	MOWSE score	MW (Da)	Protein matched
Functional category 1: metabolism/sugars and polysaccharides/amino acids/Nucleotides/Lipid				
4	lipoxygenase	1001	97490	48/40
10	β-amylase	399	56378	19/15
33	histone H3	223	12290	7/7
36	actin	204	32425	7/6
48	phosphoglycolate phosphatase	162	37420	2/2
62	Nucleoside diphosphate kinase 1	123	16489	6/5
69	Adenosylhomocysteinase; AdoHcyase; S-adenosyl-L-homocysteine hydrolase	113	53769	5/4
80	actin depolymerizing factor 4	103	16368	1/1
81	methionine synthase	102	84401	9/5
84	Glycine-rich RNA-binding protein	100	15894	3/3

Table 5.3: (continued)

93	Acidic endochitinase; Flags: Precursor	95	27933	2/2
99	urease accessory protein UREG	91	30179	1/1
Functional category 2: Energy/ATP synthase/Glycolysis/Electrontransport/Gluconeogenesis/Photosynthesis/Pentose phosphate				
8	Ribulose-1,5-bisphosphate carboxylase	652	53056	48/35
9	AtpB	406	51944	16/14
14	AtpA	324	54044	12/10
16	seed maturation protein	312	17907	13/11
21	Enolase	232	48127	11/7
22	alcohol dehydrogenase 1	127	20101	5/3
25	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	256	37130	10/7
26	phosphoglycerate kinase	246	42294	12/9
28	seed maturation protein	107	26533	2/2
29	chloroplast chlorophyll a/b-binding protein	233	33241	6/6

Table 5.3: (continued)

38	Ribulose-1,5-bisphosphate carboxyase/oxygenase (RuBisCO)	200	19252	6/6
44	PsbB	184	48718	7/7
47	Fructose-bisphosphate aldolase	164	41958	3/3
49	ADP ribosylation factor	141	20710	3/3
50	Fructose-bisphosphate aldolase	143	38599	4/4
53	triosephosphate isomerase	148	27415	5/4
64	Ribulose bisphosphate carboxylase chain 1	120	20288	4/2
65	chloroplast light- protein	117	24199	2/2
71	malate dehydrogenase	111	33823	3/3
72	AtpE	111	15655	3/2
74	cytoplasmic ribosomal protein L18	108	20998	2/2
75	RuBisCO large subunit-binding protein subunit alpha; 60 kDa	108	52461	2/2
77	seed maturation protein	106	38256	2/2

Table 5.3: (continued)

79	Pyruvate, phosphate dikinase, chloroplast	104	100900	4/4
82	Phosphoglucomutase, cytoplasmic	100	63513	4/2
83	chloroplast light-harvesting complex I	101	29559	2/2
85	35 kDa seed maturation protein	99	35320	4/3
88	cytosolic malate dehydrogenase	97	35817	3/2
94	Glyceraldehyde-3-phosphate dehydrogen.	95	35660	8/3
Functional category 4: Transcription/mRNA				
52	EM1 (*Phoenix dactylifera* L.)	149	8746	5/5
Functional category 5:Protein synthesis/Translation factors				
18	protein disulfide isomerase-like protein	308	58963	19/12
61	elongation factor Tu	108	43423	6/2
63	eukaryotic translation elongation factor, putative	122	94981	11/8
68	TufA(elongation factor Tu)	113	44470	8/6

Table 5.3: (continued)

70	eukaryotic initiation factor 4A	112	47187	3/2
78	40S ribosomal protein S4	96	29719	4/3
92	putative 60S acidic ribosomal protein P0	96	34367	2/2
Functional category 6: Protein destination and storage/Storage protein				
2	Glycinin	2436	54927	99/86
3	alpha subunit of beta conglycinin	1624	63184	74/52
11	chloroplast protein precursor LI818R	347	26530	9/8
13	allergen Gly m Bd 28K	328	52780	9/9
17	(heat shock cognate 70 kDa protein 1); ATP binding isoform 1	312	71420	13/10
35	Ca+2-binding EF hand protein	208	27134	6/6
57	16.5 kDa oleosin	141	17463	7/5
58	7S globulin	140	67008	12/6
60	Peptidyl-prolyl cis-trans isomerase 1	136	18395	7/5

Table 5.3: (continued)

76	chaperonin hsp60	106	61654	4/3
Functional category 7: Transporters/Transport ATPases				
5	Sucrose-binding protein	878	60884	42/34
40	Putative spindle disassembly relat. protein	199	90645	10/7
87	Potential calcium-transporting ATPase 11	98	114855	2/2
Functional category 10: Signal transduction				
41	Calmodulin-related protein	199	21214	5/5
Functional category 11:Stress responses/Disease/defence/pathogenesis-related protein				
7	Seed biotin-containing protein	654	67894	24/20
31	dehydrin	227	23774	6/6
39	Late embryogenesis abundant protein (LEA) Protein	200	49484	8/6
55	LEA Protein	143	10408	3/3
73	early flowering protein 1	109	17076	7/5

Table 5.3: (continued)

functional category 12:Unclear classification				
6	unnamed protein product	855	22972	25/24
12	unnamed protein product	341	47117	13/11
19	Unknown protein (from glycine max)	299	43082	5/4
20	Putative histone H2B	284	14338	6/6
24	unknown	259	32097	12/11
27	RuBisCO activase	242	44544	7/7
30	unknown	203	19015	10/10
34	Poly [ADP-ribose] polymerase 3	209	92771	11/8
42	unknown	191	27847	11/7
43	unknown	189	36000	12/9
51	14-3-3 protein	150	30038	4/4
54	unknown	144	16808	7/6

Table 5.3: (continued)

66	unnamed protein product	115	54175	2/2
90	unknown	96	25129	5/4
91	RF12	96	30778	2/2
95	expressed unknown protein	94	145357	13/9
97	predicted protein	93	13235	2/2
100	Calnexin homolog	90	62270	4/3

The one hundred most abundant proteins identified in this study were classified into nine different groups according to their functions using the categories described by Bevan et al. (1998). The different functional group classifications and the percentage of the 100 proteins that fall into these groups are summarised in Figure 5.13. Several of the proteins identified have been reported before and have known functions. It is not practical to discuss the function of all one hundred proteins, so only a selection is discussed below. Those chosen for further discussion are the top twenty most abundant proteins from Table 5.4.

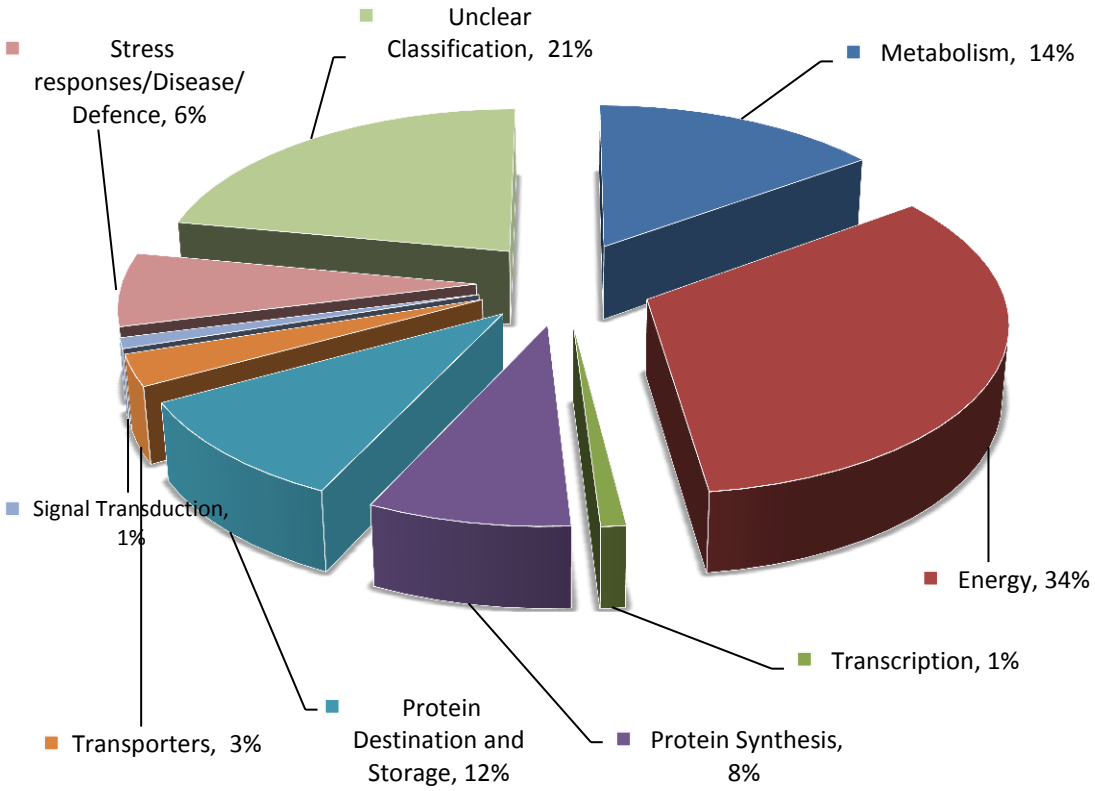

Fig 5.13: The percentage of the identified date seed proteins to functional categories according to Bevan et al. (1998).

Table 5.4: Twenty most Abundant date palm seed proteins identified by Liquid-chromatography coupled mass spectrometry (LC-MSMS).

HN	Proteins description	MOWSE score	MW (Da)	Protein matched
2	Glycinin	2436	54927	99/86
3	alpha subunit of beta conglycinin	1624	63184	74/52
4	lipoxygenase	1001	97490	48/40
5	Sucrose-binding protein	878	60884	42/34
6	unnamed protein product	855	22972	25/24
7	Seed biotin-containing protein	654	67894	24/20
8	ribulose-1,5-bisphosphate carboxy.	652	53056	48/35
9	AtpB	406	51944	16/14
10	beta-amylase	399	56378	19/15
11	chloroplast protein	347	26530	9/8
12	unnamed protein product	341	47117	13/11
13	allergen Gly m Bd 28K	328	52780	9/9
14	AtpA	324	54044	12/10
16	seed maturation protein	312	17907	13/11
17	HSC 70 kDa protein 1	312	71420	13/10
18	protein disulfide isomerase	308	58963	19/12
19	unknown protein	299	43082	16/13
20	putative histone H2B	284	14338	2/2
21	Enolase	232	48127	11/7
22	alcohol dehydrogenase 1	127	20101	5/3

Functional category 1: metabolism/sugars and polysaccharides/amino acids/Nucleotides/Lipid

4 Lipoxygenase

Lipoxygenase is an iron-containing enzyme that catalyses the formation of hydroperoxides in fatty acids that contain a pentadiene segment (Andreou & Feussner, 2009). Lipoxygenases are very common in plants and have been linked to a number of functions such as plant growth, resistance to pests, senescence and responses to damage (Vick & Zimmerman, 1987).

10 β-amylase

This enzyme is found in plant seeds that have starch as the primary storage polysaccharide. In plant seeds it functions to break down starch into maltose when carbohydrate is required for glycolysis during plant growth. β-amylase hydrolyses maltose units from the non-reducing end.

Functional category 2: Energy/ ATP synthase/ Glycolysis/ Electrontransport/ Gluconeogenesis/ Photosynthesis/ Pentose phosphate

Proteins in this category are involved with energy metabolism in the cell, and the high abundance of these proteins reflects the high energy requirements required in a germinating and growing embyo plant.

8 Ribulose-1,5-bisphosphate carboxylase (RuBisCo)

RuBisCo is one of the most abundant proteins on Earth (Feller et al., 2008). RuBisCo is also identified elsewhere in table 5.3 at HN 75. The biological function of RuBisCo is to catalyze two reactions: the carboxylation of D-ribulose 1,5-bisphosphate, the primary event in carbon dioxide fixation and the oxidative fragmentation of the pentose substrate in the photorespiration process. Both reactions occur simultaneously and in competition at the same active site.

9 ATP synthase B subunit

14 ATP synthase A subunit

ATP synthase is an enzyme complex that provides energy in cells through the the synthesis of ATP from ADP (Boyer et al., 1973). The enzyme is composed of two regions, the F1 and F0 regions each of which contain a number of subunits. The A and B subunits are found in the F0 region which in plants is embedded in thylakoid membrane of the cholorplast or in mitiochondria (McCarty, 1992). Here the A and B subunits take part in

proton movement across the membrane, whilst the F1 subunit which sits inside the chloroplast or mitochondrian is involved with the ATP/ADP reaction (Boyer et al., 1973).

16 Seed maturation protein

Seed maturation proteins are part of a group of proteins known as Late embryogenesis accumulating (Lea) proteins (Dure et al., 1989). Some seed maturation proteins are involved in control of embryo progression into seedling growth (Rosenberg & Rinne, 1988) whilst others are involved in seed resistance to dessication and drought (Blackman et al., 1991).

21 Enolase

Enolase, also known as phosphopyruvate hydrolase is an enzyme that catalyses the penulitimate step of the the glycolysis cyle, the conversion of 2-phosphoglycerate to phosphoenolpyruvate (Zhang et al., 1997).

22 Alcohol dehydrogenase 1

Alcohol dehydrogenases occur in many organisms. In plants they catalyse the conversion of ketones or aldehydes to alcohols which also involves the oxidation of the co-factor NADH to NAD (Chang & Meyerowitz, 1986). The primary function of this reaction is to ensure a constant supply of NAD+ for use elsewhere in the cell.

Functional category 4: Transcription/mRNA

52 EM1 (*Phoenix dactylifera* L.)

EM1 was not one of the twenty most abundant proteins, but it was the most abundant in functional category four (Transcription/mRNA synthesis) which represents 1% of the total proteins in the sample. It was also one of the few proteins that was positively identified in the NCBI database as being from *Phoenix dactylifera* L. EM1 (embryogenesis abundant) is one of the stress induced proteins that are expressed in times of drought to protect cells from dehydration stress at the molecular level (Sham & Aly, 2012).

Functional category 5: Protein synthesis/Translation factors

18 Protein disulfide isomerase−like protein

Protein disulfide isomerase (PDI) is known to be a multifunctional protein (Pihlajaniemi, 1991; Noiva and Lennarz, 1992; Wang and Tsou, 1993). PDI or PDI-like proteins are found in the endoplasmic reticulum of cells and their

main function is to ensure the correct folding of proteins by catalysing the formation of disulphide bonds (Yang et al., 2014). Most of the functions for PDI have been identified in animals where it is part of the microsomal triglyceride transfer protein complex (Wetterau et al., 1990), which controls lipid transfer between membranes in the liver; it has been identified as being identical to the β-subunit of prolyl hydroxylase (Vuori et al., 1992) an enzyme that catalyzes the formation of 4-hydroxyproline in collagens; it is identical with thyroid hormone binding protein (Fliegel et al., 1990), is identical to glycosylation site binding protein (La Mantia et al., 1991), and has dehydroascorbate reductase activity (Wells et al., 1990). Whether it has any of these functions in plants is not known. Morjana and Gilbert (1990) found that the isomerase activity of protein disulfide isomerase can be inhibited by many peptides. In plants PDI appears to have a major role in controlling endosperm development through the control of the type and abundance of seed proteins (Kim et al., 2012).

Functional category 6: Protein destination and storage/Storage protein

2 Glycinin

3 Alpha subunit of β-conglycinin

Glycinin and beta conglycinin are the two most abundant proteins identified in our date seed sample. These two proteins are also known to be major storage proteins in most seeds, and in particular in soy beans (Utsumi et al., 1997). The relationship between the molecular and functional properties of glycinin and β-conglycinin subunit has been investigated in several studies (Jiang et al., 2011; Maruyama et al., 2004; Utsumi et al., 1997). It has been found that beta conglycinin has very good emulsifying properties and is a better emulsifier than glycinin (Molina et al., 2001). This is due to β-conglycinin having a larger number of hydrophobic groups with higher molecular flexibility compared to other protein fractions (Bernard et al., 2001). The functional properties of these two proteins will be discussed further in Chapter 6.

11 Chloroplast protein precursor LI818R

Chloroplasts are complex plant organelles (plastids) that perform many essential processes in the plant cell (Keegstra and Cline, 1999). The complexity of function of the chloroplast is illustrated by the fact that the plastid genome encodes 100 proteins (Sugiura, 1989), which can be classified

into six distinct groups found in different compartments of the plastid and which carry out such diverse essential functions as amino acid and fatty acid biosynthesis and photosynthesis.

17 Heat shock cognate 70 kDa protein (HSP70)

Heat shock protein (HSP) 70 was detected in date palm seed, this was not surprising since these proteins are abundant at the late stages of seed maturation in particular embryo maturation, their accumulation caused by mediate desiccation, tolerance acquisition and seed dehydration (Karuna et al., 2000). Heat shock proteins are expressed as a response to high temperature or other environmental stresses. They are named according to their molecular weight (e.g. HSP70 is a 70 kDa protein) (Li & Srivastava, 2004). Heat shock proteins act as molecular chaperones, which are proteins that protect other proteins against denaturation during heating or other denaturing conditions (Ellis & van der Vies, 1991).

Functional category 7: Transporters/Transport ATPases

5 Sucrose-binding protein

When carbohydrate is transferred long distances through the vascular system of a large number of higher plants it is in the form of the disaccharide sucrose (Contim et al., 2003). The cellular control of sucrose uptake across cell membranes is mediated by sucrose-binding protein (Overvoorde et al., 1996). Sucrose-binding protein genes have been isolated from roots, shoots and leaves of spinach (Warmbrodt et al., 1989; 1991), from pea (Contim et al., 2003), from young leaves of the tobacco plant (Pedra et al., 2000), and from soybean (Contim et al., 2003).

Functional category 11: Stress responses/Disease/defence/pathogenesis-related protein

Dakhlaoui-Dkhil et al. (2013) report that 16.6% of the identified proteins of Date Palm (*Phoenix dactylifera* L.) leaf are defence-related proteins which include defence regulated proteins and resistance proteins, those involving detoxification, stress responses, cell rescue and cell death (Bevan et al., 1998).

7 Seed biotin-containing protein

Biotin-containing proteins are found in plants and animals. Biotin is a B group vitamin (vitamin B7) that acts as a coenzyme (coenzyme R) in carboxylase enzymes (Mock, 2006). Plants have been shown to contain five

types of biotin-containing protein (Nikolau et al., 2003). Four of these are enzymes that catalyse carboxylation, decarboxylation or transcarboxylation reactions. A fifth form, seed biotin-containing protein is found in large quantities in seeds, has no enzymic activity and is most likely used to control biotin storage (Nikolau et al., 2003). The recommended amount of biotin for humans is 30 µg per day (Mock et al., 2003). A lack of biotin in food results in changes in the hair and skin in mammals (Hytonen, 2005), followed by emaciation and eventually leading to death (Gyorgy, 1939). Johnson et al. (1980) reported that there is a link between low biotin and infant death. Seed biotinylated protein has been found in the seeds of plants such as pea but is absent from roots, pods, stems, flowers and leaves (Hytonen, 2005). Similar biotin-binding proteins are found in animals and bacteria. Avidin is a biotin-binding glycoprotein found in egg white, while streptavidin is found in bacteria (Hytonen, 2005). These are relatively heat stable proteins with denaturation temperature of 83–85˚C. However, when biotin is bound to avidin or streptavidin the denaturation temperature reaches 188˚C (Gonzalez et al., 1999; Nordlund et al., 2003).

Functional category 12: Unclear classification

6 Unnamed protein product

12 Unnamed protein product (possibly 7S globulin basic subunit)

19 Unknown protein (possibly formate dehydrogenase)

20 Putative histone H2B

The NCBI database of proteins which was searched using Mascot during the analysis of the proteomics results is the largest store of experimentally identified biological macromolecular structures available (Nadzirin & Firdaus-Raih, 2012). However, in this database there are a large number of proteins that have uncharacterized functions (Berman et al., 2002). Unnamed or hypothetical proteins are often those that have been identified based on genome sequencing of an organism, but the protein for which the gene codes has not been identified, named and characterised in the plant or animal. Dakhlaoui-Dkhil et al. (2013) reported that 29.4% of protein detected in date palm leaf was hypothetical protein.

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CHAPTER SIX

Functional Properties of Date Palm Seed Protein

6.1 Introduction

Functional properties of proteins are defined as those properties that determine behaviour of proteins in a food system during production, processing, storage and consumption. Extraction and isolation of proteins from plant seeds is only the first step to integrating these proteins into food products. If they are to be of use as food ingredients they have to have sufficient functionality for them to be used in place of current food proteins such as milk, egg and soy proteins. Studies of the functional properties of new protein sources can provide valuable information on the potential effectiveness of the proteins in food products (Yada, 2004). The important functional properties of proteins in food applications are solubility, swelling and water & fat holding capacity, emulsifying activity and emulsion stability, foaming ability and foam stability and gelling capacity (Zayas, 1997; Yada, 2004).

6.1.1 Solubility

The solubility of proteins is defined as the ability of protein to associate with water. This can be affected by various conditions such as pH, temperature, solvent, ionic strength and the presence of cosolutes such as sugars.

Proteins can be completely or partly soluble or completely insoluble in water (Zayas, 1997). There are a number of stages involved in dissolving a protein powder in water. When the powder is first added to water it must be able to sink into the bulk of the water phase and not sit on the surface of the water. Thus sink ability is important. Secondly, the powder particles must be easily wetted. The phenomenon of wetting relates to how easily water penetrates into the pores of the powder particles.

The wettability influences the final stage which is dissolution where the protein particles disintegrate and soluble protein is released into solution. It is not easy to follow all of these stages experimentally so it is usual to measure solubility as the concentration of protein found in solution and to express this as a percentage of the total protein in the original powder sample added. The solubility of proteins in food grade powders is influenced by the presence of sugars. Sugars can affect the solubility of proteins in a number of ways. In milk powder production the milk sugar lactose crystallises at the surface of powder particles, modifying the surface structure and altering the wettability

of the particles (Thomas et al., 2004). During processing at high temperature (or long term storage at lower temperature) reaction between the reducing end group of sugars and reactive amino groups on lysine side chains can lead to the Maillard browning reaction (as shown in Figure 6.1) which reduces the solubility of the protein. Heat processing in general reduces the solubility of many proteins if they are heated for long enough above their denaturation temperature. In this respect, the presence of sugars in protein powders can be beneficial because they can protect proteins against heat damage (Semenova et al, 2002; Gu et al., 2008).

Sugars act as kosmotropic agents. That is they promote the formation of water-water interactions in solution and therefore strengthen interactions within proteins in solution at the same time. The net effect of this is that sugars increase the temperature at which proteins will denature in solution and therefore have a protective effect during the drying process that is part of protein powder production.

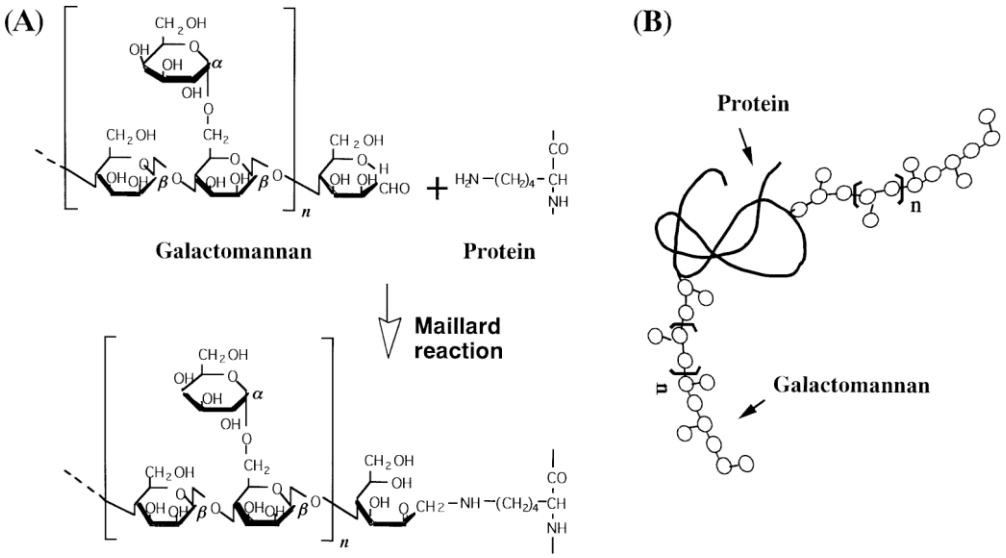

Fig 6.1: Schematic view of the Binding mode of Polysaccharide with Protein through Maillard Reaction (A) and the Resulting Protein-Polysaccharide Conjugate (B) (Kato, 2002).

6.1.2 Emulsifying Ability and Emulsion Stability

Emulsions are two phase systems of liquids (water and oil) with one of the liquids dispersed in the other (Yang & Baldwin, 1995). There are two types of emulsion. One type is where oil droplets are dispersed within an aqueous continuous phase and is known as an oil-in-water emulsion (o/w), with

common food examples being milk and mayonnaise. The second type of emulsion is the water-in-oil emulsions (w/o) where water droplets are dispersed in an oil continuous phase, for example butter and margarine (Dalgleish, 2006; Raikos et al., 2006). The stability of emulsions in foods is crucial to the stability of formulated products, since the presence of free fat in foods (for examples milk beverages) is not desirable to the consumer. There are three main mechanisms by which emulsions destabilise, namely flocculation, creaming and coalescence (McClements, 2005). Coalescence is the irreversible breakdown of an emulsion where two droplets merge to form one. Ultimately, this leads to oil phase separation and product breakdown.

Creaming is when emulsion droplets rise to the top of a container through buoyancy forces. It is driven by the difference in density between oil and water, and is faster with larger droplets. Thus, coalescence speeds up creaming by forming larger droplets. Creaming also speeds up coalescence because the creamed droplets form a layer at the top of the emulsion where droplets are closer together, thus increasing the chance of the coalescing.

Flocculation refers to a weak reversible association of emulsion droplets, where the droplets do not merge together. Although flocculation can be reversed by stirring it does speed up the rate of creaming and coalescence because the associated droplets (or flocs) behave as if they are larger droplets and so cream and coalesce more quickly. Proteins are often used by food manufacturers to aid in the formation and stabilization of emulsified fat in foods. To describe the emulsifying properties of protein in a food system the characteristics used are emulsifying ability and emulsion stability (Zayas, 1997).

Emulsifying ability refers to how good a protein is at forming small emulsion droplets under specific conditions, and most methods for measuring it involve a direct or indirect measure of the particle size of the droplets formed. Emulsion stability refers to how good the protein is at preventing destabilization of the emulsion. Proteins stabilise emulsions by forming an adsorbed layer around the emulsion droplets that prevents coalescence of the droplets. Proteins achieve this by a mechanism called steric stabilization (McClements, 2005). In steric stabilization if two emulsion droplets approach each other and their adsorbed layers overlap, this leads to both an elastic and osmotic repulsion between the droplets. The method used to

measure emulsion stability varies depending on the type of instability that is being measured. A common method is to look at the change in particle size with time, and this is the method we use here based on a protocol originally proposed by Pearce and Kinsella (1978). It is important to realise that emulsifying ability and emulsion stability are highly dependent on various conditions such as pH, temperature, type and size of emulsifiers, food compositions and homogenization pressure (Peamprasart & Chiewchan, 2006) and thus it is only a useful test when the emulsifiers are compared under the same conditions (Carpenter & Saffle, 1964; Tornberg & Hermansson, 1977).

6.1.3 Water Holding Capacity (WHC) and Oil Holding Capacity (OHC)

Water holding capacity (WHC) of foods is defined as the ability to retain water (either added or naturally present) during the application of heating, pressing forces or centrifugation (Kenefel et al., 1991; Zayas, 1997). Generally, there are two types of water held in a protein structure, the water trapped in the protein matrix or a corresponding co-matrix (fat, polysaccharide) and 2) the water explicitly bound to the molecule that is no longer available as a solvent. These are often called bound or unbound (or free) water. The unbound water can be regarded as retained water and the bound as absorbed water (Kneifel et al., 1991).

Water holding capacity is influenced by several conditions such as temperature, pH, protein size and shape, lipid and carbohydrate associated with the protein (Han & Khan, 1990). The structure and amino composition of the protein also has an influence on water binding. The more polar amino acids present in the proteins the more hydrogen bonds can be formed with water and the higher the bound water and WHC. Animal proteins tend to have a higher WHC than plant proteins because they have a higher proportion of amide groups in the proteins (Zayas, 1997). Ionic strength of salts and pH also affect the WHC. At or close to the isoelectric point of a protein, the WHC will be reduced due to loss of charged groups on the protein that would normally hydrogen bond with water. Similarly, increasing ionic strength leads to a screening of charged groups, and a gradual dehydration of the protein, and drop in WHC as the protein charged groups and ions in solution compete for waters of hydration. Fat or oil binding in proteins is likely to

occur through physical entrapment of the lipid in the protein structure, probably associated with hydrophobic regions rather than a specific interaction with amino acid residues, and so hydrophobic or insoluble proteins will have a higher OHC (Zayas, 1997). OHC has been correlated with surface hydrophobicity. OHC is also strongly dependent on the protein content of the powder and a higher quality of protein (greater percentage protein) gives a higher OHC.

6.1.4 Gelation Properties

The gelation properties of proteins are exploited in many food applications. A gel can be defined as a semi-solid structure where liquid is trapped in a continuous protein network of macroscopic dimensions (Oakenfull et al., 1997). The most common way of forming a protein gel is to heat a protein solution so as to denature molecules of the protein. The thermal treatment is required to expose the reactive functional groups of the protein, followed by aggregation of the protein via intermolecular interactions. This involves both bonded and non-bonded interactions, including hydrogen bonding, van der Waals, electrostatic and hydrophobic interactions, and covalent disulfide bond formation. To form a protein gel requires a minimum protein concentration which is usually around 4–5% (w/v) or above. The gelation concentration is itself a function of pH, temperature and ionic strength (Aussanasuwannakul, 2004; Elssa, 2005).

6.1.5 Foaming Ability and Stability

Proteins are used as natural surfactants in many applications where foam production is required (Doucet, 2004). Foams are colloidal dispersions where air phase bubbles are dispersed in a solid or liquid phase (Yang & Baldwin, 1995). Liquid foams consist of air bubbles dispersed in a continuous phase of liquid, whereas solid foams consist of air bubbles dispersed within a continuous solid phase. In the food industry, whipped cream and ice-cream are typical liquid foams, whilst cookies, breads and cakes are examples of solid foams. Solid foams are generally more stable compared to liquid foams. Liquid foams start to destabilise immediately after formation (although this may be relatively slow), whereas, solid foams may have stability for many days. A pure liquid cannot form stable foams unless surface active materials are present. To build the stabilising film of

food foam, proteins are often used as a surface active component to adsorb at the gas liquid interface of the air bubbles (Raikos, 2006; Foegeding et al., 2006). The structure and physicochemical properties of the proteins used to form the foam, including dynamic surface properties of the protein solutions, play an important role in the ability of the foaming agent to create and stabilise foam (van Kalsbeek & Prins, 1999). At the interface, a range of intermolecular interactions is possible including electrostatic, hydrophobic, disulfide bond formation, hydrogen bonding and van der Waals interactions (Prins et al., 1998). The structure of foam consists of tightly packed air bubbles, surrounded and stabilized by protein films (Figure 6.2) (Raikos, 2006).

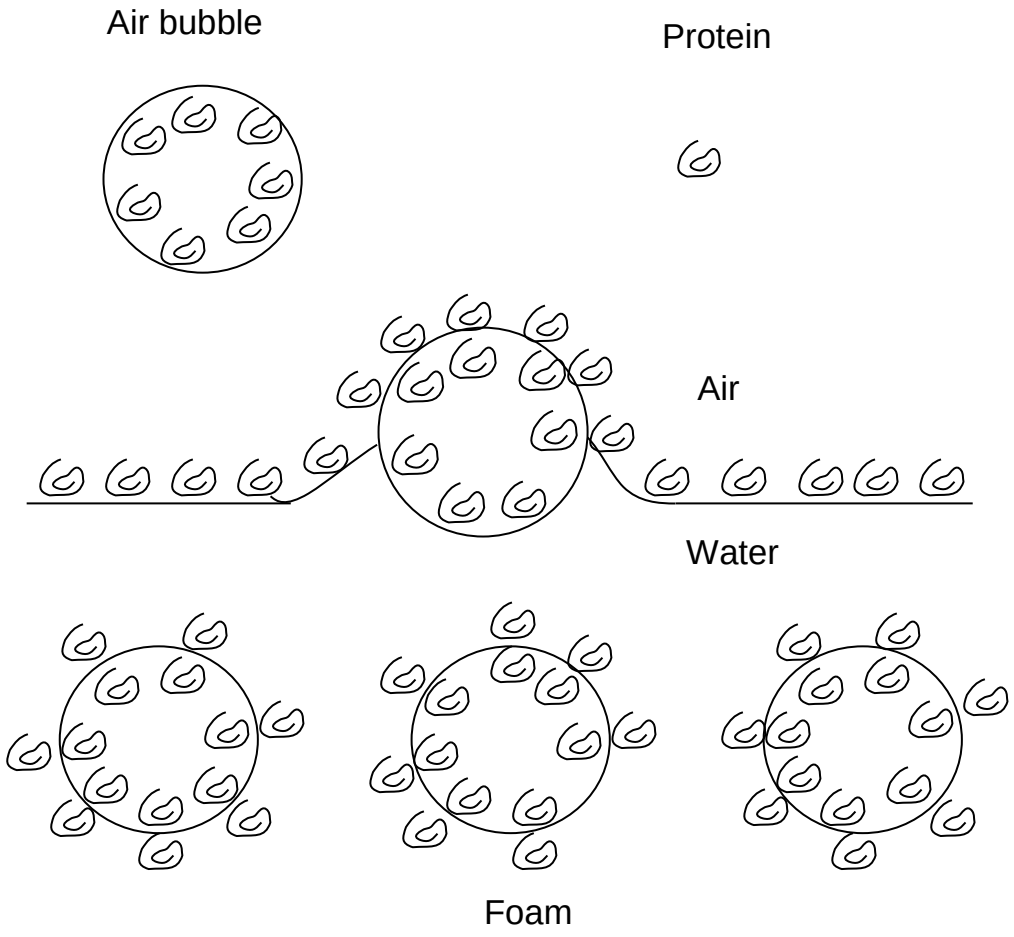

Fig 6.2: Protein-stabilised foam structure (Raikos, 2006).

In the following sections of this Chapter results relating to the functional properties of the extracted date seed proteins are presented and discussed. For comparison, results for whey protein concentrate (WPC) and soy protein isolate (SPI).

6.2 Results and Discussion

Previous results in Chapter 3 indicated that the highest protein content of DPS was obtained by extraction using method 5 (Ph/TCA) so, we used this protein powder to test date seed protein functional properties. For convenience this powder has been called date palm seed powder isolate (DPSPI).

6.2.1 Proximate Composition of Date Palm Seed Protein Isolate (DPSPI)

The proximate composition of DPSPI was determined before testing functionality (Figure 6.3). The main component of the powder was protein at 68.24% (w/w) with the remainder comprising 2.94% moisture, 4.63% total ash, 7.82% crude fibre and the total carbohydrate (calculated by difference) found to be 16.40%. A negligible amount of fat/oil was found in the powder.

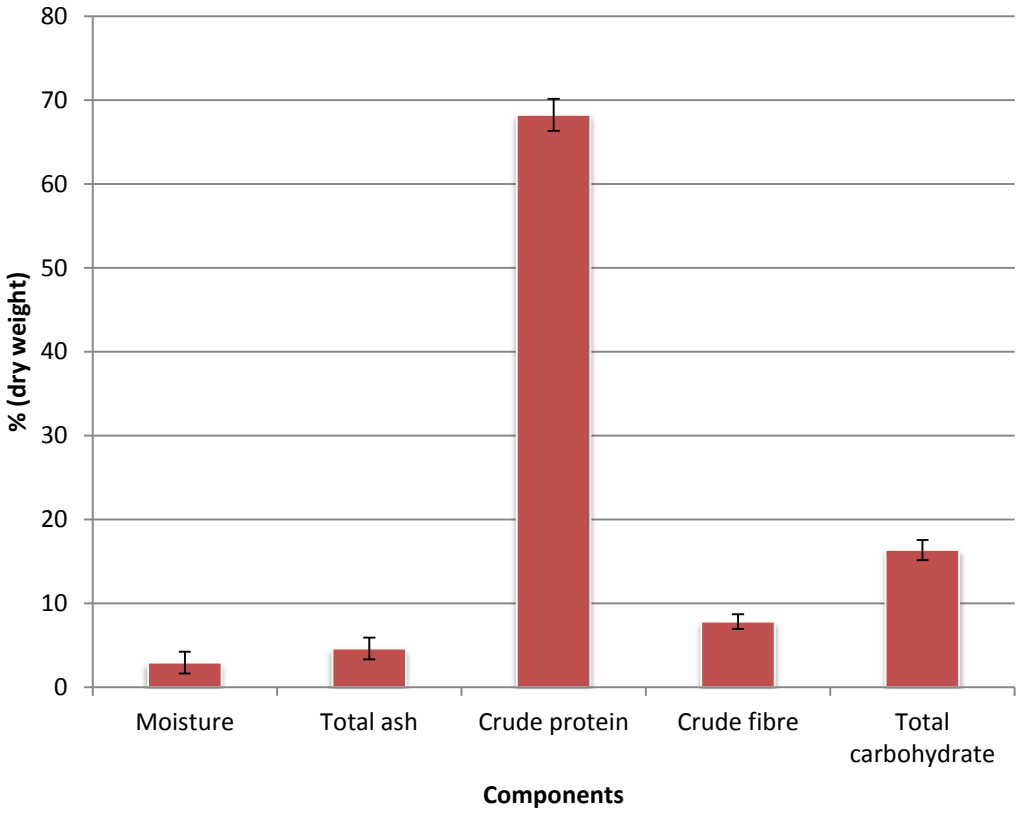

Fig 6.3: Proximate Composition of the DPSPI powder.

6.2.2 Amino Acid Composition of Date Palm Seed Protein isolate (DPSPI)

The amino acids composition of DPSPI is presented in Table 6.1. Data were expressed as mg amino acids/g protein. Sixteen amino acids were detected and identified in the DPSPI sample. Most of the essential amino acids such

as isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine and histidine were present except for tryptophan. Leucine was the present in the highest concentration of the essential amino acids at 88.63 mg/g protein while the histidine was present at the lowest concentration of 14 mg/g protein. Overall glutamic acid (217 mg/g protein) was the amino acid present in the highest concentration in DPSPI.

Table 6.1: Amino acid composition (mg/g protein) of DPSPI

Amino acids	**Extracted DPS protein**
Essential amino acids:	
Isoleucine	34±9.7
Leucine	88±12.0
Lysine	55±9.7
Methionine	22±3.9
Phenylalanine	53±13.0
Threonine	27±5.6
Valine	61±15.9
Histidine	14±1.2
Non-essential amino acids:	
Aspartic acid	100±20.2
Glutamic acid	217±29.8
Serine	48±16.6
Arginine	123±16.1
Glycine	61±15.6
Alanine	56±13.9
Proline	38±10.8
Tyrosine	32±2.9

These were results were consistent with the amino acids composition of the full fat date palm seed powder which was discussed in Chapter 3 (Table 3.2) and also broadly in line with those reported by Al-Showiman & BaOsman (1992) and Bouaziz et al. (2008). The results indicate that the DPSPI could be a useful source of amino acids for human nutrition.

6.2.3 The Thermal Characteristics of Date Palm Seed Protein Samples

Differential scanning calorimetry (DSC) measures the heat required to raise the temperature of a sample compared to that required to increase the temperature of a reference sample. It can be used to determine when a change of state occurs in a sample, and whether the process is exothermic or endothermic. For proteins DSC can reveal structural and conformational changes of proteins during heat treatment. A DSC thermogram exhibits one or more peaks corresponding to the unfolding transition of one (or more in a mixture) protein. The maximum of the denaturation peak characterises the protein thermo-stability. The peak maximum is the point at which 50% of the protein is considered to be denatured, and the temperature at which this occurs is often quoted as the denaturation temperature for the protein. The denaturation enthalpy (ΔH, the integrated area under the heat flow curve) can be used as an indication of hydrophobic/hydrophilic interactions in proteins and compactness of structure (Ma & Harwelkar, 1991; Hua et al., 2005).

Water has a higher heat capacity than proteins in solution due to the large number of hydrogen bonds and its highly ordered structure. When proteins are dissolved in water they become surrounded by a hydration shell of water, where the water close to hydrophobic patches on the protein becomes more ordered and closely packed. This occurs because the water cannot hydrogen bond with the protein in this region and so becomes locally more ordered (Shinoda, 1977). As the protein is heated this hydration water starts to disorder (melt) and this gives rise to the anomalously high values of heat capacity measured for protein solutions. The water heat capacity is subtracted from the protein solution heat capacity by simultaneously measuring heat flow in a blank sample of the solvent buffer and subtracting this from the protein solution heat flow data. The resulting heat flow data include a contribution fron the heat capacity of the protein itself, and from the effect that the protein has on the water (Freire, 1995).

To understand more about the thermal properties of the extracted date seed protein and the effect of extraction method on this, several samples of the date seed protein, with varied levels of purity were investigated by differential scanning calorimetry (DSC). These samples were crude date palm seed powder (6% protein), protein concentrates prepared using lab methods 2 (11.79% protein), 4 (38.20% protein and 5 (68.24%) and a sample pre-treated

with Mannaway enzyme and then extracted using lab method 2 (31.46% protein). The DSC thermograms obtained from these protein samples are shown in Figures 6.4–6.8.

We have already seen in Chapter 5 that the most abundant proteins in date seed are glycinin and β-conglycinin. The denaturation temperature of the major protein components of the date seed are reported as 90˚C for glycinin and 72˚C for β-conglycinin although multimeric forms of glycinin may retain their quaternary structure and in this state are very stable when heated at 100˚C (Mori et al., 1982; Nakamura et al., 1984).

The DSC thermogram of crude date palm seed powder (Figure 6.4) shows a single broad peak. The peak starts at about 40˚C and reaches a maximum at 88.73˚C (which corresponds to the denaturation temperature), and a ΔH of 235.6 J/g. This does not correspond to the reported denaturation temperature of either of the major protein fractions, glycinin or β-conglycinin, but is midway between the two. For such a crude sample of protein (only 6% of the powder is protein) this is perhaps not unexpected, and the thermogram is likely to reflect not only a mixture of more than one protein type but also the contribution from other components of the powder, particularly the carbohydrates. In addition, there are also many other proteins in the sample, which although they may not be present at as high a concentration as glycinin and β-conglycinin, will nonetheless contribute something to the overall thermal porpoerties of the sample.

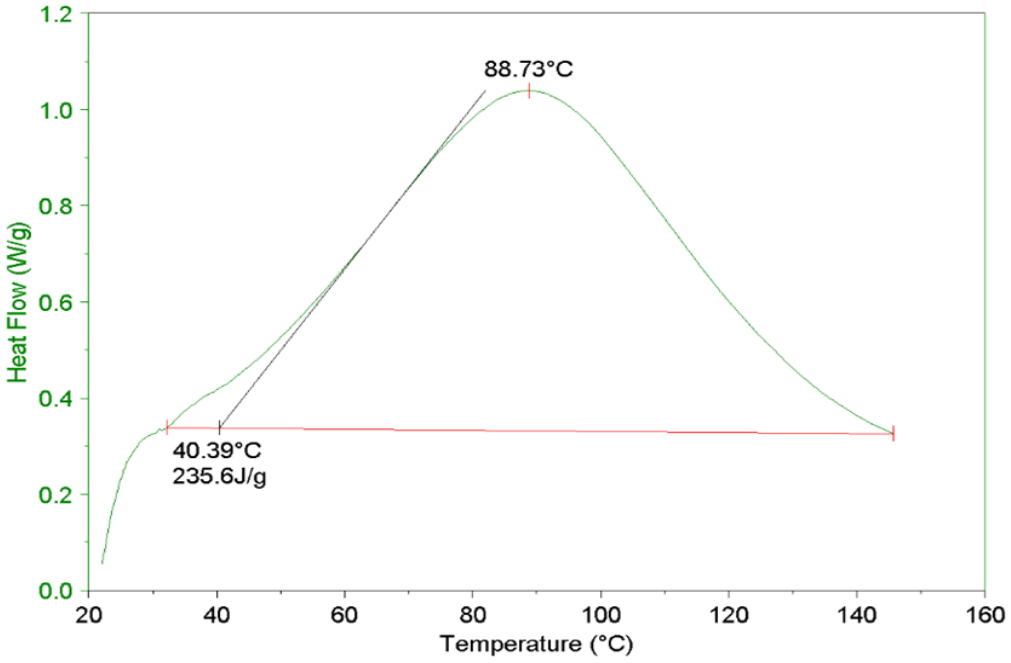

Fig 6.4: DSC Thermogram of Crude Date Palm Seed Powder (6% protein).

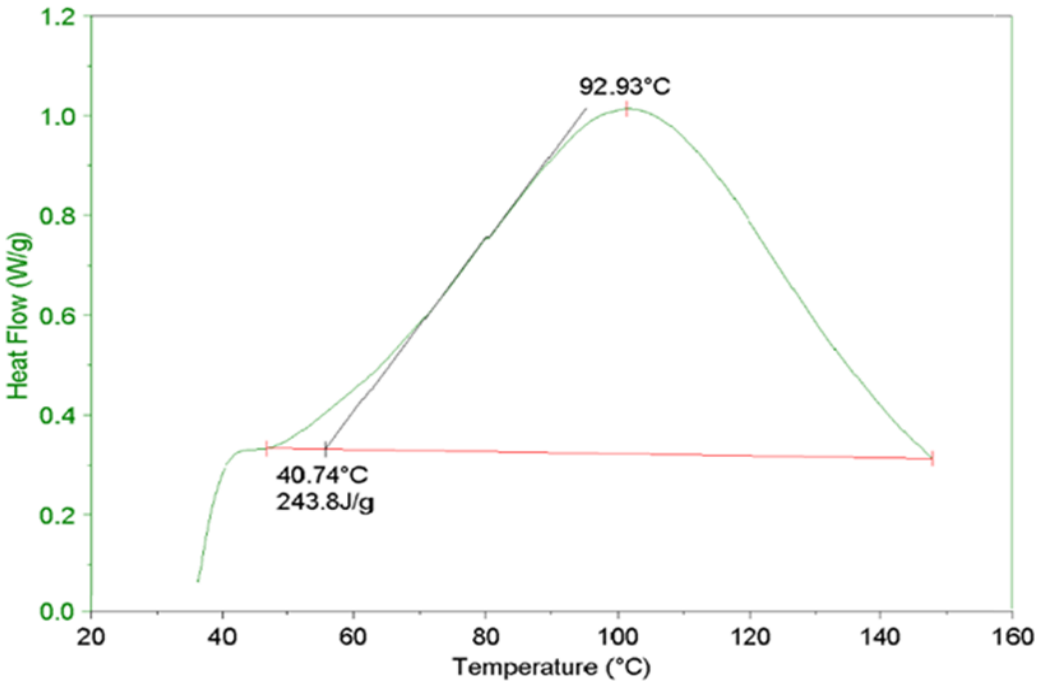

Fig 6.5: DSC Thermogram of Date Palm Seed Protein Concentrates (Lab method 2) (11.79% protein).

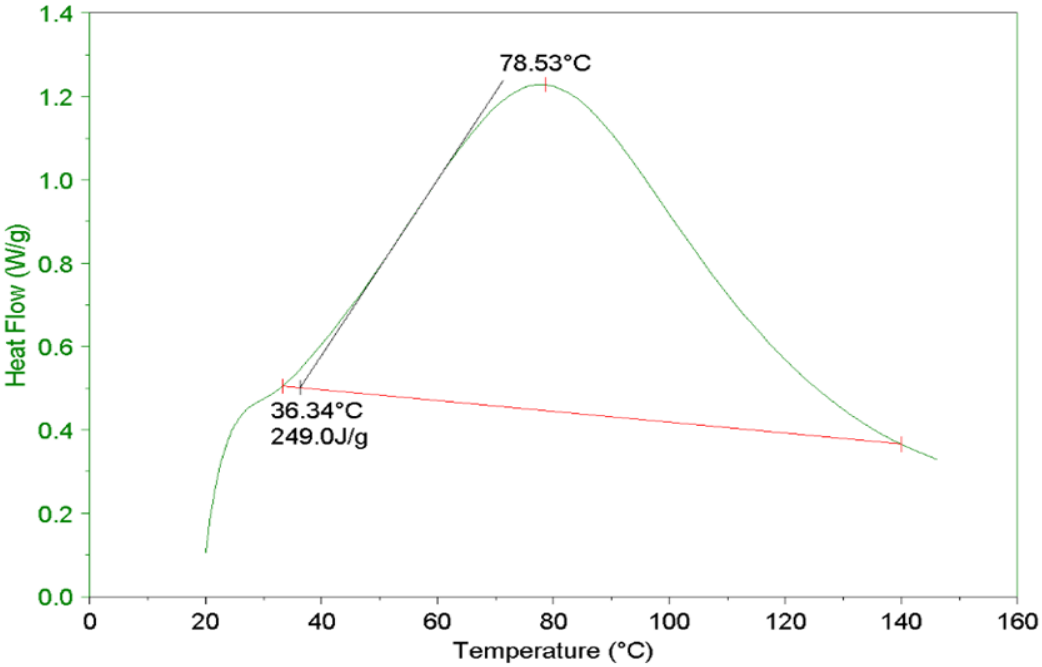

Fig 6.6: DSC Thermogram of Date Seed Protein Concentrates (Lab method 4) (38.20%protein).

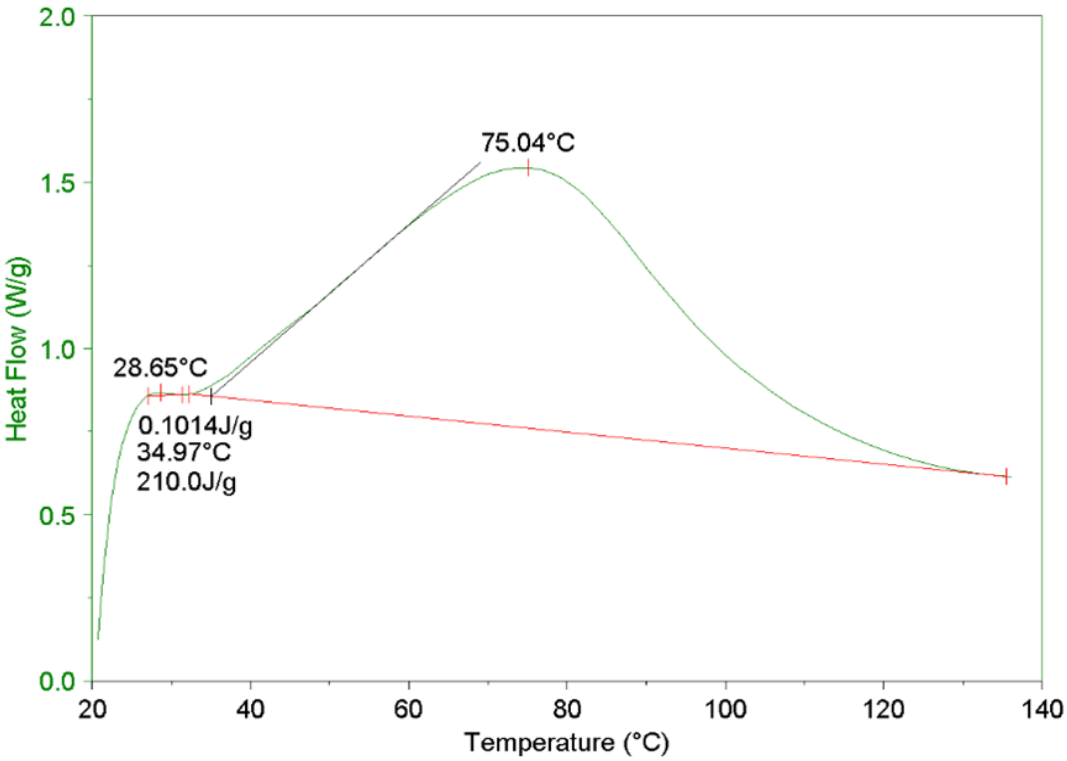

Fig 6.7: DSC Thermogram of Date Seed Protein Concentrate (treated by Mannaway enzyme) (31.46% protein).

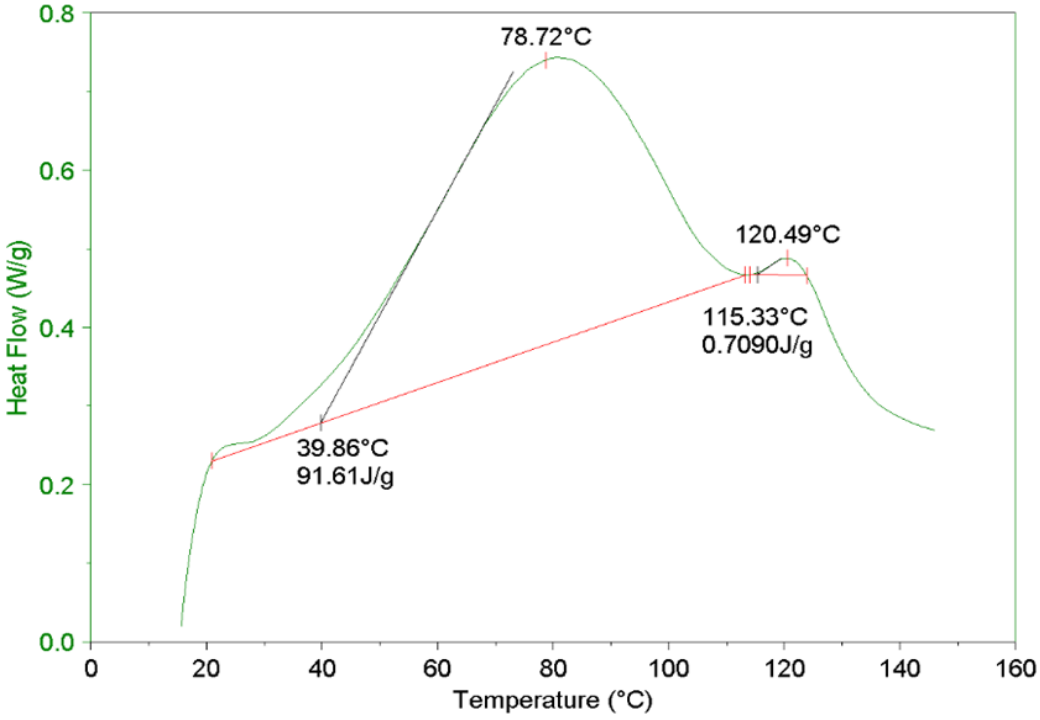

Fig 6.8: DSC Thermogram of Date Seed Protein Isolates (Lab method 5) (68.24% protein).

A similar broad peak with denaturation starting at 40.47˚C and a peak denaturation temperature of 92.93˚C (enthalpy (ΔH) of 243.8 J/g) is also observed for date seed protein prepared using lab method 2 (11.79% protein Figure 6.5). As the protein content of the powder increases, the peak denaturation temperature shows a trend of reducing to values closer to 75–78˚C. For the powder produced by lab method 4 (38.2% protein, Figure 6.6) the denaturation temperature was 78.53^{o}C, for lab method 2 after pre-treatment with Mannaway (31.46% protein, Figure 6.7) denaturation temperature was 75.04˚C, and for lab method 5 (68.24% protein, Figure 6.8) two denaturation peaks were observed, one at 78.72˚C and one at 120.49˚C.

Our results for the DSC analysis of the different protein powders suggest a number of things. Firstly, the crude low protein powders contain a mixture of protein and non-protein components that most likely all contribute to the thermal denaturation behaviour of the powders. This will explain why a single very broad denaturation peak is observed, and why these do not correspond closely to the expected denaturation behaviour of the major protein components of the seed powder (glycinin and β-conglycinin). Powders that have a higher protein content, where the contribution to the thermal behaviour of the proteins still have a single denaturation peak, suggesting a mixture of proteins but this has a reduced maximum in the range 75–78˚C. We know from Chapter 5 that the major protein components of the protein of date seed are glycinin and β-conglycinin. The thermal denaturation results may suggest that as the non-protein components of the powders are removed and the protein content increases, the thermal properties become dominated by the glycinin and β-conglycinin thus giving a thermal denaturation temperature between the reported denaturation temperatures of the two proteins (Mori et al., 1982). For the highest protein content powder, the powder made using a Ph/TCA extraction two peaks are observed, with a second peak appearing at higher denaturation temperature (approximately 120˚C).

The SDS PAGE analysis of the protein component of crude date seed protein and dates seed protein isolate (prepared using method 5) identified high molecular weight protein fractions in the date seed protein isolate, but not in the crude date seed protein powders. We speculated that these may be glycinin and β-conglycinin multitmers. This would explain the second

(high temperature) denaturation peak if these multimers corresponded to glycinin aggregates, since it has been noted previously that the quaternary structure of glycinin is highly stable even when heated at 100˚C (Nakamura et al., 1984).

6.2.4 Functional Properties of Date Palm Seed Protein

6.2.4.1 Solubility Properties of Date Palm Seed Protein

Protein solubility is one of the most important functional properties of a protein due to its effect on all other functional properties of the protein (Kinsella, 1979; Bian et al., 2003). Put simply, if a protein is not soluble it will not dissolve in an aqueous phase and will not be able to emulsify, foam or gel. The solubility of a protein is affected by many different factors such as pH and ionic strength but primarily the exposure to heat (Kinsella, 1979; Nazareth, 2009). Heating of protein solutions will denature the the protein, which in turn leads to aggregation of the proteins and a decrease in the protein solubility compared to native protein (Mine, 1995; Kim, 1998 & Langendorff et al., 1999).

6.2.4.1.1 Solubility as a Function of pH at Room Temperature

The effect of pH in the range 2–12 on protein solubility at room temperature in crude date palm seed (CDPS), date palm seed protein isolates (DPSPI) and soy protein isolates (SPI) is presented in Figure 6.2. The results indicate that the protein solubility for all samples is at a minimum at around pH 4.0 where we believe the isoelectric point of all samples will be.

At the isoelectric point the protein-protein interactions increase because the charge on the protein is zero. This means that electrostatic repulsive forces are lowest and the attractive van der Waals forces will predominate (Pelegrine & Gasparetto, 2005). In contrast the highest solubility for all three protein samples occurred at high pH. The increase in solubility either side of the isoelectric point can be explained by the protein having negative or positive net charge under these conditions and therefore a net repulsive electrostatic interaction between the proteins under these conditions. The similarity between the date seed and soy proteins reflects the similarity in nature of the proteins. Both soy proteins and date palm seed proteins are seed

storage proteins. Soy proteins are a complex mixture of proteins, but principally are composed of glycicnin (11S fraction) and β-conglycinin (7S fraction), which together make up 70% of the total protein in soy (Utsumi et al., 1997). These two proteins also are major constituents of date seed protein (Chapter 5). The isoelectric point of glycinin is 4.90 and of β-conglycinin 4.64 (Koshiyama, 1983). These values fit well with the observed solubility minimum for both the soy protein and date palm seed protein in the region of pH 4 (Figure 6.9).

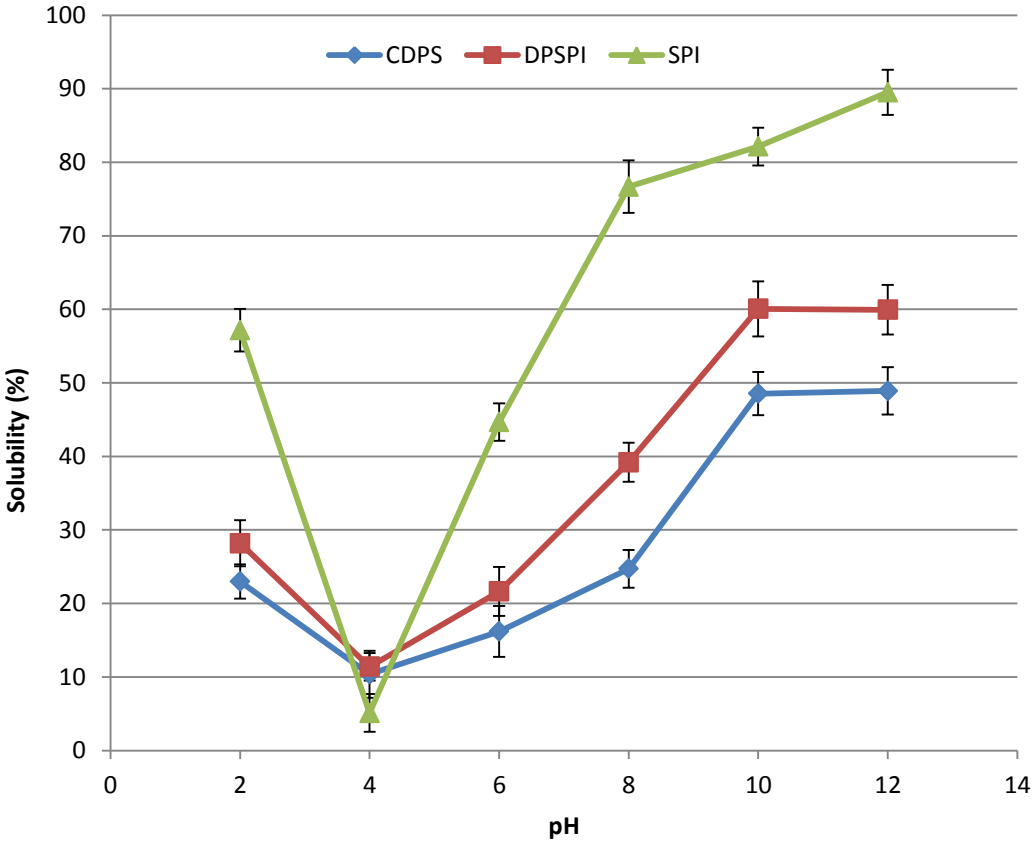

Fig 6.9: Effect of pH on Protein Solubility at Room Temperature.

The isoelectric point obtained of CDPS, DPSPI and SPI are also consistent with those reported by Tsukada et al. (2006) and Jiang et al. (2010) for soy protein. Other studies on different seed proteins have also found the same pH minimum in protein solubility, suggesting that seed protein composition, and thus the functionality is dominated by glycinin and β-conglycinin or storage proteins of a similar structure. Lqari et al. (2002), Sanchez-Vioque et al. (1999) and Goncalves et al. (1997) report isoelectric pH of 4.3, 4.3 and 4.5 for seed proteins from Lupinus angustifolius, chickpea and rapeseed protein respectively. At pH values other than the isoelectric point the solubility of

the date seed protein is lower than for soy protein and the solubility of the crude date seed protein sample lower than the purified sample. The difference in solubility of SPI compared to CDPS and DPSPI under the condition used here could be related to differences in the molecular structure of the proteins, the method of preparation and/or the presence of other biomolecules such as polysaccharides with which the protein may be associated.

6.2.4.1.2 Solubility as a Function of pH at Temperatures of 55˚C and 85˚C

In Figure 6.10 and 6.11 the effect of heating to 55˚C or to 85˚C on the solubility of CDPS, DPSPI and SPI at different pH is plotted. The solubility profiles obtained for CDPS, DPSPI and SPI were similar to those at room temperature in that they showed the same U-shaped profile with a minimum in solubility at pH 4, and the solubility increased in the order SPI > DPSPI > CDPS.

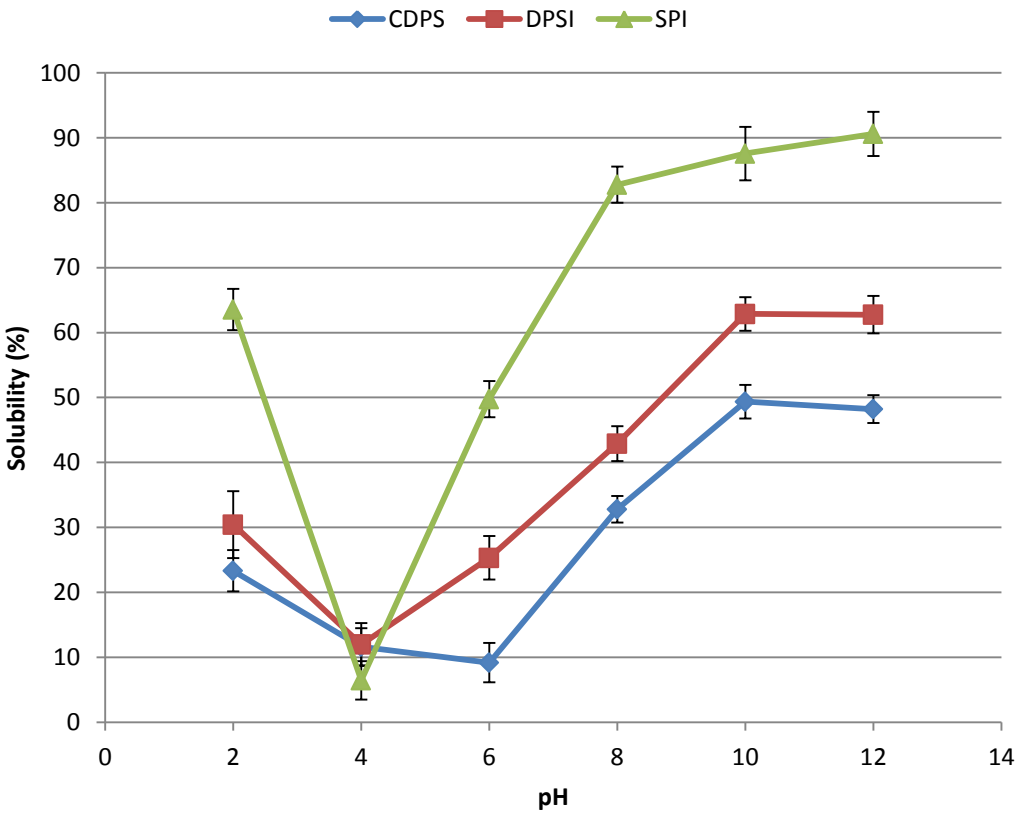

Fig 6.10: Effect of pH on Protein Solubility at 55˚C.

When the change in solubility upon heating to 55˚C or 85˚C (relative to solubility at room temperature) is plotted in Figures 6.12 and 6.13 it can be

seen that the effect of temperature on solubility is complex and pH dependent. Heating to 55˚C increases the solubility for all three protein powders (Figure 6.12), but the effect is small-no more than 8% increase in solubility for any powder or pH condition. The change in solubility for all powders at the isoelectric pH around 4 is low, suggesting that some factor other than charge neutralisation may contribute to the reduced solubility at this pH.

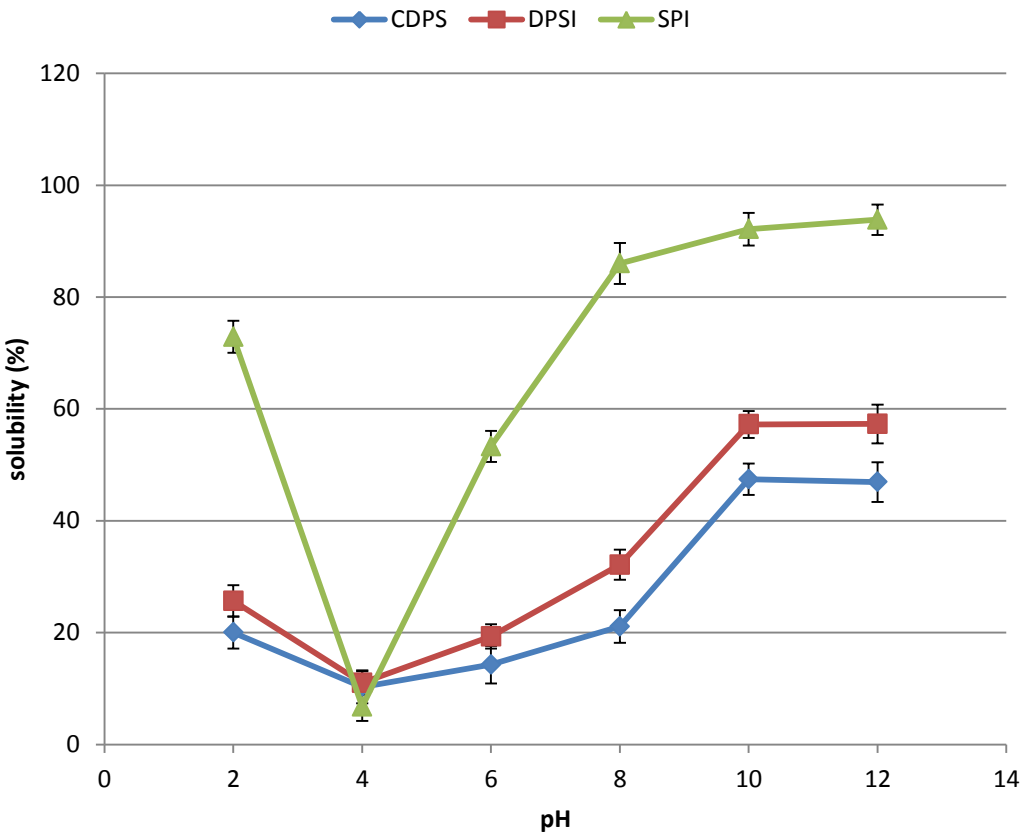

Fig 6.11: Effect of pH on Protein Solubility at 85˚C.

In general the increase in solubility on heating to 55˚C is greatest for the SPI sample, which probably reflects the higher overall protein content (purity) of the SPI sample (about 90% protein) over the date seed powders. Similar effects have been observed for the solubility of milk whey protein. Whey protein solubility was observed to increase as the temperature increased from 40˚C to 50˚C, but decreased above the denaturation temperature around 80˚C, where the proteins will be denatured by the effect of heat treatment on secondary and tertiary structure (Wong et al., 1996; Pelegrine & Gomes, 2008).

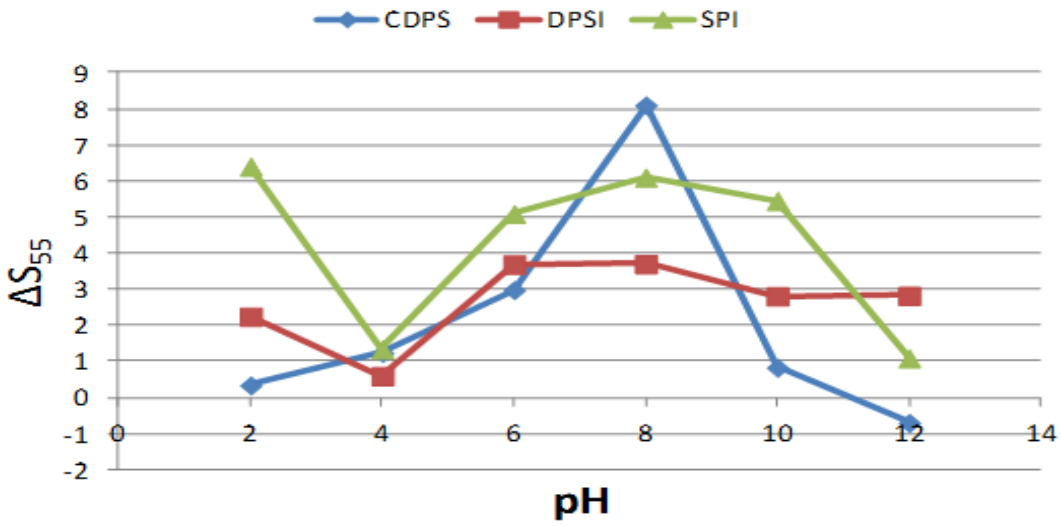

Fig 6.12: Effect of pH on change in Solubility on Heating at 55˚C Compared to Solubility at Room Temperature (ΔS_{55}).

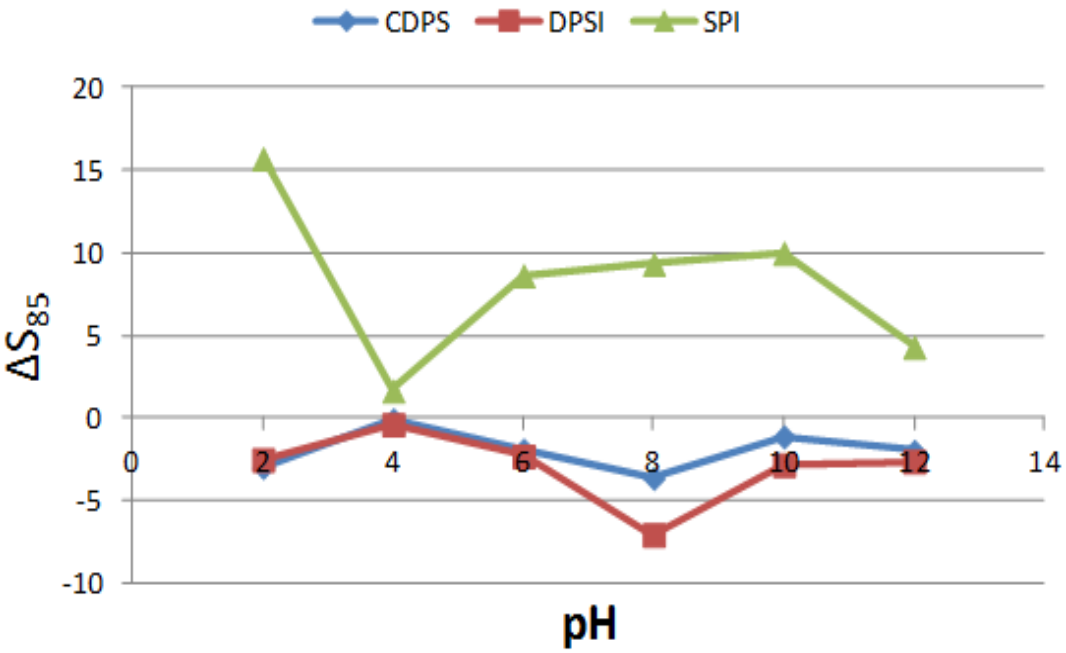

Fig 6.13: Effect of pH on Change in Solubility on Heating at 85˚C Compared to Solubility at Room Temperature (ΔS_{85}).

When the protein solutions are heated to 85˚C the SPI again shows an increase in solubility which is greater than observed at 55˚C, and is also a

minimum at pH 4, The date seed protein samples, however show a consistently lower solubility at all pH values than is seen when they are solubilised at room temperature. This would suggest that when heated at 85˚C the date seed proteins denature and precipitate thus reducing the solubility, whilst the soy proteins remain undenatured. The DSC profiles in Figures 6.4 to 6.8 do suggest that a significant proportion of the proteins in date seed may denature below 85˚C. The denaturation temperatures of glycinin and β-conglycinin are reported to be around 90˚C and 70˚C respectively (Hermansson, 1978), although trimers and hexamers of the soy proteins are highly stable when heated to 100˚C (Nakamura et al., 1984). One explanation of the difference in solubility of the SPI and date seed proteins at 85˚C could be that in SPI a larger proportion of the proteins are in the more stable quaternary structures (oligomeric forms). This might arise due to the different methods for separating and purifying the protein powders.

6.2.4.2 Emulsifying Activity and Emulsion Stability of DPSPI Compared to SPI and WPC

Figure 6.14 shows the emulsifying activity index of DDPSP and DPSPI at different pH within the range 2–12. The emulsifying activity (EAI) of both DDPSP and DPSPI are a minimum at the isoelectric point, and increase either side of this as pH increases. The EAI for the both crude and purified date seeds were similar, although the EAI for the crude sample was always slightly higher than for the purified samples at all pH values. The emulsions were made at a constant protein content, which means that the crude DDPSP samples will contain other component in high concentration (probably polysaccharides). The EAI results suggest that these non-protein components contribute to the emulsifying activity of the sample.

The emulsion stability (ESI, Figure 6.15) shows a similar dependence on pH for the date seed protein samples, i.e. it is a minimum at the isoelectric point. However, the emulsion stability for the crude DDPSP is much lower than for the DPSPI at all pH values, and at alkaline pH the difference is considerable (52.61min for DDPSP compared to 168.91 min for DPSPI).

In Figures 6.16 and 6.17 the emulsifying activity and emulsion stability of DPSPI is compared to those of soy protein isolate (SPI) and bovine whey

protein concentrate (WPC). WPC is considered an excellent emulsifier and is a common protein emulsifier in formulated foods (Euston & Hirst, 2000).

In Figure 6.16 it can be seen that the EAI of WPC is significantly greater than that of SPI which is in turn significantly greater than that of DPSPI at all pH values. The WPC and SPI EAI results also show a minimum at pH 4–5, which is consistent with the reported isoelectric point of whey protein which is reported to be about pH 4.8 (Demetriades et al., 1997), and soy glycinin and β-conglycinin (pH 4.9–5.2 and 4.7–5.0 respectively) (Golubovic et al., 2005). Even though there are large differences in the EAI between the DPSPI, SPI and WPC, the emulsion stability (ESI, Figure 6.17) does not show the same trends. The ESI for all three protein samples are very similar across the pH range tested, and for WPC it is slightly lower than for DPSPI at all pH values.

The emulsion stability is influenced greatly by the size of the oil droplets in the emulsion. Since the EAI is an indirect measure of droplet size we might expect there to be a correlation between the EAI and the ESI for the emulsions made with the different samples, since larger emulsion droplets are known to be, in general, less stable than smaller droplets.

In Figure 6.18 the ESI is plotted against EAI for emulsions made with all protein samples tested for EAI and ESI (DDPSP, DPSPI, SPI and WPC). As expected there is a linear relationship between ESI and EAI for the four protein samples, with more stable droplets being associated with greater EAI (smaller particles size). Moreover, the DDPSP and SPI samples show the same relationship between ESI and EAI (i.e. the data fall on the same line). The DPSPI data, however, show a different behaviour, with the slope of the EAI vs ESI graph being greater (i.e. the emulsion stability is higher for a given EAI) than for the DDPSP and SPI samples. The WPC sample on the other hand shows the opposite behaviour, where the EAI is high in comparison to the date seed and soy proteins, but gives a lower ESI for a given EAI. The relationship between EAI and ESI is still linear for WPC, however, and has a very similar slope to that for the SPI. The functional properties of proteins are also expected to be related to the solubility of the protein (Theerakulkait et al., 2006).

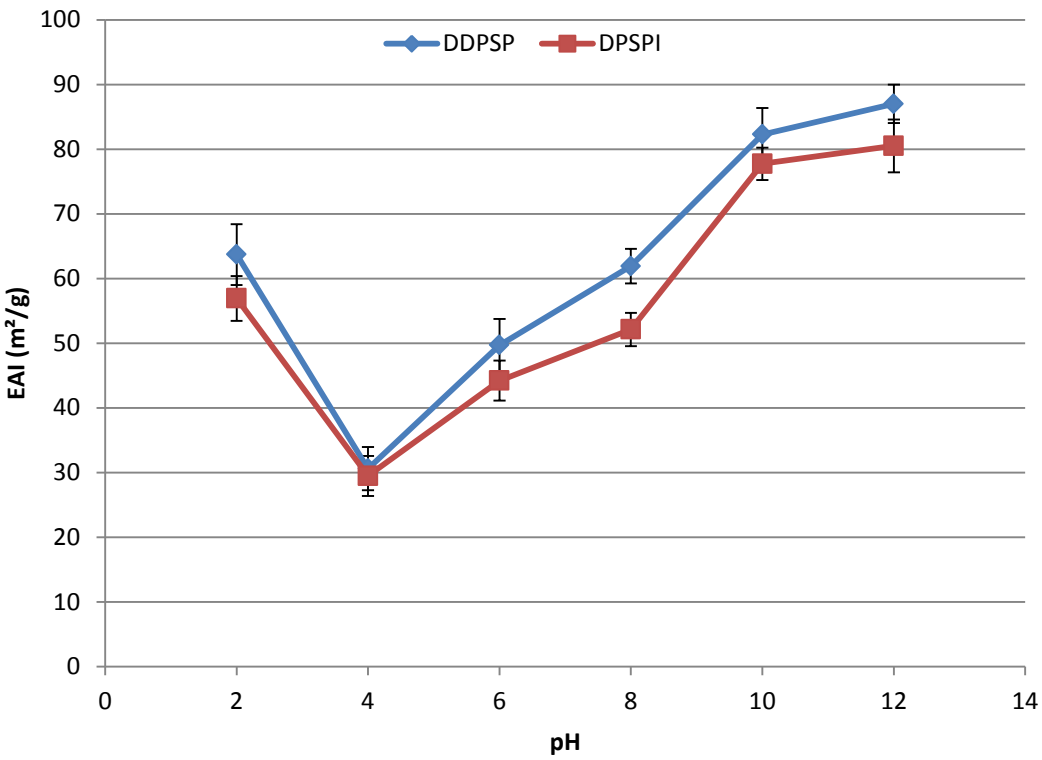

Fig 6.14: Emulsifying Activity Index of DDPSP and DPSPI.

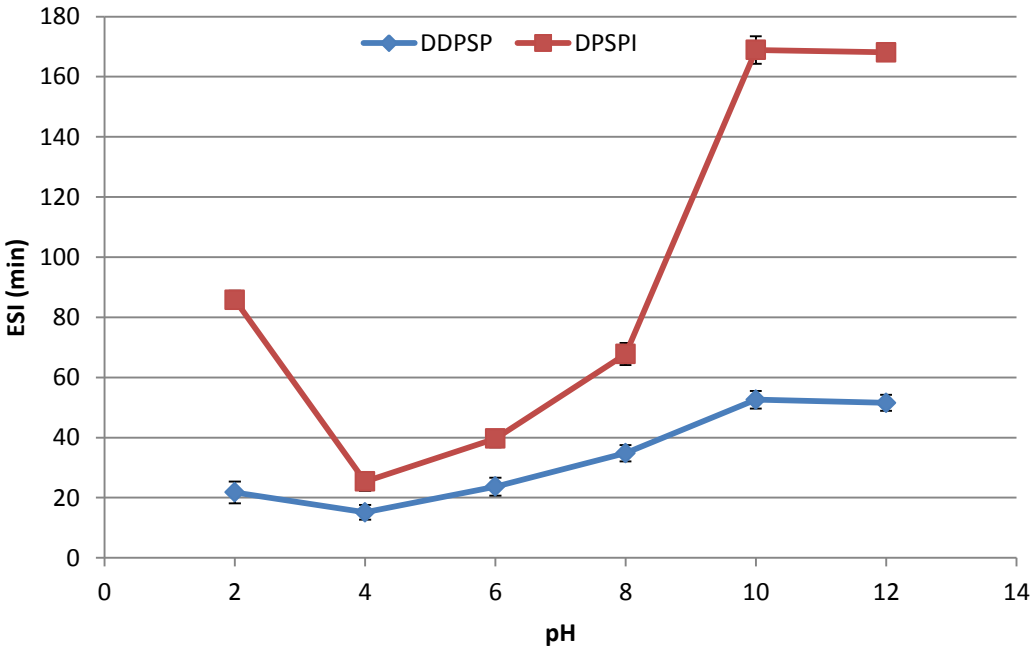

Fig 6.15: Emulsifying Stability Index of DDPSP and DPSPI.

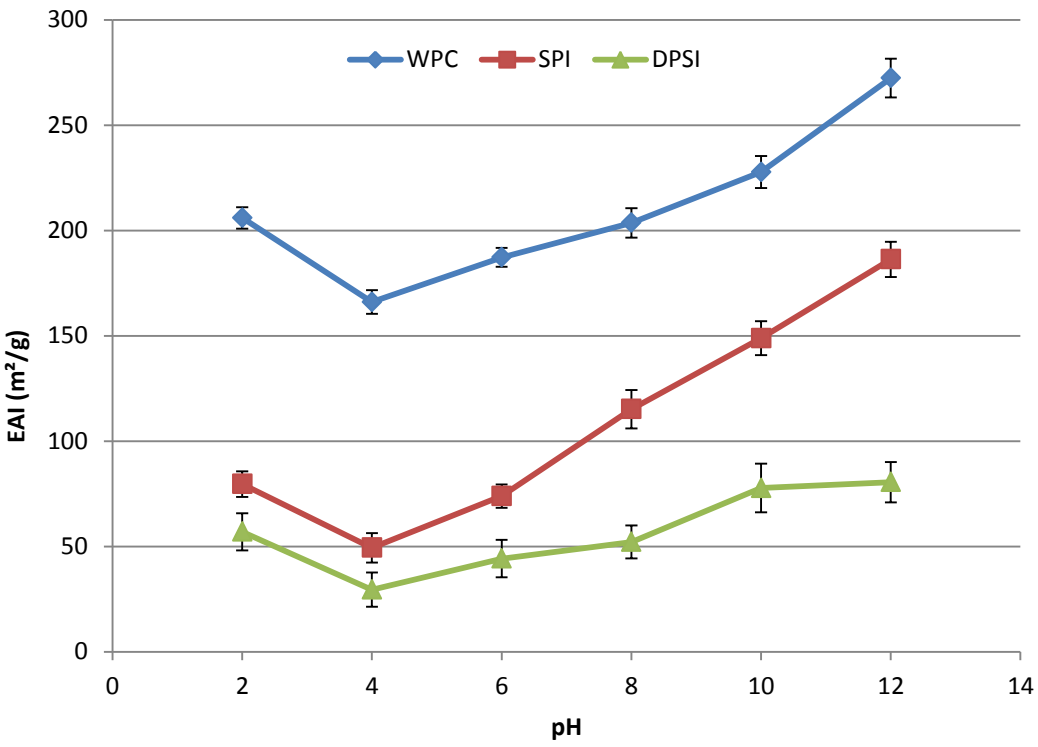

Fig 6.16: Emulsifying Activity Index of DPSPI compared to WPC and SPI.

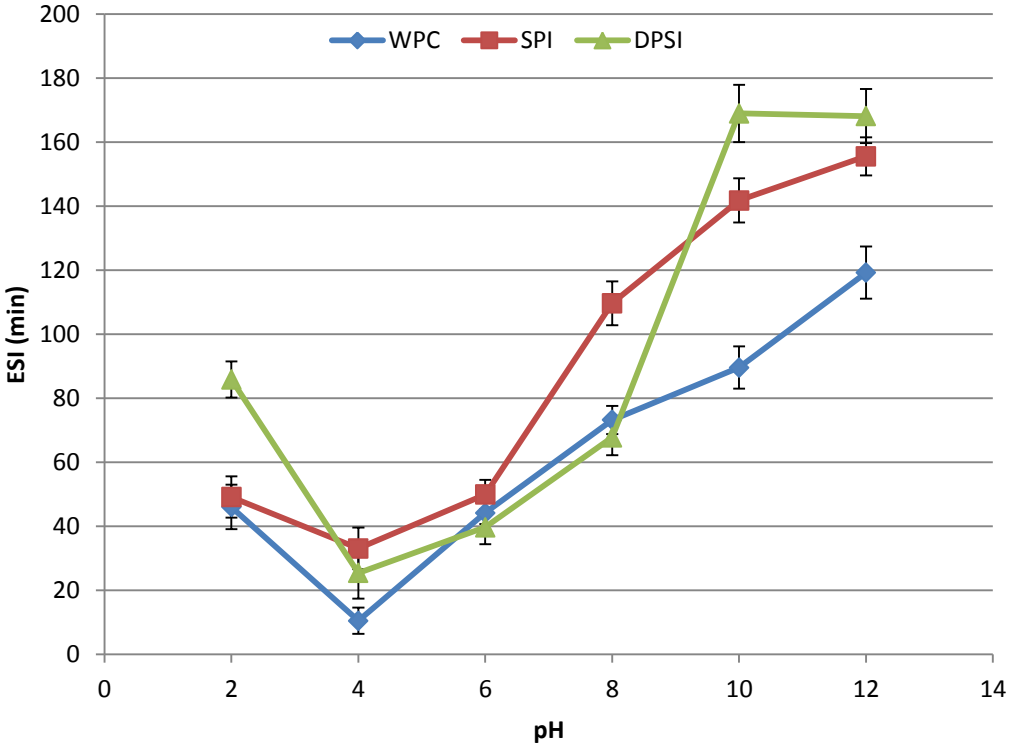

Fig 6.17: Emulsifying Stability Index of DPSPI compared to WPC and SPI.

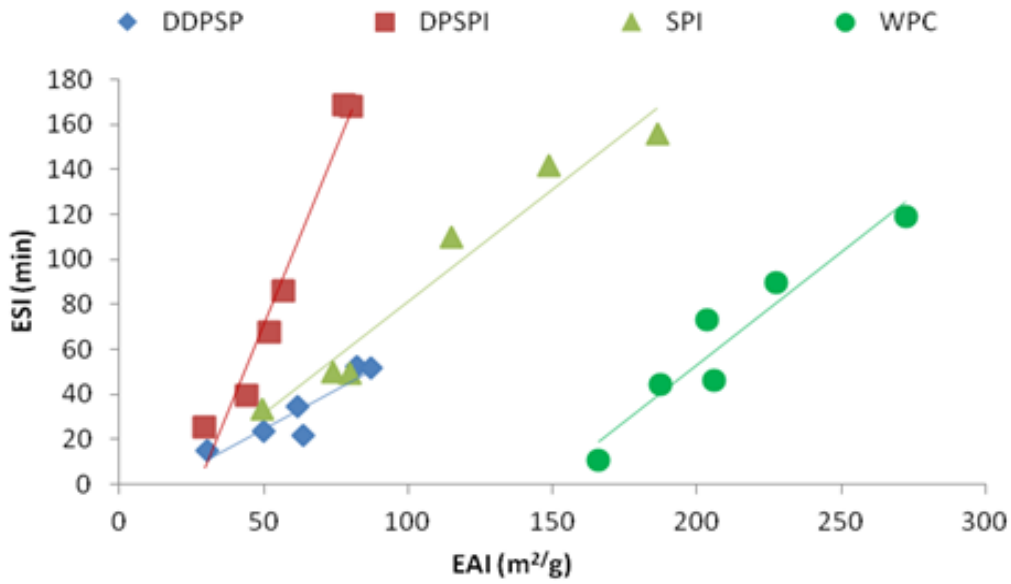

Fig 6.18: Relationship between Emulsifying Activity and Emulsion Stability

In Figures 6.19 and 6.20 the EAI and ESI are plotted against the solubility of the proteins at the corresponding pH, solubility data for the WPC was not measured and so the data for this protein sample is not included in Figures 6.19 and 6.20. Up to a solubility of 60% the EAI values for all three samples fall on a single straight line. Only SPI powder has solubility above 60%. The EAI values for SPI above 60% solubility fall on a second straight line with a slope greater than that for the data below 60% solubility.

For ESI vs solubility on the other hand, the crude DDPSP and SPI data below 60% solubility fall on the same straight line, but the DPSPI data have a higher ESI for a given solubility than the other two protein powders. The emulsifying properties of proteins are influenced by a number of factors. The structure of protein has a large bearing on emulsifying properties, as does the aggregation state, and environmental factors such as pH and salt content, and the presence of other biomolecules (e.g. sugars, polysaccharides etc.).

Much of the research on the emulsifying properties of seed storage proteins has been carried out on soy bean protein. We have noted earlier in this chapter that soy proteins are mainly of glycinin and β-conglycinin (70% of the total protein), and so we would expect their emulsifying properties to be dominated by these two proteins. Given that we have shown in Chapter 5 that date seed protein also contains high levels of glycinin and β-conglycinin we would also expect these same proteins to play a large part in the emulsifying

behaviour of date seed protein as well. Glycinin has a complex quaternary structure, and is found as a hexamer (molecular weight in the range 300–380 kDa) in the plant seed, and is made up of combinations of five distinct subunits (Staswick et al., 1981, 1984). At different pH and ionic strength combinations glycinin hexamers can dissociate to form a trimeric (7S) or a monomeric (3S) form (Peng et al., 1984).

Keerati-u-rai & Corredig (2010) have found that soy proteins adsorb to the surface of oil droplets with a layer that is 30–40 nm thick. This compares to the adsorbed layer for the milk whey protein β-lactoglobulin that is only 4–6 nm thick (Atkinson et al., 1995). This suggests that soy proteins adsorb as aggregates rather than individual proteins as is the case with β-lactoglobulin. The subunit composition of the hexameric glycinin has been found to affect the emulsifying properties of the protein (Maruyama et al., 2004). Given that the subunits composition of the glycinin hexamer can vary, and that dissociation of the hexamer can occur, then it is likely that emulsifying ability will also be variable depending on the particular make-up of the glycinin quaternary structure.

β-conglycinin is also a multimeric protein of molecular weight in the range 150–200 kDa consisting of three subunit chains identified as α, α' and β subunits (Thanh & Shibasaki, 1979). Like glycinin the subunit composition of β-conglycinin is variable. β-conglycinin has been shown to be a better emulsifier than glycinin, because it is able to adsorb and spread more rapidly at the emulsion droplet interface (Utsumi et al., 1997; Bernard et al., 2001; Molina et al., 2001). The type of subunit in β-conglycinin has also been demonstrated to affect the emulsifying ability with the α subunit being the best emulsifier followed by α' and then β (Utsumi et al., 1997). Therefore, the balance between glycinin and β-conglycinin and the relative proportions of different subunits in seed protein powders is likely to influence the relative emulsifying properties of those powders. We can hypothesize that this may explain the differences in emulsifying activity and emulsion stability observed between the SPI and date protein samples.

In Chapter 5 the protein composition of the date seed isolate and crude date seed protein and the SPI protein powders used in the emulsifying study was characterised with SDS-PAGE electrophoresis.

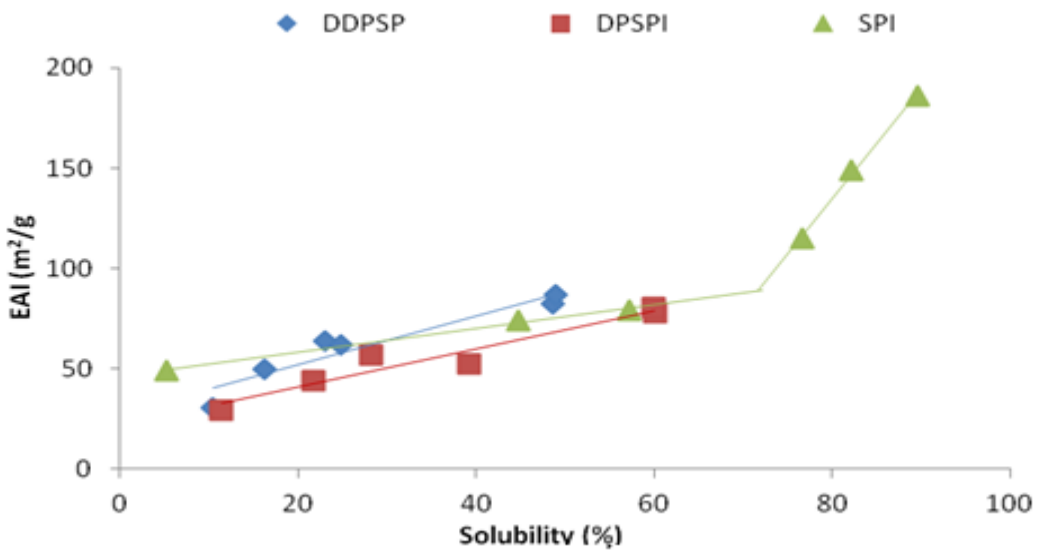

Fig 6.19: Relationship Between Protein Solubility and Emulsifying Activity

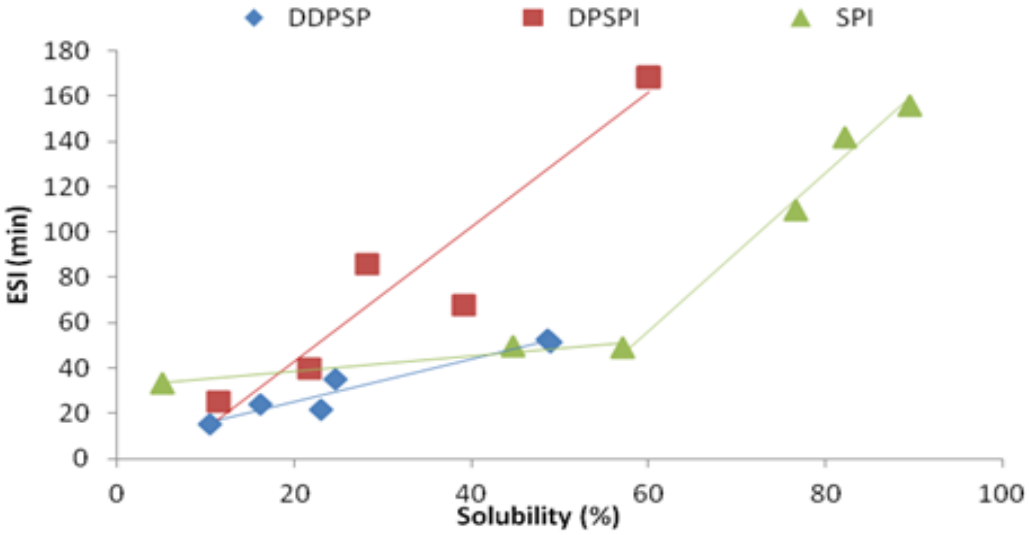

Fig 6.20: Relationship Between Protein Solubility and Emulsion Stability

Distinct differences in the protein molecular weight profile between all three samples was noted (Table 5.2, Chapter 5). For the date seed proteins, high molecular weight protein fractions that may correspond to the glycinin and β-conglycinin multimers that were found in the date seed protein isolate, but not in the crude date seed protein where the highest molecular weight was

70kDa. This suggests that the extraction method, where DDPSP was extracted via alkaline extraction and DPSPI via an acidic Ph/TCA extraction, alters the quaternary structure of the glycinin and β-conglycinin fraction of the date seed protein, which subsequently impacts on the emulsifying properties. The higher molecular weight of the proteins in the DPSPI can also be used to explain why emulsions made with this fraction are more stable than the DDPSP and SPI emulsions for a given EAI (Figure 6.18 and 6.20).

Euston & Hirst (2000) investigated the emulsifying properties of aggregated and non-aggregated milk protein products. They found that large aggregates of proteins were often poorer emulsifiers than non-aggregated proteins, but the latter led to a greater stability of the emulsions under certain conditions. They explained this by suggesting that aggregates of proteins were less able to unfold at the oil droplet interface and so were unable to spread and stabilise the newly formed emulsion droplet surface in the homogenizer, thus leading to larger droplets and a lower emulsifying activity. On the other hand, the large aggregates led to a very thick, dense adsorbed layer which increased the emulsion stability.

The emulsion stability was most likely increased through both a steric mechanism and a change in the effective density of the droplets. The dense adsorbed layer forms a greater steric barrier to coalescence than would a thinner layer of less aggregated protein, and would slow coalescence. Creaming instability of the emulsions would be slowed due to the aggregated protein layer increasing the effective density of the oil droplets. Oil droplets rise to the top of the emulsion under the force of gravity (buoyancy) due to the difference in density between oil and water. This is described in simple terms by Stokes equation:

$$v_S = \frac{2(\rho_p - \rho_f)gr^2}{9\eta} \qquad (1)$$

where the velocity (v_s) at which the particle rises is proportional to the density difference between particle (ρ_p) and suspending fluid (ρ_f), proportional to the square of the radius (r) and inversely proportional to the viscosity of the fluid (η) and g is the gravitational constant. Euston & Hirst

(2000) proposed that the main effect of aggregated proteins on emulsion droplet stability was to increase the effective density of the oil droplet, thus decreasing the density difference and slowing the rate of creaming. We can hypothesize that a similar effect may be happening in our emulsions, and that the greater stability of the DPSPI at the same EAI is due to the presence of adsorbed aggregates. The presence of protein aggregates in the DPSPI is confirmed by the SDS-PAGE results in Chapter 5. This also why WPC has relatively low emulsion stability but very high emulsifying activity. Whey proteins only form loosely associated oligomers (Iametti et al., 1998) in solution and these break up in the homogenizer so that only protein monomers adsorb. Therefore they form a thin, monomeric protein layer at the surface of the emulsion droplet, which will not have the same effect on droplet density as larger glycinin and β-conglycinin aggregates, and will also have a lower steric stabilising ability as well.

6.2.4.3 Water and Oil Holding Capacity

The water holding capacity (WHC) and oil holding capacity (OHC) of full fat and DDPSP prepared using the different lab-scale preparation methods were determined and compared to soy protein isolate (SPI) (Table 6.2). Soybean protein isolate (SPI) was found to have the highest WHC of 6.37ml of water/g powder compared with that of full fat seed powder (3.99 ml/g), DDPSP (4.12 ml/g), method 1 (1.98 ml/g), method 2 (2.41 ml/g), method 3 (3.11 ml/g), method 4 (3.96 ml/g) and with that of method 5 (2.47 ml/g). In general, the WHC of date seed and their extracted protein powders ranged from 1.98 ml/g to 3.99 ml/g. Lin & Zayas (1987) reported that WHC of commercial protein concentrate and isolate ranged from 1.5-2.5 ml/g. Our results for WHC of all samples fall within this range. Some seed protein concentrates show higher WHC, such as sesame protein concentrates with reported WHC of 3.5 ml/g, and SPI of 6.1 ml/g (Onsaard et al., 2010), with the later confirmed by our results in Table 6.2.

Aletor et al. (2002) report that the WHC ranged from 1.49 ml/g to 4.72 ml/g in viscous food such as gravies and soups, and thus our seed powders could in principle be used as a thickener in this type of food system. We noted that DDPSP had higher WHC than that of full fat seed powder and all other lab methods. This could be due to the higher fibre contents, since complex

polysaccharides are known for their ability to bind water and thus have higher WHC (Elleuch et al., 2008). In addition, the WHC increased as the protein concentration increased from extraction method 1 (8.65%) to extraction method 4 (38.2%, Table 6.2). Proteins are also able to bind water strongly, and therefore it is expected that WHC will be linked to protein content (Kinsella, 1979; Ogunwolu et al., 2009).

Table 6.2: Water and Oil Holding Capacity of Laboratory Prepared DPS Protein Concentration and Isolates.

Samples	Water absorption capacity (ml/g)	Oil absorption capacity (ml/g)
Full fat seed powder (5.64 % protein)	3.99±0.10	3.39±0.22
Defatted seed powder (5.64% protein)	4.12±1.01	3.51±0.78
Method 1 (8.65% protein)	1.98±0.63	4.76±0.19
Method 2 (11.79% protein)	2.41±0.93	5.55±0.44
Method 3 (21.11% protein)	3.11±0.52	5.69±1.08
Method 4 (38.20% protein)	3.96±0.21	6.42±0.91
Method 5 (68.24% protein)	2.47±0.89	2.14±0.75
SPI (90% protein)	6.37±0.77	3.08±0.92

On the other hand, the protein powder with the highest protein content (method 5, Ph/TCA extraction) has a lower WHC than method 4 which has lower protein content. Presumably this extraction method removes some component that might contribute more to the WHC than the protein, possibly some of the carbohydrate. There are many components that could affect the WHC and OHC such as the presence of lipids and carbohydrates as well as the nature of the protein itself e.g the distribution of polar and non-polar amino acids between the surface and interior of the molecule (Moure et al., 2001).

The trends in oil holding capacity of the protein concentrates showed similarities and differences to the trends seen for WHC. Unlike for WHC, the OHC for the crude full fat and DDPSP were lower than for the extracted

protein powders. For the extracted powders the OHC increased as the protein content increased i.e. the same trend as for the WHC data. Again, the exception to this was the date seed protein isolated by method 5. These results were in agreement with those reported by Campbell et al. (1992) who found that the OHC of sunflower and soya protein increased as the protein content increased.

The OHC of date palm seed protein isolate by method 5 (2.14 ml/g) was lower than those of SPI (3.0 ml/g), this could be due to the low solubility of date seed protein compared to SPI or to presence of hydrophobic residues in the protein. Sze-Tao & Sathe (2000) found that the OHC of almond protein isolates (3.60 ml/g) was higher than that of SPI (2.93 ml/g) and they noted that this could be due to the presence of an increased proportion of hydrophobic residues on the protein surface. High oil absorption is very important in many food applications such as mayonnaise, salad dressings and cake batters. Gelation and foaming ability and stability properties of date palm seed protein isolate (DPSPI) has been not mention here due to the lack of protein sample of (DPSPI).

6.4 References

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CHAPTER SEVEN

Conclusions and Recommendations

7.1 Conclusions

The cost of some key protein food ingredients is a major factor in the price of some manufactured food ingredients. Protein ingredients such as egg and milk proteins are particularly volatile in terms of both price and supply and this presents a challenge to food manufacturers using these ingredients because they have to ensure sufficient quantities of protein are available, but also to adsorb cost fluctuations in their process.

There is also a wider question of sustainability of proteins from animal sources, and questions are being asked as to whether feeding plant proteins to animals can be justified when they could be used directly for human consumption. For this reason, manufacturers are looking for alternatives to animal proteins based on both sustainability and economic considerations. Another area where new sources of protein could be found are the waste materials from many plant protein processing operations. Such waste includes the peel, skin and seeds of fruits and vegetables, materials which are normally discarded or used in low value commodities such as animal feed. In this thesis we have investigated whether useful protein can be extracted from the seed of the date palm fruit which could subsequently be used in human food formulations.

When considering the viability of protein isolation from a new source, several factors have to be considered. A major factor is whether the protein can be isolated easily and cheaply enough, and in sufficiently high quantities for it to be sold for a cheaper or the same price as the protein it will be replacing. Thus both the yield of the extracted protein and the protein quality (percentage protein in the final powder) become important. A second factor is whether the final protein powder has the functionality required to replace other proteins.

Based on these criteria we can make use data for the total number of dates seeds produced per year, the data from this dissertation for protein content of date seeds and data from this thesis for protein yield and content of date seed powders to estimate the potential quantities and quality of date seed protein powders that could be produced.

Results for the physical characteristics of the dates used in this study show that the date palm seed comprises about 10% of the date's weight (Chapter 3, Table 3.4). The global production of dates is around 7 million tons annually

(Chao & Krueger, 2007). We have measured the protein content of date seed as about 6% by weight (Chapter 3, Figure 3.1). From this it can be calculated that around 700000 tons of seeds will be discarded as a waste. Based on these figures the total amount of protein (ktonnes) and the total final powder that would be produced using some of the extraction methods used here has been estimated and is given in Table 7.1.

Table 7.1: Estimated Protein Production from Date Seed Using Various Extraction Methods

Extraction Method	**Total Protein (ktonnes)**	**Total Powder (ktonnes)**	**Protein Content (%w/w)**	**Protein Yield (%w/w)**
Method 1	61	229	8.65	32.67
Method 2	83	190	11.79	27.18
Method 3	148	223	21.11	31.81
Method 4	267	367	38.2	52.49
Method 5	478	311	68.24	44.45
Control	79	175	11.35	25.06
Pectinase	116	142	16.56	20.31
Cellulase	137	222	19.54	31.72
Viscozyme	155	449	22.16	64.21
Protease	189	236	26.97	33.71
Mannaway	220	371	31.46	52.99
Mannaway+Viscozyme	232	455	33.17	64.99
Pectinase+Cellulase	247	381	35.26	54.39
Mannaway+Viscozyme +Pectinase+Cellulase	289	236	41.35	33.72

The highest recovery of protein occurs when enzymes such as Mannaway and Viscozyme are used. However, these give protein powders which typically are only composed of a third or less of protein, with the remainder presumably solubilised carbohydrates.

The highest protein content obtained was for Method 5, the Ph/TCA extraction which is not feasible for food grade protein extraction, but gave a protein powder of sufficient protein content for functional testing.

Of the non-enzymic methods the highest protein yield of 53% was obtained by using ultrafiltration along with the alkaline extraction (lab method 4). Here the protein content of the final powder was 38% and was improved by the removal of low molecular weight contaminants during ultrafiltration. Although it was not tried in this study it is possible that a combination of enzyme digestion, alkaline extraction, and ultrafiltration could be a route to the production of a high yield, high purity protein powder.

Proteomics analysis of the date seed protein obtained from the Ph/TCA extracted sample using LC-MSMS revealed a large number of proteins in the date seed protein sample. As would be expected the majority of these proteins (70% by number) were associated with metabolic processes in the seed and seedling, followed by 15% (by number) of storage proteins such as 11S and 7S globulin (glycinin and β-conglycinin).

The two most abundant proteins in the date seed sample were glycinin and β-conglycinin, the seed storage proteins. Although it was not possible from the LC-MSMS results to determine the absolute proportions of the different proteins in the date seed sample (i.e. the weight %) from comparison with the SDS-PAGE results (Chapter 5, Figures 5.3, 5.5 and 5.7) we expect most of the protein to be comprised of these two proteins. In soy bean they make up 70% of the total protein (Utsumi et al., 1997). If this is the case with date seed protein, and our results do suggest a high wt% of the 11S and 7S globulins, we would expect this to be reflected in the functional properties of the date seed proteins.

It was not possible to study the gelation properties of the date seed proteins directly, since the amount of protein obtained using the Ph/TCA extraction method was not sufficient for the high concentration solutions needed for this test. Instead, we studied the thermal properties indirectly using DSC. The denaturation peak for all date seed protein samples was a broad peak which suggested a mixture of proteins. None of the peaks corresponded directly to the denaturation temperatures for the two major protein components i.e. 90˚C for glycinin and 72˚C for β-conglycinin. This is not inconsistent with a mixture of proteins dominated by glycinin and β-conglycinin but cannot be

taken as conclusive proof on its own. For the date seed protein with the highest purity (68%) a second peak appeared in the thermogram at 120˚C.

A possible explanation is that this peak may be due to high molecular weight multimeric aggregates of glycinin and β-conglycinin which are known to be highly stable when heated at temperatures up to 100˚C (Nakamura et al., 1984).

The emulsifying properties of the date palm seed protein isolate (DPSPI) made using the phenol/TCA extraction method was compared to SPI and WPC. The DPSPI had a lower emulsifying activity (EAI) than both SPI and WPC at all pH values tested (Chapter 6, Figure 6.16). This could have been partly a result of the lower protein content of the DPSPI since based on the probable composition from proteomics and SDS-PAGE results we would have expected the DPSPI emulsifying behaviour to have been similar to SPI which also has a high proportion of glycinin and β-conglycinin.

Somewhat surprisingly, the DPSPI emulsion showed greater emulsion stability (ESI) at a given EAI than did SPI and WPC (Chapter 6, Figure 6.18). The reason this is surprising is because the EAI is generally accepted as a measure of emulsion droplet size (larger EAI = smaller droplets) and droplet size is a major factor in emulsion stability (larger droplets = lower stability). However, when the EAI of the protein emulsions is plotted against protein powder solubility at each pH a linear relationship is observed where results for both DPSPI and WPC fall on the same line, along with some of the SPI results. This confirms the strong link between emulsifying properties and protein solubility. A similar plot of ESI against solubility shows a more complex behaviour where the enhanced stability of the DPSPI proteins is again evident.

In conclusion, the protein from date seed has proven difficult to extract with a high yield and high protein content using methods that might produce food grade protein product. However, when the protein is purified it has functionality and has emulsifying properties similar to SPI, and also adequate water and oil holding capacities.

It was not possible to test the foaming and gelation properties directly due to the small amounts of extracted protein that were available. However, based on DSC results it would be expected that the DPSPI would be able to form thermally induced gels if heated in solution at a high enough concentration.

On balance, it currently would be too difficult to isolate and purify the date seed proteins on a commercial scale, since the complex process required would lead to too great a cost for the powder. A large part of the problem would appear to be the close association between seed protein and the polysaccharide matrix of the seed, both endosperm and seed coat, which in date seed contains a high proportion of insoluble polysaccharide. Any further progress in this directs protein separation of date seed would need to await advances in the technology used to separate proteins from complex seed matrices.

7.2 Recommendations For Future Work

If date seed protein were to become a viable option as a protein source for food manufacturers progress will have to be made on the extraction of proteins from plant sources where they are in intimate contact with insoluble polysaccharides. Further research in this area would also be of general benefit for the extraction of protein from other plant sources. Any method that could be used successfully for the commercial extraction of the protein would need to be relatively cheap and use non-toxic chemicals to provide food grade materials. Further investigation of the use of enzymic digestion of the polysaccharides may be a profitable way forward, combined with ultrafiltration to remove the resulting soluble carbohydrate from the solution.
In addition, we were unable to carry out a thorough study of the functional properties of the date seed protein isolate because of the low quantities we were able to extract. Before date seed protein can be considered for use in foods the functional properties, including foaming and gelation, will also need to be characterised over a wider range of protein contents, and combinations of pH and salt that are relevant to food systems. They would also need to be tested in food formulations.

Appendix

Extraction and Characterisation of Protein Fraction from Date Palm Fruit Seeds

Ibrahim A. Akasha, Lydia Campbell, Stephen R. Euston

***Abstract*—**Date palm (Phoenix dactylifera L.) seeds are waste streams which are considered a major problem to the food industry. They contain potentially useful protein (10-15% of the whole date's weight). Global production, industrialisation and utilisation of dates are increasing steadily. The worldwide production of date palm fruit has increased from 1.8 million tons in 1961 to 6.9 million tons in 2005, thus from the global production of dates are almost 800.000 tonnes of date palm seeds are not currently used [1]. The current study was carried out to convert the date palm seeds into useful protein powder. Compositional analysis showed that the seeds were rich in protein and fat 5.64 and 8.14% respectively. We used several laboratory scale methods to extract proteins from seed to produce a high protein powder. These methods included simple acid or alkali extraction, with or without ultrafiltration and phenol trichloroacetic acid with acetone precipitation (Ph/TCA method). The highest protein content powder (68%) was obtained by Ph/TCA method with yield of material (44%) whereas; the use of just alkali extraction gave the lowest protein content of 8%, and a yield of 32%.

***Keywords*—**Date palm seed, Phoenix dactylifera L., extraction of date palm seed protein

I. Introduction

PROTEINS are valued by the food manufacturer for their functional properties (emulsification, gelation, foaming etc.) and for their nutritional value. Of late there has been concern over the sustainability of some food protein sources such as fish meal protein, and the rising cost of others such as egg proteins and soy proteins. This has led to the investigation of alternative protein sources for food use which can be used to either partially or fully replace more expensive proteins.

There is also an advantage in using material that was previously considered to be waste to recover useful functional proteins. Such waste includes the peel, skin and seeds of fruits and vegetables, materials which are either discarded or used in low value commodities such as animal feed. One waste product we are investigating to see if useful protein can be extracted is the seed of the date palm fruit.

There is limited published work focused on date seeds even though they contain potentially useful quantities of protein and fat/oil, in this study we attempted to recover a high proportion of the soluble protein from date seed. Previous studies by [2]; suggest that at least 53% of the total protein can be recovered using simple aqueous extraction techniques. We tried to improve on this by using methodologies that are standard in the protein industry, and are commonly used for protein powders derived from other seed products (e.g. soya bean protein). Protein extraction from seeds is problematic due to the relatively low proportion of protein and the high proportion of complex carbohydrates.

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II. Materials And Methods

A. Materials

Date palm fruit (Phoenix dactylifera L.) at Tamr stage (complete maturity) of the commercially available date palm *Deglet Nour* variety were purchased from a local supermarket. The seeds were manually isolated, soaked in water, washed to remove any remains date flesh, air dried for a week, and then were further dried overnight at 40ºC. The seeds were ground into a fine powder and defatted by extraction with hexane using a Soxhlet apparatus. The defatted powder was dried to form a date seed powder (DSP). All chemicals and solvents used in this study were Analar grade.

B. Methods

1. Physical analysis

Firstly, calyxes were removed to measure the physical characteristics including: weight of whole fruit, flesh and seed, flesh: whole fruit ratio and seed: whole fruit ratio. The weight of one hundred fruits and seeds was measured, and then the average of single fruit and seed were calculated.

2. Chemical analysis

Proximate analysis of date palm fruit (DPF) and date seed powder(DSP) including moisture and total solids, Total ash, Crude fat, Protein, Crude fibre were carried out according to[3]. The percent yield of protein was calculated using a formula published by [4]:

$$\text{Percent yield} = \frac{\text{Extracted sample (g)} \times \%\ \text{Protein in extracted sample}}{\text{Starting sample (g)} \times \%\ \text{Protein in starting sample}} \times 100$$

Total carbohydrate was calculated by difference as total percent value using the following formula: Total carbohydrates = 100 – (%moisture + % ash + % protein + % fat + % crude fibre).

All analytical determinations were carried out in triplicate and the final data were expressed on a dry weight basis.

C. Laboratory preparation of date palm seed protein extract

Five methods were use to extract protein from DSP.

- *Method 1*

Method 1 was based on the procedure for concentrating the protein from soybean proposed by [5] with some modifications. This involved solubilisation of non-protein contaminants in defatted DSP using water/HCl, pH 4.5, 40ºC, 60min, centrifugal separation, neutralization of the sediment with 1M NaoH and freeze drying to form a protein concentrate.

- *Method 2*

Method 2 was based on the method by [6]. This involved solubilisation of non-protein contaminants in DPSP using water/NaOH, pH 10, 55ºC, 60min, centrifugal separation, washing of the sediment with distilled H2O, centrifugation,

resolubilization of pellet in 1M NaoH at pH 7 and freeze drying to form a protein concentrate.

- *Method 3*

Method 3 was based on method by[7]. DPSP was mixed with water/NaOH, pH 10, room temperature, 60min, and then centrifugally separated. The supernatant was kept for further treatment. The pellet was mixed with distilled water/NaOH, pH 10.0 centrifuged. The two supernatants were combined and ultrafiltered (10 kDa membrane) and freeze dried.

- *Method 4*

The same as method 2 except that after the pellet was solubilised the slurry was then ultrafiltered (10 kDa membrane) and freeze dried.

- *Method 5*

Method 5 was based on a method from [8], [9]. DPSP was mixed with cold acetone, vortexed and centrifuged. The pellet was washed with cold acetone twice and allowed to dry at room temperature. The pellet was ground to fine powder, rinsed with 15% w/v TCA in acetone, vortexed and then centrifuged. This was repeated 3x. Finally, the pellet is washed with cold 15% w/v TCA in water and another three 3x with cold 80% v/v acetone.

The pellet was suspended in a 1:1 mixture of Tris-buffer, pH 8.0 and dense SDS buffer (2%w/v SDS, 5%w/v sucrose 0.1M Tris-HCL, pH 8.0, 5% v/v β-mercaptoethanol), vortexed and centrifuged.

The pellet was re-suspended in the same buffer and centrifuged. Both supernatents were mixed and precipitated at 4 ºC overnight with five volumes of cold 0.1M ammonium acetate in methanol, and centrifuged The pellet was then washed three times with 0.1M ammonium acetate in methanol and centrifuged as above followed by the same process with cold 80% v/v acetone.

The pellet was mixed with aqueous 24%w/v TCA, vortexed, precipitate on ice for 30min, and centrifuged. The pellet was washed with cold acetone, incubated 15min on ice and centrifuged and dried.

III. Results and Discussion

A. Some physical measurements

The physical characteristics of date palm fruit provides important information to the date industry that helps trading, processing and storage of dates.

Table I summarizes the physical measurements, for the date variety used in this study. The data indicate that seed and flesh comprised 10 and 90% respectively of the mass of the whole fruit.

The results were higher than those reported by [10], which is most likely due to a difference in the variety of dates used, as well as differences in agriculture treatments and environmental conditions.

TABLE I
SOME PHYSICAL MEASUREMENTS OF DATE PALM (FLESH&SEED)

Measurements	Average
weight of whole fruit(g)	9.51*
weight of date flesh(g)	8.53
weight of date seed(g)	0.98
Flesh: whole fruit ratio	89.70
Seed: whole fruit ratio	10.30
Number of fruit in Kg	106.00

*All values are means of three determinations.

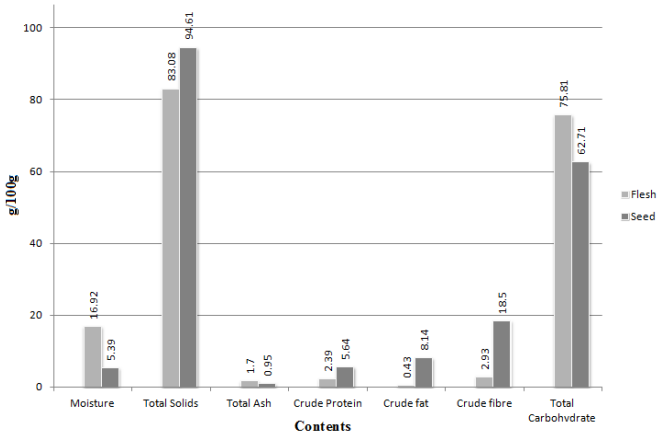

Fig. 1 proximate analysis of date palm (flesh and seed)

B. Proximate Composition of DPSP and DPF

Figure (1) presents the chemical composition of date palm flesh and seed. The total solids, crude protein, crude fat and crude fibre of date seed were higher than those in date flesh, whereas, moisture, total ash and total carbohydrate were lower than those in date flesh. Crude protein and crude fat were higher in the seed than in the flesh. The results of both (seed and flesh) contents were agreement with those reported by [11], [12] and [13], higher than those reported by [14], [15] and lower than those reported by [16], [17] and [18]. Those differences are most likely due to the variability between cultivars, as well as stage of maturity [19].

C. Chemical compositions of full fat DPSP and defatted DPSP

Prior to protein extraction, removal of the oil fraction from the seed is desirable. This changes the chemical composition of the powder. The chemical composition of full fat and defatted of date palm seed is presented in Figure 2. The fraction of all non-oil components increases slightly, as expected. In addition the color of defatted DSP was lighter than the original DSP.

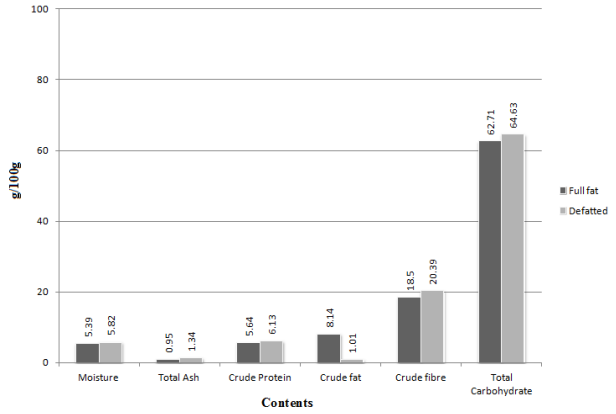

Fig. 2 Chemical compositions of full fat and defatted DSP

D. Protein extraction of date palm seed

Figure 3 presents results for the protein content and yield for the five procedures used to prepare date palm seed protein concentrates. illustrating the difficulty in removing protein from plant seeds. All methods were able to extract protein at a higher concentration than in the original powder. The protein content in DSP increased from 8.65% (Method 1) to 68.24 (Method 5) while the lowest protein extract was obtained by alkaline extraction (Method 1) The yield of protein extracted from the DSP ranged from 27-52%; 52.5%, with the highest protein yield obtained by using UF along with the alkaline extraction (Method 4) whereas, using just the alkaline extraction (Method 2) gave the lower protein yield. these The differences in protein content and yield are likely due to the presence of polysaccharides or other high molecular weight contaminants present free in solution or conjugated to the protein.

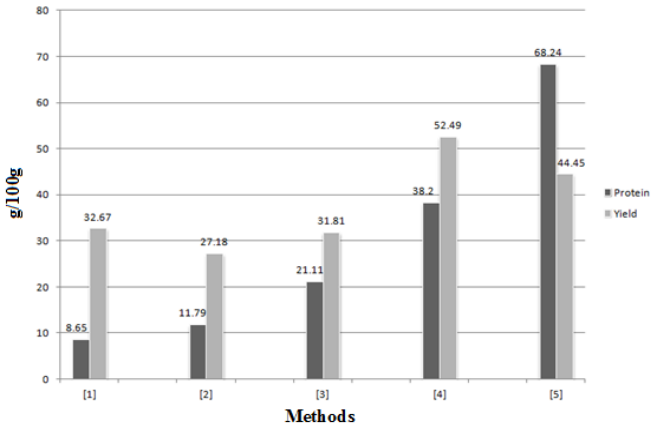

Fig. 3 Laboratory methods to extract date palm seed proteins

IV. Conclusions and Future Work

The increasing cost of foods particular food protein from animal source in the world has encouraged the food scientists to develop new source of plant proteins; date palm (seed or flesh) could be one of these sources. It is clear from the current results that protein of a relatively high quality can be recovered DSP, although it remains to be seen whether this can be done on a commercially viable scale. Any such protein could be used for animal feeds.

Additional experiments are under way in our lab to improve extraction using enzymatic methods to breakdown complex polysaccharides that interfere with the extraction of high purity powders. We are also carrying out studies to identify the proteins in of concentrated date palm protein powders using techniques such as 2D electrophoresis combined with Maldi-tof mass spectrometry. Further work will look at the functional properties of seed proteins to see if they may be of use as ingredients (emuslifiers, foamers or thickening/gellling agents in formulated foods.

Acknowledgment

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