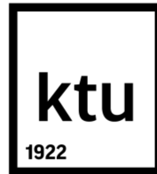

KAUNAS UNIVERSITY OF TECHNOLOGY

FACULTY OF CHEMICAL TECHNOLOGY

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PROTEIN FUNCTIONAL PROPERTIES CHANGES IN MODIFIED RAPESEED (*Brassica napus*) PRESS-CAKE

Master's thesis

Supervisor

Prof. habil. dr. Gražina Juodeikienė

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"PROTEIN FUNCTIONAL PROPERTIES CHANGES IN RAPESEED (*BRASSICA NAPUS*) PRESS CAKE"

DECLARATION OF ACADEMIC INTEGRITY

07 06 2017

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Santrauka

Didėjanti žmonių populiacija pasaulyje verčia ieškoti naujų maisto šaltinių, nepadidinant maisto gamybos ir tiekimo grandinės. Rapsų sėklos yra antri pagal apimtis plačiausiai auginami aliejiniai augalai. Tačiau juose esantys palyginti dideli kiekiai toksinių ir antimikrobinių faktorių tokių, kaip gliukozinolatai, fitatai ir fenoliai, riboja jų platų panaudojimą, kaip baltymų šaltinį, žmonių mityboje. Šio darbo tikslas buvo modifikuoti rapsų sėklų išspaudas, praturtinant klasifikavimo oru būdu baltymais turtingas frakcijas. Eksperimento metu tirtos rapsų sėklų miltai, rapsų sėklų išspaudos ir koncentratai. Baltymų tirpumas (procentais) buvo analizuotas esant pH ribose nuo 1 iki 10 ir pastebėta, kad tirpumas baltymų padidėja tolstant nuo izoelektrinio taško ribų (pH 3-5). Nustatytas atvirštinis ryšys tarp vandens įgėrimo ir riebalų surišimo gebos.

Antroje tiriamojo darbo dalyje ruošti rapsų sėklų išspaudų mišiniai su *T. molitor larvae* priedu (9:1), kurie buvo ekstruduoti ir tirtas jų baltymų tirpumas. Nustatyta, kad ekstruzijos proceso metu sumažėjo rapsų baltymų tirpumas.

Kolli Sahana. PROTEIN FUNCTIONAL PROPERTIES CHANGES IN MODIFIED RAPESEED (Brassica Napus) PRESS CAKE. Master's thesis in Food Science and Technology/ supervisor Prof., habil. dr. Gražina Juodeikienė. The Faculty of Chemical Technology, Kaunas University of Technology.

Research area and field: Technological Sciences, Food Technology.

Key words: *rapeseed, air-classified, protein solubility, WBC and FBC, T.molitor larvae, Extrusion.*

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SUMMARY

Increasing number of people is the driving force for growing demand for food, which is however not being accompanied by increase in food supply. Rapeseed is the second most extensively grown oilseed crop. However, due to presence of considerable amount of toxic and anti-nutritive factors such as glucosinolates, phytates, and phenols, which reduces its capacity to serve as potential protein source to humans. Hence, aim of the project is to modify rapeseed press cake by enriching protein fractions by air-classification. The physiochemical properties of air classified samples along with RPF, RPC and concentrates were determined. The percent protein solubility of samples were analysed over a pH range of 1-10 and it has been observed that protein solubility increases on moving away from the isoelectric point (pH 3-5). The water binding capacity (WBC) and fat binding capacity (FBC) of samples were found to be negatively related.

In second part of the project, protein solubility of Rapeseed press cake was studied under the influence of digestive enzymes of T.molitor larvae (subjected to starvation for 0hrs, 24 hrs and 48 hrs) were cake in combination with extrusion. Protein solubility has decreased considerably due to formation of disulphide linkages which has resulted in protein aggregation while extrusion.

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ABBREVATIONS

BSA-Bovine Serum Albumin

NaOH- SodiumHydroxide

HCl-Hydrogenchloride

RPC- Rapeseed press cake

RPCD- rapeseed press cake defatted

RPF- Rapeseed four

AC- Albumin concentrate

GC- Globulin concentrate

AI- Albumin isolate

GI- globulin isolate

Mw. molecular weight

Kd- kilo dalton

WBC- Water binding capacity

FBC- Fat binding capacity

SDS-PAGE- Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

APS- ammonium per sulphate

DES- Defatted extruded samples

NDES- Non-defatted extruded samples

RPC_C- Rapeseed press cake control

RPCD_2 Rapeseed press cake at pH 2

RPCD_8 Rapeseed press cake at pH 8

INTRODUCTION

The world's population is estimated to be 34 percent higher than the present conditions, it is expected to reach 9.1 billion at the advent of 2050. In today's time, almost one person out of seven people around the world are subjected to malnutrition [1]. It can be supported by the reports suggested by United Nations Food and Agriculture Organizations (FAO) that about 795 million out of 7.3 billion people in the world are undernourished (FAO 2014). Sub-Saharan Africa puts up an unmatched population growth rate and even if we suppose the fertility rate will show decrement it is outlined that population would be more than twice its today's population, by 2050 [1] . Increasing number of people is the driving force for growing demand for food, which is however not being accompanied by increase in food supplies. Furthermore, urbanization and industrialization has shaped world food economy, consequently shifting the pattern of food consumption towards livestock products. In the developing part of the world, milk and dairy products are consumed at 3.4-3.8 percent p. a. whereas meat is consumed at 5-6 percent per p. a. The immense dependency on livestock, not only has severe implications on forest land but also is the prime factor driving exhaustion of farming land, responsible for producing supplies like cereals, oilseeds, vegetables and fruits. Subsequently, incorporation of higher portions of animal proteins in our diet impacts global hunger and malnutrition since it imposes additional produce and grains to incline towards feeding livestock resulting in feeding lesser people [3]. Furthermore, it is creating dysfunctionality in the crop-livestock synergies existing in mixed farming systems [3]. The livestock sector has serious environmental implications which to an extent is due to policy falsifications and failing to adhere to rules and regulations. In countries like Brazil, for instance, the major cause for deforestation has been over utilisation of productive land for livestock development and in few other countries it is replaced by overgrazing. In 2006, according to the survey conducted by The United Nations Food and Agriculture Organization, livestock is estimated to emit 18 percent of greenhouse gases, such as methane and nitrous oxide, which is even higher than transport and are 25 and 300 times more poisonous than carbon dioxide. Soaring levels of greenhouse gases are extenuating conditions such as rising temperatures, melting ice caps and glaciers, shifting ocean currents and weather patterns, creating threat to the existence of human race, reduction in biodiversity and depletion of natural resources [3].

LITERATURE REVIEW

One of the favourable substitutes to reduce the dependency on meat consumption and overcome malnutrition is by partially replacing with plant proteins, where legumes and oilseeds are an important source of these macromolecules (Boye, J.I., Aksay, S., Roufik, S., Ribéreau, M., Mondor, M., &Farnworth, E. (2010a). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation technological. Food Res. Int., 43: 537-548). There is a well-established stature of plant proteins in functional foods, as food additives, in nutraceuticals, promoting good health and disease risk reduction. Soybeans, Rapeseed, cotton, sunflower and groundnut are the most common sources of vegetable proteins and accounts for 69, 12.4, 6.9, 5.3 and 2.8% of world production of vegetable proteins, respectively (Moure, A.J., Domínguez, H. & Parajó, J.C. (2006). Functionally of oilseed protein products: A review. Food Res. Int., 39: 945-963).After extraction of oil, the press cake, especially from oilseeds, have potential protein sources that are being explored worldwide. The negative image of soy production and allergenicity attached to soy along with the increasing demand for gluten-free products opened a clear market potential for rapeseed proteins to be winner in this area (OECD-FAO Agricultural Outlook 2015–2024). Rapeseed is the second most extensively grown oilseed crop. Canada ranks first in the world for large scale-production of high quality rapeseed, which are usually characterized with low uric acid (<2%) and glucosinolates (<30 Imol/g). In Australia, it is imported and produced as the principle oil seed crop and captures 96% of the total oilseeds production. The canola seed contains 40% oil and about 17-26% protein whereas rapeseed press cake (by product of rapeseed after oil extraction) contains 50% of protein on a dry basis. However, due to presence of considerable amount of toxic and anti-nutritive factors such as glucosinolates, phytates, and phenols, which reduces its capacity to serve as potential protein source to humans. Therefore, large portion of rapeseed proteins after oil extraction is being directed towards livestock nutrition. Hence, there is a need for a technology to transform plant proteins into palatable products.

Origin and evolution

Rapeseed *(Brassica napus*), an important oilseed crop, member of the mustard family Brassicaceae (also called as *Cruciferae*). In Europe it is called with the term Rapeseed or double low rapeseed, whereas it is preferably termed as “Canola” in Canada and Australia. The name

rape is gained from the Latin word “rapum” which means turnip. It has been suggested that Rapeseed proteins is known for their nutritional value as well as their functional properties from early 80’s. In present day scenario, rapeseed is grown primarily for its healthy oil seeds and used particularly in Salad dressing and cooking purposes. However, it is indicated from early records that initially, rapeseed oil was used in oil lamps for illumination and later got its purpose as cooking oil during ancient civilization in Asia and along the Mediterranean. Moreover, it is reported that B. juncea and B. campestris are the two species which have a range of morphotypes and were cultivated in India over 3,000 years ago, much before the Christian era, for cooking, frying, as seasoning ingredient and also for religious ceremonies (Mehra, 1966). History suggests that roots of rapeseed cultivation are from India, china and Japan. The Greek, Roman and Chinese writings of 500-200 BC highlights the medicinal value of this crop. Archaeological findings have witnessed impressions of seeds of B. juncea from the findings of Indus valley Civilization ca 2300-1750. Species of genus Brassica were cultivated in ancient Rome and Gallia, and during Bronze Age the seeds belonging to these species were found in German graves and Swiss constructions too. Although, there is no clear picture of which species were cultivated. However, at the outbreak of Second World War, rapeseed oil gained immense popularity because it was an excellent lubricative agent for marine engines. During the last 35 years, the world production of rapeseed has reached 6 times the production volume in 1980. Today, after soybean, Rapeseed is the second most widely cultivated oilseed crop (OECD-FAO, 2015). Although, it’s grown mostly for its wholesome healthy oil, it has potential to be used as biofuels, cosmetics and in other industrial products. Our interest lies in the fact that rapeseed generates 10-14 million metric tons of plant protein in parallel with 20-30 million metric tons of oil, which is primarily benefitted by the livestock industry as feed in the form of de-oiled rapeseed meal.

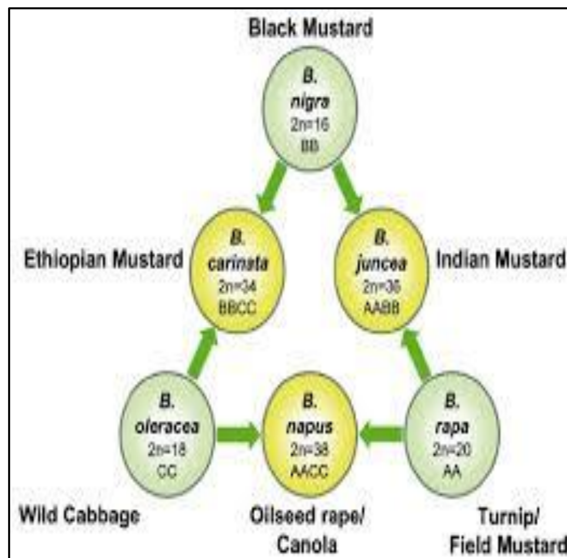


Figure 1: relationship between various Brassica species. Adapted from Ahuja et al. (2010).

Composition, Nutritive and Biological value of Rapeseed meal

Amino acid profile

Rapeseed proteins, present in the rapeseed press cake after oil extraction, have the capacity to reach 40% on a dry basis depending on its variety. The quality of proteins can be determined by protein efficiency ratio (PER) which is gain in body mass with respect to the amount of protein intake. PER below 1.5 considers the protein to be of poor or low quality; between the range of 1.5 and 2.0, it is described as protein of intermediate quality and PER higher than 2.0, protein is defined as good /high quality protein. In that case, rapeseed proteins are expected to have PER of about 2.64, which leaves Soybean meal behind which exhibits PER of 2.19 (Tan et al., 2011a). Rapeseed proteins show a well-balanced amino acid composition and this have been reported in various papers. Glutamine, glutamic acid, arginine and leucine are present in higher quantities whereas like other oil seeds it is limited in lysine. Additionally, they are also rich in sulfur containing amino acids such as cysteine, methionine, glutathione and homocysteine, which effectively regulates antioxidant defence processes (Fleddermann et al., 2013) (http://www.canolacouncil.org, 2015b). However, amino acid composition strongly depends on extraction process. Nevertheless, 30% of the total protein can be extracted in alkaline medium but the successive purification steps at industrial scale changes the protein composition of the resulting meal (Chabanon, Chevalot, Framboisier, Chenu, & Marc, 2007). Reports presented by shahid unveil that rapeseed meal which were analysed had high glutamic acid (16.77-18.63% w/w protein) but lower concentrations of tyrosine, methionine and cysteine were found. Two-phase solvent extraction method did not show any effective results in altering the amino acid compositions of rapeseed meal. However, there was a slight increase in the cysteine levels in other varieties of rapeseed meal, when two-phase solvent extraction was performed.

Protein quality can also be evaluated by calculating protein digestibility corrected amino acid score (PDCAAS) value. It has been investigated that rapeseed meal satisfies the requirement for non-essential amino acids in adults and 10-12 years old by having a score > 1.00 as reported by Klockeman et al. (1997). However, it is also reported that, reference values were < 1.00 for infants and children between 2-5 years old with lowest scores attributing to “methionine and cysteine” in former case and “lysine” in the latter case. Besides this, work done by Fleddermann et al., (2013) conveyed that PDCASS for rapeseed proteins is equal to 0.83-0.93 which can be considered in close proximity with animal proteins such as egg (1.00), milk (1.00) and fish (1.00). Additionally, nitrogen digestibility of rapeseed is quiet appreciable as it shows 93%-97%, higher than wheat (91%) and beans (78%) and hence it can be regarded with proteins derived from animals such as egg (98%), casein (95%) and collagen (95%) (Fleddermann et al., (2013)).

Major protein fractions

For rapeseed to be dispositioned as a potential value-added food and nutraceutical ingredients in the long run it is important to explore and elucidate the fundamental properties of rapeseed proteins. The major rapeseed proteins of interest are seed storage proteins as they are available in generous amounts in the embryo and their identification and characterization attracts economic interests (Shewry et.al. (1995)). They constitute of 80% of the total proteins (Hoglund, Rodin, Larsson, & Rask, 1992; Mieth et al.1983) and were differentiated into four fractions for the very first time by Osborne (1924), depending on their solubility in water, diluted salt, aqueous alcohol and weakly acidic or alkaline solutions. According to solubility characteristics, they are namely albumins, which are water soluble; globulins, which are salt soluble; prolamins, which are soluble in ethanol; and lastly glutelins, which are not soluble in any of the solvents mentioned above. Over time albumins, globulins and prolamins are most promising due to their growing economic importance. They can be differentiated from each other based on sedimentation coefficients measured in Svedberg units (S), indicates the speed of sedimentation of a macromolecule in a centrifugal field. Out of all the major protein fractions mentioned above, Cruciferin and Napin are the two majorly studied groups of rapeseed storage proteins, which are different from each other because of their sedimentation coefficients, molecular weights and type of molecular interactions that regulates structure stabilization. These factors collectively determine their functionalities in food systems (thesis).

The main seed storage proteins which are focussed in the present work, are the major N-storage proteins, Cruciferin (11S globulin) and Napin (2S albumin), constitute ca. 60% and 20% of the total protein, respectively, although the variation in ratio is viable between cultivars (Höglund et al., 1992). It has been reported that, particularly in European genotypes which are low erucic acid and glucosinolates, cruciferin to napin ratio ranges from 0.6 to 2.0.

In *Brassica napus*, 11S globulin, Cruciferin, is an important storage protein whose accountability is approximately 60% of the total seed storage protein. Cruciferin is characterized under cupin super family, having a hexameric quaternary structure of 300-310 kDa. Rapeseed Cruciferin is a group of proteins that are derived from as many as five different subunits namely CRU1, CRU2, CRU3, CRUA and CRU5 (Sjödahl, Rödin, & Rask, 1991; Wanasundara, 2011). Rapeseed cruciferin typically exists as a hexamer (6 subunits) of 50 kDa with two polypeptide chains, each comprising of an acidic heavy chain (α=40 kDa) and a basic light chain (β=20 kDa) (Wu and Muir, 2008). Additionally, the α- chain holds approximately 254 to 296 amino acids whereas β-chains holds approximately 189-191 amino acids. Variation in the number of α- and β- chains is possible in every subunit due to number and type of amino acid residues. The crystal structure of protein cruciferin is acquired as a result of X-ray diffraction analysis which conveys that

A3B4 promoter in soyabean glycinin is similar to cruciferin, which also acquires 25-27 β- sheets and 7 α- helices that forms two jellyroll β-barrel domains and two extended α- helix domains by mechanism of folding (Adachi et al., 2003). The hexameric form dominates at higher ionic strength and mildly alkaline conditions. However, alteration in pH and ionic strength can foster reversible dissociation of the polypeptides (Schwenke et al., 1983). In the tertiary structure of cruciferin α- chains are supposed to come in contact with the soluble environment. On the other hand, β- chains are presumed to be hidden within the structure of the molecule. The quaternary structure is composed of two trimers that provides Cruciferin the shape of a hexamer. Each of these trimers are characterized with IE and IA faces, which denotes that IE face contains inter-chain disulfide bonds and IA face is known to contain intra-chain disulfide bonds. Disulfide bonds play a very crucial rule in stabilising the structure of Cruciferin as, it is compulsory for two trimers to join together via IE face in order to form hexamer. Hexamer formation is also aided by various other bonds predominantly non-covalent bonds, followed by hydrophobic, electrostatic, hydrogen, van de walls and hydrogen- bonded salt bridges (Adachi et al., 2003). Structural homology can be established with other 11/12-S seed proteins. Like other oilseeds, dissociation of proteins under the influence of external factors can be witnessed. The process is shown below.

Cruciferin is characterized as “neutral protein” with isoelectric point equal to 7.25±0.10. It is reported to exhibit a wide range of isoelectric points from pH 4.75 to 9.15 when dissociated to several subunits. Isoelectric point and secondary structure can be influenced by the type of cultivar as well, which has an impact on the ratio of acidic to basic amino-acids. This can further effect the secondary structure, which is mostly characterized by circular dichroism. According to Tan et al., (2011a) its α-helix is relatively low when compared to its high β-pleated structure.

Napin is a major 2S albumin with molecular weight staging between 12 and 17.7 KDa (Monsalve and Rodriguez, 1990). The synthesis of napin is initiated in membrane-bound ribosomes with prepronapin precursor as its first form in the process of becoming a mature napin. In the second stage, translocation into lumen of endoplasmic recticulum and detachment of signal peptide to pronapin occurs which is connected by intra chain disulfide bonds (Ericson et al., 1986). In the last stage, proteolytic cleavage in the PSV leads to the formation of mature napin that consists of subunits, i.e. the short chain and long chain (Ericson et al., 1986; Murén, Ek, Björk, & Rask, 1996). These polypeptide chains are linked to each other via two inter-chain disulfide bonds; wherein the cysteine residues present in the long chain are linked through two intra-chain disulfide bonds. Similar to Cruciferin, the expression of napin is synchronized by

multiple genes which range from ten to sixteen (Scofield & Crouch; 1987; Raynal, Depigny, Grellet, & Delseny, 1992). The secondary structure of *B. napus* reveals greater percentage of α-helices (~ 48.6 to 59%) and fewer β-sheets (7 to 15%) over an extensive pH range of 3 to 12. The three dimensional structure is displayed in a loop region distinguished with an "up and down" topology by four helical motifs. A right- handed super helix is formed by the long chain and short chain, where the long chain forms three helices namely HII, HIII and HIV and the short chain tends to form a split helix by assembling two short helices namely HIa and HIb with few amino acid residues which are distinct.

Napin is a water-soluble, basic protein with the isoelectric point in the pH range of 9-10 As mentioned above, as disulfide links are considered as major stabilizing bonds, they ensure maintenance of structural confirmation at high and low pH points and also with various electrolyte concentrations (Wanasundara, 2011) (Mieth et al., 1983). Due to high level of amidation of amino acids, napin is identified with strong basic character. Napin have been resulted as a hydrophilic protein when measured through Fluorescent probes (Jyothi etal. , 2007).

Carbohydrates, Vitamins and Minerals

Carbohydrates matrix is quiet complex and possess 15% of the total rapeseed meal which consists of starch, free sugars and non-starch polysaccharides. The rapeseed carbohydrates are presumed to contribute significantly to the digestible energy but its potential gets hindered as they appear to be guarded by the cell wall. It is mostly composed of pectins (50%). Cellulose (24.1%) and pentosans (25.9%) (Bell, 1984). Rapeseed meal consists of 8-10% sucrose, 2-3% oligosaccharides, 20-22% non-starch polysaccharides (NSP) and 5-8% lignins and polyphenols. D-glucose, D-fructose, D-galactose, sucrose melibiose, raffinose, maninotriose and stachyose are soluble sugars which comprise of about 48% of low molecular weight carbohydrates. Although these sugars are removed during processing because of their solubility, these compounds are the main reason of flatulence (Bell.1984). In comparison to Soybean, rapeseed meal comprises of 30 percent of dietary fibre in the hulls fraction, which unlike soybean is not eliminated during processing. Unlike Soybean meal, the separation of hulls from the seed is challenging since the hulls fraction stays with the seed portion and moreover, the hulls fraction in rapeseed is comparatively higher than its counterpart. Due to its poor digestibility rapeseed meal is mostly used as a secondary feed to livestock. Also, ratio of NDF (neutral detergent fibre) to acid detergent fibre (ADF) is relatively low which enhances the convenience of feeding rapeseed meal to ruminants. In this situation, to boost the digestibility and metabolizing energy of rapeseed meal, de-hulling prior to processing can prove significant in removing the dietary fibre Rapeseed meal is a very good source of Selenium and phosphorus. Like, other plant sources of

phosphorus, it is present as phytate, in considerable amounts. Besides this, rapeseed meal contains modest amounts of vitamins such as Choline, biotin, folic acid, niacin, riboflavin, and thiamine. Mineral content basically depends on the soil quality, geographical and climatic parameters (Newkirk, 2011).

Anti-nutritive Factors and phenolic compounds

Glucosinolates

The presence of Glucosinolates in Rapeseed cultivars (120-150 µmol/g) is relatively more in comparison with glucosinolates in Canola (10-12 µmol/g). Total Glucosinolate content of Canola meal in Canada has been reduced to about 7.2 µmol/g which is one of the major improvements brought by breeders. Glucosinolates are hydrolysed into toxic compounds such as nitriles, isothiocyanates, and thiocyanates which is catalysed by the presence of an endoenzyme, myrosinase. Moreover nature of glucosinolates and myrosinases causes variation in the pattern and amount of hydrolysed compounds. Higher levels of aglucon portion of glucosinolates arises conditions such as reduced feed intake, enlarged thyroid, reduced plasma thyroid hormone levels, and may also attack kidney or liver. However, considering the negative implications associated with the presence of glucosinolates, a two- solvent oil extraction method was developed to reduce the amount of glucosinolates in rapeseed meal to negligible levels (Naczk et al., 1985). Interestingly, it is also reported that low level of glucosinolates, and its metabolites have the ability to exhibit different kinds of biological activities (Bernhoft, 2010). For instance, Song and Thornalley (2007), reported that as much as 0.61 umol/g of glucosinolate found in broccoli can reduce the risk of cancer. Nevertheless, it is mandatory to focus on possible ways to reduce glucosinolates level and improve the potential of extracted rapeseed proteins for human consumption.

Component	Average
Starch (%)	5.1
Sugars (%)	6.7
Sucrose (%)	6.2
Fructose + glucose (%)	0.5
Cellulose (%)	4.5
Oligosaccharides (%)	2.2
NSPs (%)	15.7
Soluble NSPs (%)	1.4
Insoluble NSPs (%)	14.4
Crude fiber (%)	11.7
ADF (%)	16.8
Acid detergent lignin (%)	5.1
NDF (%)	20.7
Total dietary fiber (%)	32.3
Calcium (%)	0.64
Phosphorus (%)	1.12
Magnesium (%)	0.56
Copper (mg/kg)	6.2
Zinc (mg/kg)	68.2
Iron (mg/kg)	188
Manganese (mg/kg)	55
Biotin (mg/kg)	0.96
Choline (mg/kg)	6500
Folic acid (mg/kg)	2.3
Niacin (mg/kg)	156
Pantothenic acid (mg/kg)	9.3
Pyridoxine (mg/kg)	7
Riboflavin (mg/kg)	5.7
Thiamine (mg/kg)	5.1
Vitamin E (mg/kg)	13

NSPS, non-starch polysaccharides
ADF, acid detergent fiber
NDF, neutral detergent fiber

Figure 2: Representation of Carbohydrate and mineral content of rapeseed meal. Referred from Bell et al. (1999); Downey (1990); Newkirk (2011).

Phytic acid

Like many plants and oilseeds, phosphorus is stored in the form of phytic acid (phytates) in rapeseed, which acts as an anti-nutritive factor by inhibiting the bioavailability of proteins and minerals by reducing absorption. It forms complexes with the basic proteins and results in protein –phytate complexes which restrain the action of enzymes that are responsible for digestion such as pepsin, trypsin and α- amylase (Rodrigues et al., 2012). This results in reduced protein digestibility and amino acid availability (Thompson 1990).Furthermore, it has been found that it also lowers starch digestion and absorption as a result of poor amylase activity (Yoon and others 1983). On the other hand, it is a strong chelating agent that binds with di- and trivalent metals, such as calcium, magnesium, zinc, and iron, to form compounds which are not absorbed from the intestine because of their poor solubility (Liener, 1994). It is reported that 0.53% is phytate bound protein out of 1.22% of total protein in rapeseed meal.

Phenolic Compounds

According to Nowak et al. (1992), rapeseed proteins contains highest amount of phenolic compounds in comparison with other oil seeds. Phenolic acid esters concentration in rapeseed is considered to be 30 times more prevalent than Soybean. Total content of phenolic acid ranges from 6400- 18400 µg/ g which relies on the variety and processing condition. Rapeseed meal without hulls has lower amount of phenolic acid in comparison to rapeseed meal with hulls. Growing condition and extent of maturation also effects the phenolic composition. Phenolic acids that are present in the free form, constitute (6.5-9.0%), esterified forms may constitute as much as 99% of total phenolics. Sinapine, is the prominent phenolic ester which is produced by esterification of sinapic acid with choline, sinapolycholine, followed by sinapolyglucose. Vanillinic acid, p-hydroxybenzoic acid and syringic acid are various other phenolic acids which form ester bonds as well (Kozlowska et al., 1983b). Sinapate esters provides bitter taste, dark colour to rapeseed meal and also to extracted proteins which reduces its significance in the market. Rapeseed meal and insoluble bound acids and are compounds that are formed from benzoic acid and cinnamic acids (Naczk et al., 1998b).

Nonetheless, Rapeseed proteins are nutritionally valuable ingredients in rapeseed meal for food and feed applications and there is a need to develop processing steps which will minimize the aforementioned secondary plant metabolites which not only enhance the nutritive value of rapeseed proteins but also impair sensory characteristics.

Protein Extraction

Rapeseed oil production stands second, behind soybean among all the commercial oilseed crops. Generally, plant proteins contain various undesirable compounds which affects the organoleptic/ functional properties of proteins. For instance, as stated earlier rapeseed proteins like other oilseed proteins contain phenolic compounds and phytates that make them unsuitable for human consumption due to substandard organoleptic properties and poor functional properties. Rapeseed meal contains high levels of secondary metabolites such as phenolic compounds and phytic acid, which tend to interfere during extraction of proteins. Depending on the variety of rapeseed, extraction and processing conditions can vary which will ultimately affect protein functionality. Hence, to develop a new protein food ingredient, it is important to select the extraction process, that suffice our need to characterize protein of interest. Commonly, prior to extraction of individual proteins, "rape-seeds" are grounded by milling process and are subjected to defatting once the meal consistency is attained. Defatting is usually carried out by Soxhlet apparatus and mostly hexane is used as solvent in removal of fat from the meal. Defatted meal is subjected to drying either by using a fume hood or vacuum oven at 40° C. Defatted and dried meal is allowed to pass through a 40-mesh or 60-mesh screen in order to ensure consistency and assure thorough mixing with the chemicals during the later stages of extraction process.

Extraction and precipitation of protein by alkaline medium

According to the literature, mostly, rapeseed protein extraction is generalized to be either alkali extracted followed by acid precipitation or using the method introduced by Murray et al (1980). Preparation of rapeseed protein isolates, usually involves extraction using sodium hydroxide (NaOH), where the pH of the solvent is adjusted to strong basic conditions (pH 11-12) to have maximum protein recovery (Tan et al., 2011) and finally precipitation occurs in the presence of dilute acid (Klockeman and others 1997; Aluko and McIntosh 2001; Can Karaca et al., 2011). Apart from pH various factors affect the extraction process especially concentration of NaOH, followed by centrifugation and filtration and in the final stage, pH and type of acid used for precipitation. Work done by Sosulski (1983) and Mieth and others (1983), had maintained pH around 11-12 to obtain greater degree of nitrogen solubility and extraction rate, whereas, Tzeng and others (1988a) have reported to maintain pH by addition of 50% w/w NaOH solution. To prevent oxidation by interaction between proteins and phenolic compounds, 10% sodium bisulphite (Na_2SO_3) had been used while extraction. Besides NaOH, various other extracting solutions have been tested, one of such solutions is sodium hexametaphosphate (SHMP) introduced by Thompson and others (1976). It is found that protein isolate produced using

SHMP exhibited better colour and taste, however, with low protein recovery. To precipitate the dissolved proteins, pH of the solution is brought to isoelectric point by acidification using acids such as HCl or CH_3COOH in presence or absence of NaCl (Klockeman et al., 1997; Aluko and McIntosh, 2001). The optimum range for precipitation is considered b/w 4.5 and 5.5 (Ghodsavali et al. 2005)

PMM approach, another method which is commonly used to precipitate proteins follows the principle of salting in affect. In this case, defatted meal is stirred in the presence of salt, especially NaCl, to maintain an ionic strength of minimum 0.2M and then diluted with cold water to bring down the ionic strength to as low as 0.06-0.1M to precipitate soluble proteins. Basically PMM approach follows two steps where initially, proteins are solubilized because of elevated ionic strength and finally, the ionic strength is reduced to enhance hydrophobic interactions b/w protein molecules by diluting the solution with cold water, resulting in the development of protein micelle.

Conventionally, wet fractionation processes are widely accepted as mainstream technology for preparing pure plant protein concentrates. However, there are major drawbacks associated with wet fractionation such as usage of copious amounts of water and energy along with generation of lot of waste. Additionally, it tampers the native functionality of proteins during processing due to harsh conditions (pH and temperature) that are maintained in-order to obtain individual proteins by disentangling the entangled structure. Subsequent drying steps prove detrimental to protein functionality. By far, wet fractionation is the most promising method to produce protein isolates with high protein content (with 99% purity). However, in most cases, ingredients are selected based on their functionality rather than on its molecular purity. For instance, enriched pea flours were used in fish feed instead of fish meal, since the carbohydrate fractions present in pea flour, which were obtained by dry fractionation, acted as a binding agent. Thus, improving the quality of fish feed pellets. Hence, to produce value-added healthy products, it is important to select and experience ingredients based on their individual functionality. Hence, in our present work, we have focused on separating technology, such as air classification, where there is no usage of water or it is required in very less amount. It focuses on improving the functionality of individual ingredients instead of molecular purity.

Dry fractionation by fine milling in association with air classification is a more sustainable and promising technology than wet fractionation. At present, there are various propositions that are routed towards exploring and developing dry fractionation technology to deliver protein concentrates with high (native) functionality to add to the existing novel food proteins.

Air classification is a technique that separates smaller fine fractions of proteins from the larger and coarser fractions of starch and fibre rich fragments. Post milling, finely milled flour undergo dispersion in the presence of strong air stream that enters the conical vessel. The conical vessel is equipped with the so-called rotor classifiers attached with blades on the top. These blades counter the air-flow direction in presence of centrifugal force creating a separation zone for flour particles. Subsequently, the flour particles are separated into fine and coarse fractions depending on the particle density. The action of drag force and centrifugal force from air flow and classifier wheel respectively, is the combined reason for determining the size of fine and coarser fractions. The dominance of drag force over centrifugal force allows the particle to pass through the wheel openings and enter fine fraction, whereas, the heavier fractions are pulled towards the bottom of the classifier wheel and collected separately as coarser fractions. Also, maximum protein shift can be achieved by determining the critical value of the particle size, under which the highest protein concentration is attained. Moreover, in air classification, unlike wet fractionation, intensive dehydration is absent, retaining the native functionality of components. Interestingly, air classification is approved for organic food production and the products that are produced using this mechanical fractionation doesn't require E-numbers. For many years, fine grinding followed by air classification has been used as a technique to concentrate protein fractions of cereal grains and legumes. Moreover, air classification is more efficient in case of cereal grains which possess soft endosperm, such as in wheat and sorghum. However, air classified protein and starch rich fractions of soybean, pea, chickpea, beans, lentils etc. are being studied extensively and are tested for various techno-functional properties.

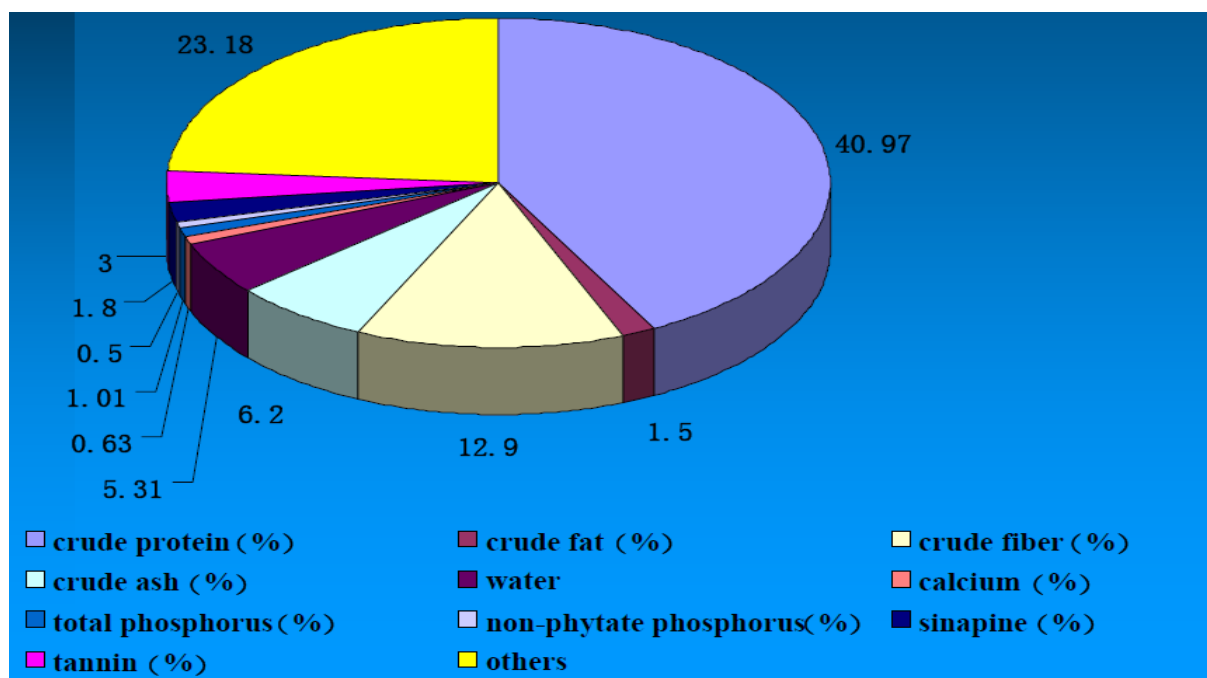


Figure 3: *Brassicaceae* oilseed proteins contain acceptable levels of all essential amino acids, including relatively high level of lysine and sulphur containing amino acids required in human nutrition (Sims, 1971).

One of the other ways to shift concentration from animal protein to a protein source which is inexhaustible such as Insects. They are rich protein source and contains high quality fatty acids and maintains a well-balanced ratio of minerals and vitamins. Furthermore, nutrient content can be compared to widely eaten animal protein source such as beef and chicken (Ramos-Elorduy and others 1997, Womeni and others 2009, Xiaoming and others 2010, Sirimungkararat and others 2010).

Insect production is greatly beneficial to economy as well as environment. There are several reports that have been investigated and reported regarding the efficacy of insects in feed conversion. Cultivation of insects would reduce the emission of greenhouse gases and NH_3 (Oonincx et al., 2010). Moreover, insect feed adapts to a wider range of plant sources than livestock such as cow or swine which can grow only on conventional feed (Durst & Shono, 2010).

Tenebrio Molitor

T. molitor commonly known as yellow mealworm belongs to Coleoptera: Tenebrionidae. The species exhibit four different stages of life mainly egg, larva, pupa and adult/ beetle. Basically, at optimal conditions, T. molitor larva stages between 6-8 months. Though it can continue up to even 2 years if favourable conditions such as temperature, humidity, feed and water availability are carefully optimised and maintained. T.molitor larvae survives longer than other Tenebroids such as Tribolium castaneum (red flour beetle). At larval stage, T.molitor consumes much more than other stages and conserves energy for later stages of life. The length of larva is about 2.0-2.5 cm. followed by hardening of larval skin, developing a hard exoskeleton for support and protection. The colour of larva changes from white to brown (Ghaly & Alkoaik, 2009).

T.molitor Proteins

As known to everyone, amino acids are the building blocks of proteins. The total protein content fresh larva is between 19% to 26 % whereas on dry basis it ranges between 48 -66%.The crude protein content exhibited by T.molitor is in parallel with conventional protein sources such as beef, chicken egg and fish. Moreover, if reared under favourable conditions their protein content is higher than above described sources. However, crude protein extracts from T.molitor have

limitations because of colour, taste and texture. Gel formation capability is very poor which limits wide usage (Belitz, Grosch, & Schieberle, 2004). For wider acceptability and usage, modifications are necessary to enhance physical and sensory properties of these extracts while keeping intact its credibility.

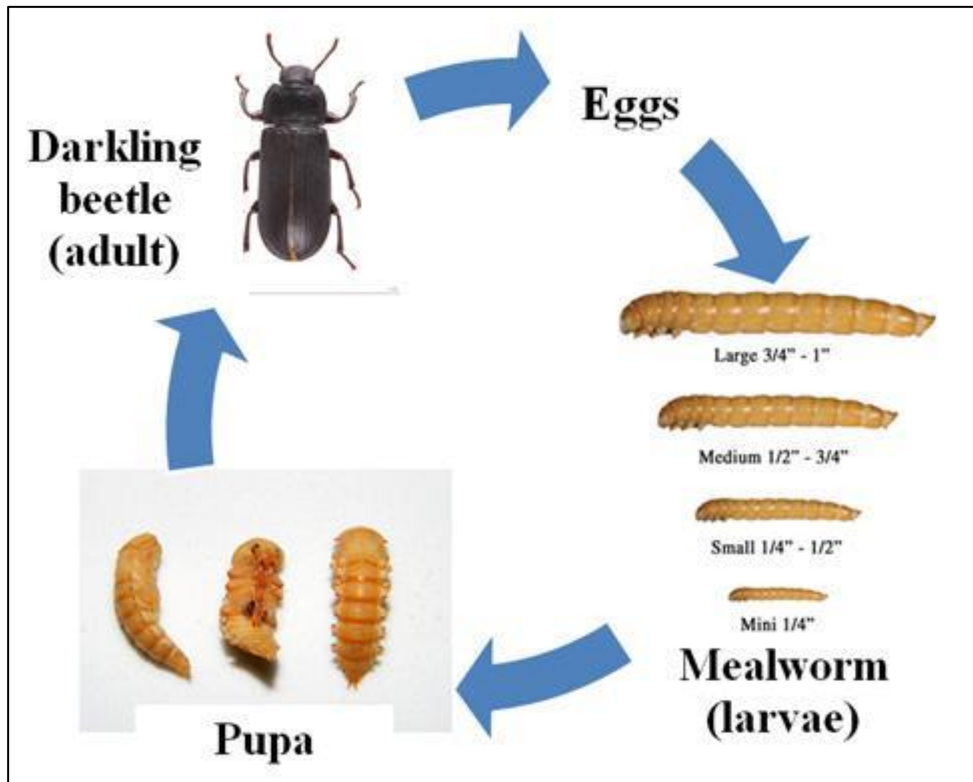


Figure 4: life cycle of T.molitor

Adapted from http://www.sugarglidertree.com/diet/diy-insect-breeding

As mentioned earlier, T.molitor particularly feed on cereals, hence reports have suggested that digestive enzymes of T.molitor are believed to specifically cleave peptide bonds of amino-acid residues of cereal proteins which comprise of prolamins (50% of the overall seed protein).

The amino acid composition of T.molitor determined by Jones, Cooper, & Harding (1972); Finke (2002) and Ramos-Elorduy et al. (2002), is compared and tabulated underneath. It depicts that essential amino acids of T.molitor fell in between 387 and 488 mg/g, higher than the daily requirement of essential amino acids (277 mg/g crude protein). Variation in feed composition can lead to variation in essential amino acids (Ramos-Elorduy et al., 2002).

T.molitor digestive Enzymes

T.molitor is stored product pest which feeds on grains and causes shortage of food grains world-wide. They are expected to have a complex as well as a unique digestive system that uses digestive enzymes specifically, digestive proteases to digest the food. Therefore, main source of dietary proteins for this group is cereal proteins. T.molitor larval gut is characterized by a complex enzymatic system. Enhanced and efficient digestion by digestive peptidases in the

larval gut is compartmentalized on the pH gradient, where the pH of larval gut varies between the regions. Digestion process greatly depends on cysteine peptidases which rely on pH for enzyme activity. The experiments have been conducted to check the presence of cysteine proteases in T.molitor midgut by proving inhibition from E-64 in reducing buffers. Thie and Houseman (1990) suggested that coleopteran species can be differentiated into minimum of three groups based on digestive proteolytic activity by either serine or cysteine proteases or both together. In the T.molitor midgut, both serine and cysteine peptidases can be found. This kind of digestion is common to coleopterans. Interesting part lies in the fact that protein digestion in T.molitor occurs partly in lumen and partly at the cell surface of posterior midgut (PM). In the anterior midgut (AM) pH is localized around 5.6 and changes to 7.9 in the posterior midgut (PM). Cysteine peptidases are found mainly in AM and serine peptidases in PM due to their acidic and neutral/ alkaline pH optima respectively. Interestingly, some of the serine proteinases may be synthesized in AM but are activated only when they reach alkaline pH of the PM. This suggests that enzyme activity is restricted in certain regions of the midgut due to their respective pH optima.

At a particular pH under reducing conditions, 64% of the total proteolytic activity (TPA) seems to be active in the AM and remaining 36% was in PM. In the AM, two- thirds of TPA is due to cysteine proteinases and serine proteinases are responsible for the remaining activity. However, similar TPA of cysteine proteinases is not witnessed in PM, higher percentage (76%) of proteolytic activity is due to serine proteinases. In PM prevalence of Trypsin-like activity with one cationic and three anionic proteinases and chymotrypsin-like proteinases with one cationic and four anionic proteinases acting as binding site are more dominant. In AM, some of the serine proteinases are synthesised but activation of proteolytic activity of these proteinases occur only once they are translocated to PM, where the pH of the environment is alkaline. Latent proteinase activity of cysteine proteinases is detected in PM, but in lower percentage. Inactivation of TPA of cysteine proteinases in the PM might be considered as a function of regulating the enzyme activity of the larval gut. Additionally, for protein digestion, cysteine peptidases represent at least four to six different enzymes, out of which cathepsin is believed to exhibit maximum peptidase activity. Barring cysteine peptidases, there are minimum of four trypsin-like and five chymotrypsin like peptidases (similar to serine peptidases) along with membrane bound aminopeptidase and soluble carboxypeptidase. Similarly, there are also insecticidal proteins, proteases such as α-amylase-trypsin inhibitors, phenolic compounds and lectins which inhibit the growth of insects and leads to improper development.

Cysteine peptidases

Cysteine peptidases are characterized mostly in the AM. These peptidases specifically target large hydrophobic and positively charged residues in P2 and P1 position respectively. As mentioned earlier, represent four to six different enzymes, out of which two major enzymes papain and cathepsin belong to C1 family and fall under clan CA of cysteine peptidases. Additionally, cysteine peptidases in T.molitor are competent enough to target glutamine-rich proteins of cereal grains and are often called as post glutamine hydrolyzing enzymes. Protein sequencing studies of cysteine peptidases has suggested that 29 protein sequences of cysteine peptidases are similar to sequences of papain C1 family and 14 and 13 sequences were similar to cathepsin L and cathepsin B and similar peptidases(B-like). However, reports indicate that orthologous cathepsin L peptidases are majorly expressed peptidases in the insects.

Serine peptidases

Compared to cysteine peptidases, serine peptidases are majorly distributed within the digestive tract of insects. Trypsin and chymotrypsin enzymes are representatives of trypsin superfamily which belong to serine endopeptidases. Generally, trypsin is found in PM of T.molitor larvae but trypsin activity is wide spread throughout the digestive tract. Secondary metabolites present in plants reduce the functionality of TPA and trypsin activity of serine proteinases in insects. Trypsin exhibits dual mechanism when it comes in contact with lectins, for instance, trypsin activity in the larval gut against a possible inhibitor may increase or decrease. Basic response of trypsin to unfavorable environment is up-regulation rather than inducing resistant isozymes. Inducing resistant isozymes may exhibit over-expression of existing proteinase isozymes which are insensitive to trypsin inhibitor. Various methods have been introduced such as non-functional trypsin which can deviate activity of trypsin inhibitor from the functional trypsin in the gut and avoid inhibition of trypsin activity due to trypsin inhibitors. Other ways are by introducing enzymes which are insensitive to inhibitors, adapting insects to consume inhibitor containing food or exploiting symbiotic microorganisms for protein digestion.

Prolyl-specific peptidases (PsP)

Prolyl- specific peptidases are hydrolases which specifically act on peptide bonds of proline and other amino acids. These proline –specific peptidases can be differentiated into subclass comprising of prolyl aminopeptidases (PAP), prolyl carboxypeptidases (PCP) and prolyl oligopeptidases/ prolyl endopeptidases (POP/PEP). In T.molitor, Goptar *et al*. reported two PSP namely, PPCP1 and PPCP2 (characterized as POP) which have maximum activities at pH 5.6 and pH 7.9 for substrate Z-Ala-Ala-Pro-pNA and Z-Ala-Pro-pNA respectively.

Carbohydrate- Hydrolysing enzymes

T.molitor larval midgut enzymes have the capacity to attack cell walls of plants, micro-organisms such as bacteria and fungi. During the enzyme activity, some digestive enzymes disappear and others are newly expressed, creating insignificant variation in both the conditions.

α- Amylase

T.molitor larvae consists of one single α- amylase that is prevalent in acidic region and preferable for cleaving starch molecules at pH optima 5.8. Activity of this acidic protein is inhibited by plant protein inhibitors. The enzyme has a molecular weight of 51.3 and likely to exhibit isoelectric point at pH 4.3, which is quiet similar to most of the proteins.

β –Glycosidases

As the name suggests, these exoenzymes cleave glycosidic bonds and act on non-reducing terminal by removing monosacharrides. T.molitor midgut is likely to possess four β –glycosidases namely: βGly1, βGly2, βGly3 and βGly4. βGly1, βGly2 has a molecular weight of 59kDa and hydrolyze disachharides, arylglucosides, alkylglucosides etc. These two glycosidases differ from each other in their protein sequences and hydrophobicity. βGly3 also hydrolyzes disachharides along with cellodextrins and β glucosides produced by plants. βGly4 shows the maximum hydrolyzing activity against β- galactosidases.

α- Mannosidases

Similar to β- glycosidases, these enzymes act on the non-reducing terminal of glycoconjugates. Major lysosomal-like α-mannosidases are present in the larval gut. It can be substantiated by the fact that the transcriptome of insect’s midgut found almost 38 α-mannosidases.

Polyphenoloxidases:

Phenoloxidases are those enzymes which are activated in response to foreign agents. It belongs to tyrosinase group and its main function is to oxidation of phenols. Basically, in insects, phenoloxidases are in inactive state (known as inactive zymogens, (proPOs)) but there are activated by serine proteases termed prophenoloxidase-activating enzyme (PPAE) at those times when response is needed against the foreign body. In their active and inactive stages, ProPOs contain two copper atoms per protein molecule with molecular weight of 50-60 kDa and 70-80kDa respectively. Moreover, polyphenoloxidases (PO) are capable enough to harm host

proteins by degrading host proteins, quinines that are cytotoxic and act against reactive oxygen and nitrogen intermediates.

Extrusion

Extrusion process/ Extrusion cooking has been defined as "the process by which moistened, expansile, starchy and/or proteinaceous materials are plasticized in a tube by a combination of moisture, pressure, heat and mechanical shear. This results in elevated product and temperatures within the tube, gelatinization of starchy components, denaturisation of proteins, the stretching or restructuring of tractile components and the exothermic expansion of the extruder "(4)(a). As a result of extrusion, plant proteins are texturized and rehydrated which gives the end product meat like appeal, flavour and texture, . Therefore, today textured plant products have found extensive appreciation in many ready-to-eat or easy- to-prepare processed food items (b). One of the major drawbacks to the direct use of plant proteins is their incapability to hold a structural integrity as that of meat proteins (b). Mechanical and thermal energy applied during the extrusion process force the protein components lose their nativity and form viscous elastic mass where the protein is in continuous phase (a)(b 96).

Extrusion equipment consists of various parts and more precisely, divided into sections. Each part has its own importance during the journey of extrudate. Overall the extrusion equipment can be divided into five, namely, extrusion drive, feed assembly, extrusion screw, extruder barrel and extruder discharge.

Extrusion Drive consists of components such as stand on which the entire extruder made of steel or cast iron is mounted and provides support to the core extruder components such as feeders, screw barrel and drive.

Feed assembly performs three major operations known as feeding, blending, and preconditioning before the actual extrusion process. It ensures uniformity and consistency of feed ingredients during the extrusion process. These operations are substantiated by supporting aids such as Hoppers/ Bins where initially the feed is stored before releasing it into feeders, where often sticky, non-flowing substances are mixed thoroughly before exiting. In case like ours, where the feed system is continuous, the individual ingredients are combined in required proportions before entering into the feeder. Lastly, the crucial step before the feed enters the extruder is preconditioning in a closed vessel where along with the feed ingredients it is mixed with adequate amount of steam/ water which is necessary to obtain the moisture and temperature levels to provide smoothness while the ingredients undergo extrusion process. It can also be

designed as a blender with pressure /atmospheric vessel for handling the pressure difference developed during the exit and entry of feed ingredients to the feed port of extrusion screw.

Extrusion Screw is considered as the central and the most significant portion in the extruder equipment while it receives the ingredients from the feed port, act as transporter, changes the structural integrity of the feed ingredients and pushes them to the discharge section of the extruder which normally holds the die, cutters and removable devices. The extrusion screw is compartmentalised into Feed Section, Compression Section and Metering Section. Feed section as the name suggests, accepts the feed from the preconditioner, characterized by deep flights and ensures sufficient amount of feed is moved down the screw and it is completely filled so that condition such as starved feeding does not occur. Followed by Compression Section, where the decrease in the flight depth, restricts the volume and increases resistance leading to compression. The combined effect of mechanical and thermal energy disintegrates the structure of feed ingredients to semi-solid plasticized mass. The end portion of the extrusion screw is known as the Metering Section which have the smallest flights, increasing the pressure and shearing rate further. This condition enables proper internal mixing of extrudate fostering temperature uniformity throughout. Pins and cut flights are employed to increase mixing and mechanical energy dissipation.

Extruder Barrel is a cylindrical structure that supports the extrusion screw. It is tightly mounted all around the rotating screw. The amount of heat transfer area is defined as the function of L/D, which usually ranges from 1:1 to 20:1. L is the length of the barrel distance from the rear edge of the feed opening to the discharge end of the barrel bore. It is made up of strong alloys such as Xaloy^R 306 and stainless steel 431.

Extruder Discharge is the last and final section of the extruder where the amorphous or plasticized dough has to pass through to undergo final transformation and come out as a finished product. Once the dough leaves the extruder screw, pressure build up at the discharge forces the viscous dough to enter the die which controls the texture and shape of the final product. Difference in viscosity of dough at the discharge and screw could be observed and it is attributed to the difference of temperature and shear rate at both the sections.

At present, protein recovery technologies from rapeseed meal are limitedly available to produce protein ingredients for food and feed uses. Improvement of functional value of rapeseed proteins by introducing to techniques such as air classification, is presumed to play a crucial role in positioning rapeseed proteins in the food ingredient market. Among these functionalities, solubility behaviour, thermal stability, surface-active properties and safety such as reduced

potential to pose allergic reactions are important. Therefore, comprehensive and detailed studies are necessary to investigate feasible recovery of storage proteins of *Brassicaceae napus* seeds with the aim to increase its competitiveness as a plant protein source.

Research Objectives

The goal of the project is primarily to discover the influence of various processing techniques on extraction of RPC proteins and to investigate the changes in solubility and composition of extracted protein fractions. Two different approaches have been adopted. In the first approach, protein enriched RPC fractions by air classification of RPC at different classifier speeds are recovered. Primarily, crude protein (N×6.25), moisture, fat and ash contents will be determined. Influence of pH on protein solubility (Bradford method) and composition (SDS-PAGE) of air-classified samples will be studied in comparison with RPF, RPC, concentrates and isolates. Further, WBC and FBC of all the samples will be analysed. Finally, air-classified samples with better protein content and low moisture are selected and analysed for colour and particle-size of the dry meal.

Second approach involved investigation of digestive enzymes of T.molitor larvae (subjected to starvation) on protein solubility of RPC in combination with Extrusion. Initially, T.molitor larvae are fed on formulated meal for 1 week and are divided equally into 3 groups. The first group will be subjected to starvation for 48 hrs, second group for 24 hrs and third group will not be subjected to starvation. All three groups of T.molitor larvae will be inactivated by storing them at -20°C for 1 week. Subsequently, Inactivated T.molitor larvae from each group will be grounded (T.molitor paste) and analysed for fat content, moisture content and protein content. 6 different formulations will be developed by mixing RPC with T.molitor paste from each group. All formulations will be different from each other as they would be adjusted to either one of the pH's- pH 2 or pH8 and will be incubated either at Room temperature or 45°C for incubation time set at either 90 minutes or 0 minutes. At last, all the formulations will undergo extrusion on a single screw extruder with priorly adjusted parameters. Extrudates of the respective formulations will be checked for changes in protein solubility and composition of RPC proteins.

MATERIALS AND METHODS

I. AIR CLASSIFICATION (1st part)

1. Recovery of test samples

Air classification it is a milling technique that is characterized by fractionation of grains/seeds into enriched starch and protein flours. Classification occurs due to high speed and high volume spiral air stream protein fraction (light fine in nature) is separated from the starch fraction (heavy coarse in nature) in the air classifier (Boye, Zare & Pletch, 2010). Test samples Rapeseed flour (RPF) and Rapeseed press cake (RPC) were provided by Kroppenstedter Ölmühle GmbH, which is one of the major rapeseed oil producers in Germany. Samples DF1500, DF1800, DF2000, HF, DF8000 and DF4000 are derived from RPC, by following a dry mechanical fractionation approach at IGV, which uses different rpm (rotation per minute) frequencies. This approach allows to characterize proteins based on protein yield since each protein fraction is a result of different rotating speed. Also, in addition to press cake, the hulls from de-hulling can also be fractionated. The process can be repeated several times to improve the separation efficiency.

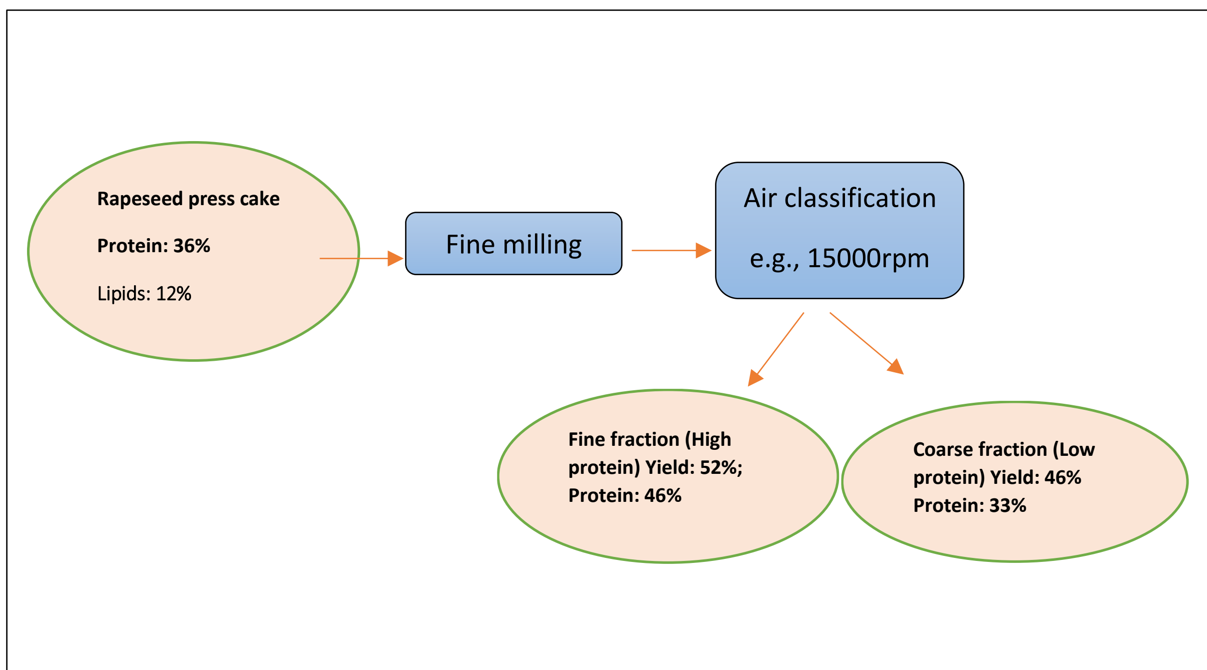


Figure 5: Air classification of Rapeseed press cake into various protein fractions with adjustment of different classifier speed. This method of classification is adapted from the project "APROPOS (Added value from high protein & high oil industrial co-streams) 2012"

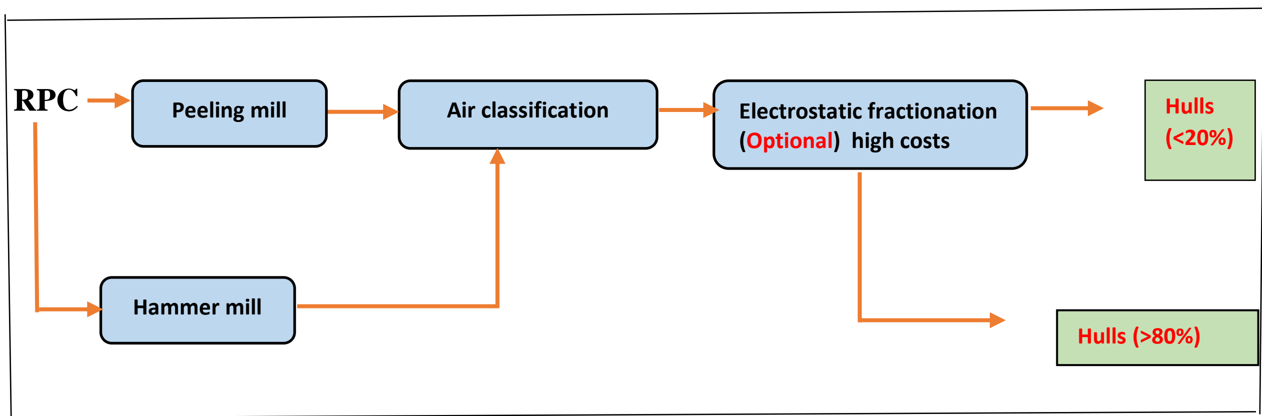


Figure 6: De-hulling of rapeseed press cake at IGV. This process is adapted from "APROPOS Added value from high protein & high oil industrial co-streams 2012"

1.1 Working of "CONDUX-High-Performance Fine Classifier *InlineStar* ™"

CONDUX-High-Performance Fine Classifier InlineStar ™ developed by NETZSCH-CONDUX is a high-performance air classifier which has been used at IGV to process rapeseed press cake into different fractions. The machine is successful due to its finest cut points in a range of d_{97} 2.6 um (based on lime stone) and *ConVor™* classifier wheel, featuring constant radial speeds ensures fineness during the classification of the product. Moreover, it's a combination of a fine grinding-mill with a downstream classifier which does not require removal of undesired ultrafine fractions, as well as separation of coarse particles or grit for pre-ground powders which happens in typical 2-stage grinding/classifying plants. It is made with steel as well as rust- and acid proof materials. Additionally, the machine is efficiently designed to handle products that are dust explosive and

Figure 7: Condux Fractionation System (IGV)

hazardous due to swing out housing door. It is well equipped to resist disturbances caused due to pressure and shock.

Samples GC and AC are concentrates with protein yield (~71.3%- 78.5%) and samples AI and GI are rapeseed isolates with protein yield (< 90%). These samples were obtained from **PPM** (Pilot Pflanzenöltechnologie Magdeburg e.V.) which is one the leading R&D Institutions in Germany dealing with oilseed research. Samples AI and GI were obtained according to Figure 4 given below and were considered as reference samples.

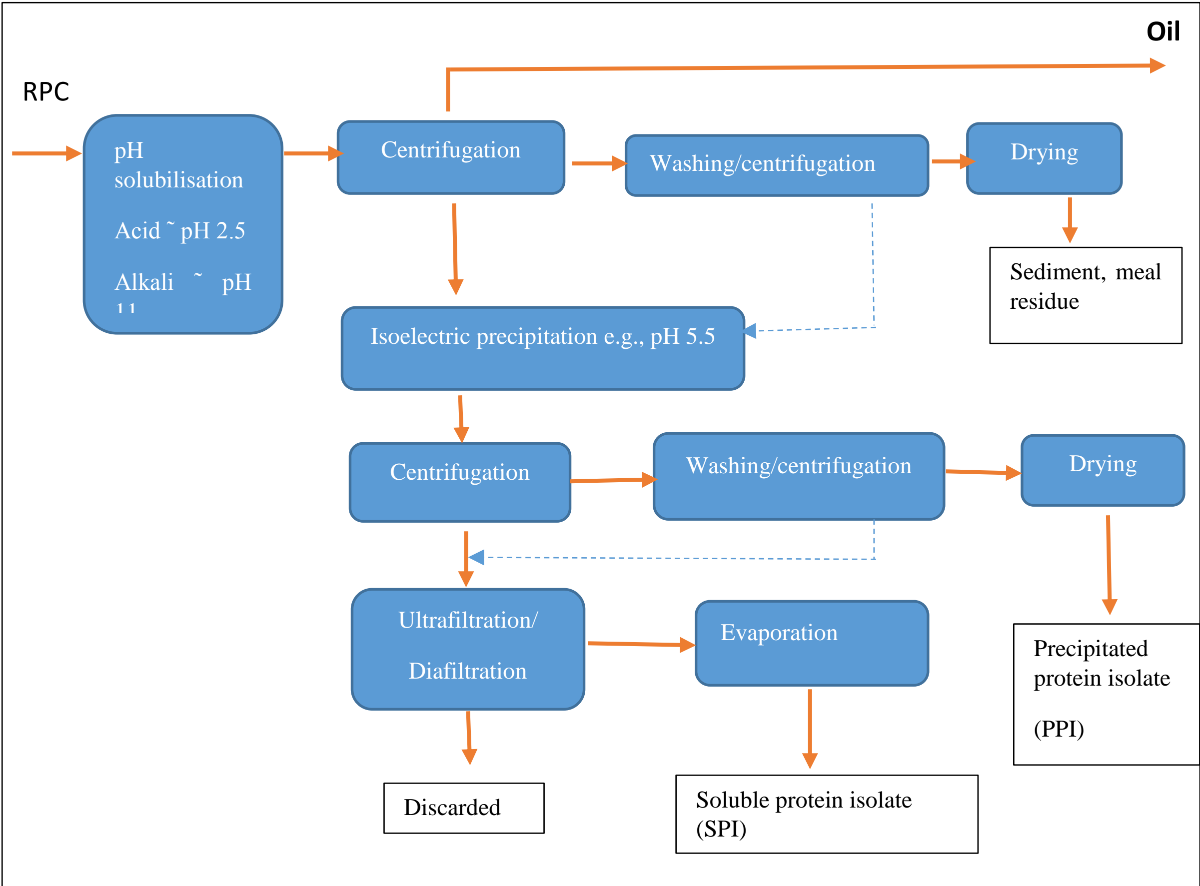


Figure 8: Process used for preparation of isolates (AI and GI) at PPM

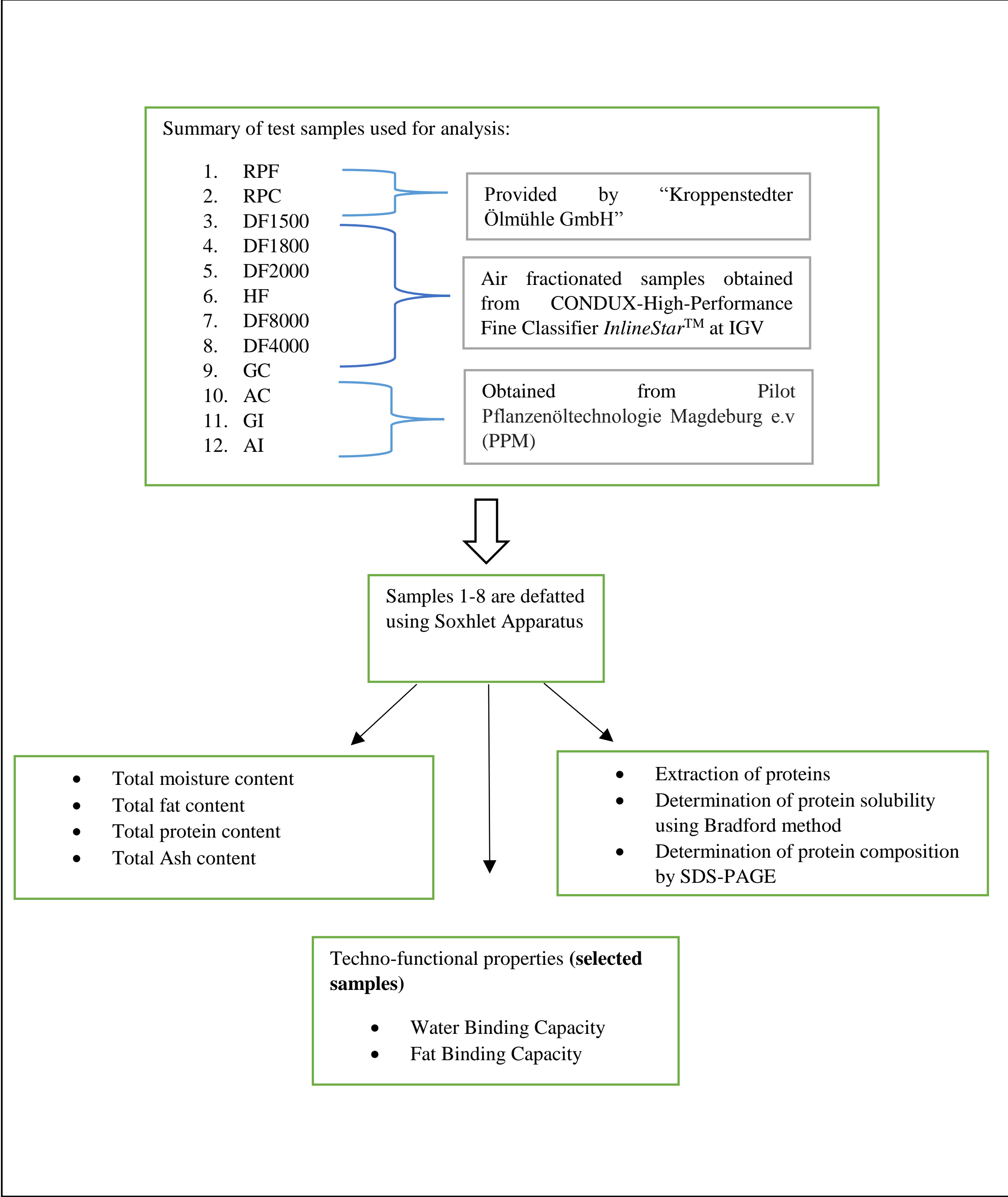


Figure 9: Overall view of samples and analysis performed under Air-classification approach.

2. Sample description

Number of samples	Codes allotted	Sample description
1	**RPF**	**Rapeseed flour**
2	**RPC**	**Rapeseed press cake**
3	**DF1500**	**Degreased, fractionated (1500 rpm)**
4	**DF1800**	**Degreased, fractionated (1800 rpm)**
5	**DF2000**	**Degreased, fractionated (2000 rpm)**
6	**HF**	**Hulls fraction**
7	**DF8000**	**Degreased, fractionated (8000 rpm)**
8	**DF4000**	**Degreased, fractionated (4000 rpm)**
9	**GC**	**Rapeseed protein isolate (globulin rich˜90% protein)**
10	**AC**	**Rapeseed protein concentrate (albumin rich˜82.7%)**
11	**GI**	**Rapeseed globulin isolate (~ 93% protein) (Control)**
12	**AI**	**Rapeseed albumin isolate(~ 98.5% protein) (control)**

Figure 10: List of samples for analysis .Sample number 1-10 are the test samples where, samples 3-8 are air classified samples. RPF and RPC are obtained from Kroppenstedter Ölmühle GmbH; sample number 9 -12 are obtained from PPM and samples 11 and 12 are reference samples.

3. Defatting of samples

Defatting of the samples is done according to the procedure described by Soxhlet in 1879 which is the most commonly used technique for separation of the lipids from the solid matrix. Initially, the cellulose thimble (Hahnemühle, Dassel, Germany) was weighed and finely ground rapeseed flour and rapeseed cake (5 g) each and samples 3-8 (16 g) each were precisely weighed and placed in the thimble holder of the Soxhlet apparatus (GERHARDT). 160 ml of Petroleum benzene (CHEM SOLUTE), used as extracting solvent, was poured into the 250 ml round bottom flask (SCHOTT DURAN) with boiling

Figure 11: "GERHARDT Soxhlet apparatus" used for defatting test sample 1-8 at *IGV*.

chips (T H. GEYER) to avoid splashing during evaporation. The temperature was set at 75°C since, the boiling point of petroleum benzene is 80°C. During the operation, the thimble holder was gradually filled with condensed fresh solvent from a distillation flask. When the liquid reaches an overflow level, a siphon aspirates the whole contents of the thimble holder and unloads it back into the distillation flask, carrying the extracted analytes in the bulk liquid. This operation was repeated and carried out for 4h.

After extraction the solvent was removed from the round bottom flask and the defatted samples in the cellulose thimble were dried at room temperature, overnight. Following morning, drying was carried out in a pre-heated oven at 103°C for $1^{1/2}$ hr till the samples attained constant weight. Post drying, samples were allowed to cool in the desiccator and the weight was determined. Without delaying, the samples were collected and stored in a dry container for further use.

Figure 12: Pictorial representation of samples post defatting.

A represents the original rapeseed and rapeseed cake; B, C and D represents samples 1-8 post defatting whereas E and F represents rapeseed concentrates and isolates

4. Determination of Physio-chemical properties

4.1.1 Determination of total ash

The "*ash content"* is a measure of the total amount of minerals present within a food sample and are based on the fact that the minerals (the "analyte") can be distinguished from all the other components (the "matrix") within a food in some measurable way

$$\textbf{Ash content (\%)} = (M_{ash} \times 100)/ M_{dry}$$

Dry ashing method was adopted to determine the total ash content of all the samples. Initially, about 1.0 g of each of the defatted samples were weighed into previously dried porcelain crucibles. Post charring, the samples were dried in an electrically heated muffle furnace (Heraeus INSTRUMENTS) at 550°C, till they attained constant weight. Percentage ash content was determined by weighing the resulting inorganic residue

Where,

M_{ash} = Mass of the ashed sample

M_{dry} = Mass of the original sample

4.1.2 Determination of fat content

The acid hydrolysis method is used to determine the total fat content of the test samples where acid hydrolysis extracts fat from the sample by subjecting it to hydrochloric acid, which disintegrates the sample and heat hydrolyses the proteins and starch, disrupting the plant cell wall, and liberating fat, which makes extraction better (Hertwig, 1923). The purpose of hydrolysis/digestion is to release bound lipids (Inkpen and Quackenbush, 1969).

Weibull-Berntrop type acid hydrolysis method is adopted where initially, 5.0 g of each of the test sample was weighed in 150 ml glass beaker attached with a lid and a stirrer. 160 ml of 4N HCl is poured into the glass beaker and is gently placed on the heating device. The resulting mixture was left for acid digestion for 30-40 minutes at 100°C. Secondly, once the mixture was cooled, the digest was allowed to pass through the filter paper and was washed thoroughly with distilled water. Drying was carried out overnight, and finally, dried samples were subjected to extraction using SOXTHERM (GERHARDT), the following day. The resulting fat residues were

determined gravimetrically after drying the filter paper in a pre-heated oven at 103°C for $1^{1/2}$ hr to bring the samples to constant weight [1].

Percent fat content was measured according to the given formula:

$$\textbf{Fat content (\%)} = \{(M_2 - M_1) \times 100\} / M_0$$

Where,

M_2 = weight of the beaker after Soxtherm extraction

M_1 = weight of the empty beaker

M_0 = weight of the filter paper after drying

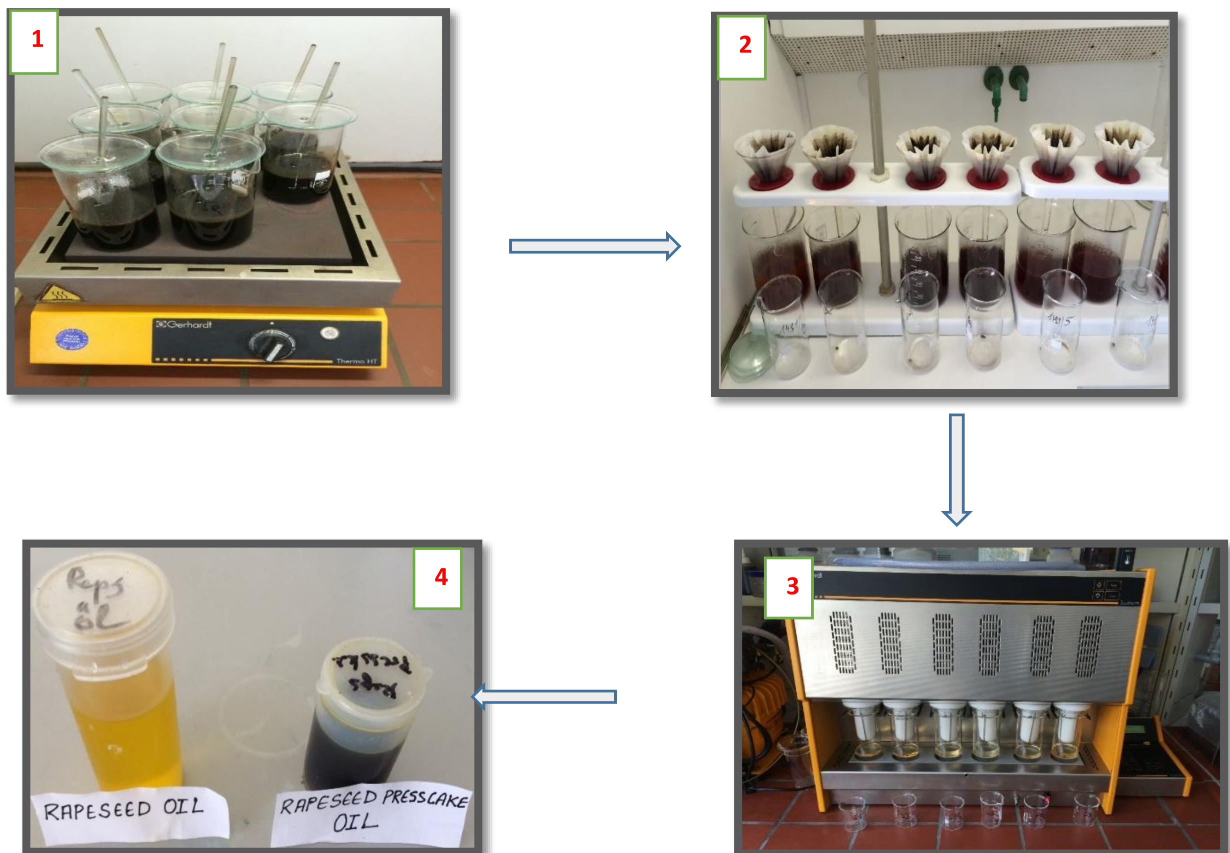


Figure 13: Pictorial representation of determination of fat content by acid hydrolysis method at IGV.

1 represents digestion of test sample using HCl; 2 represents filtration of the digested mixture post cooling; 3 represents oil extraction from digested residue of test sample; 4 represents oil extracted from RPS and RPC

4.1.3 Determination of moisture content

For determination of moisture content, initially, properly cleaned and dried glass vessel attached with a lid was taken. Empty vessel was weighed after leaving it open for at least 30 min in the desiccator and brought to laboratory temperature. 1.0 g of each of the defatted samples into the vessel was weighed and spread evenly over the whole base of the vessel and vessel was closed with its lid. All the operations were carried out as quickly as possible, ensuring there was no appreciable change in the moisture content. Secondly, the glass vessel containing the test portion was kept into the pre-heated oven at 103 ± 2°C, with the vessel lid removed. After 3hrs the lid of the vessel was closed and were kept in desiccator for cooling. As soon as the vessel attained laboratory temperature, it was weighed and returned to the oven with the lid removed. After 1 h, the operations of closing the vessel, cooling and weighing were carried out once again. This process was stopped once the difference between the two consecutive weighing's were equal to or less than 0.001 g (for a test portion of 1 g). Percentage moisture content was determined by weight loss incurred according to the given formula

$$\textbf{Moisture (\%)} = [\{(M_1 - M_2) / (M_1 - M_0)\} \times 100]$$

Where,

M_0 = the mass of the vessel, in grams

M_1 = the mass of the vessel and test portion before drying in, grams.

M_2 = the mass of the vessel and test portion after drying, in grams.

4.1.4 Determination of crude protein content

The protein content of the dietary samples have always been determined on the basis of total nitrogen content and therefore, the protein content of aforementioned defatted rapeseed test samples were determined by using the Kjeldahl method (AOAC, 2000). This method is universally accepted for determining the protein content from the determined nitrogen content by multiplying with a nitrogen- to-protein conversion factor, 6.25. The principle lies in the converting N to NH_3 by digesting the sample in sulfuric acid, using $CuSO_4/TiO_2$ as catalysts, which is distilled and further titrated.

Figure 14: Gerhardt Kjeldahl apparatus used for analysis of crude protein in test samples at IGV

% crude protein = % nitrogen × 6.25

Percent nitrogen is calculated using given formula:

$$\frac{1.4007 \times n \times K(V2 - V1)}{m}$$

Where,

n= Normality of HCl used for titration (0.1 N)

K = titration factor (1.03)

V2= volume of HCl used for titration (test sample)

V1= volume of HCl used for titration (blank sample)

5. Extraction of proteins

Distilled water was adjusted from pH 1 to 10 with 1N HCl and 1N NaOH in 50 ml falcon tubes. 250 mg of RF, RPC, DF1500, DF1800, DF2000, HF, DF8000 and DF4000 and 10 mg of GC, AC, GI and AI, were weighed into 15 ml falcon tubes, which were dissolved and extracted by adding 12.5 ml of pre – adjusted (using 1N NaOH and 1N HCl) distilled water. After mixing for 10 min at constant speed, the pH was checked and re-adjusted to required pH value, using 1N HCl and 1N NaOH. Sample mixture was constantly stirred at room temperature for 30 minutes and the pH was re-checked. Then, the suspensions were centrifuged at 6,000 × g for 30 min at 4°C. To determine the soluble protein content and composition of the dissolved proteins, aliquots of the supernatant (3×100 µl), were filled in 1.5 ml centrifuge tubes (n=3) and stored at -4°C till further use.

6. Measurement of protein concentration

Principle

The protein concentration was determined using Bradford Protein Assay in 96- well culture plates. This method uses Coomassie brilliant Blue Dye-G250 (Bradford reagent) which appears in three different stages since it absorbs at varying wavelengths. Without protein, the solution is red-brown in its acidic solution. By binding the dye with protein, the pKa of the dye shifts, it changes from a cationic to an anionic state and causes the colour of the dye to become blue from red. The Coomassie blue –dye binds to arginyl-and lysyl side chains of proteins. The dye is measured at 595 nm and the absorption change is proportional to the protein concentration. (Roti®- Quant instructions for use, Bradford protein Assay 96 well plate protocol).

The standard curve was prepared according to the dilution table given underneath using the BSA standard solution (1mg/ml).

Dilution Table:

	Water/ buffer (µl)	BSA- Sol (µl)	End conc (µg/ml)
1	400	0	0
2	380	20	50
3	360	40	100
4	320	80	200
5	240	160	400
6	80	320	800

Chemicals

Bio-Rad Bradford Reagent (conc):- Dilution: 1 part reagent + 4 parts of distilled water

Analytical procedure

One of the previously stored 300 µl aliquots of each of the test samples RF, RPC, DF1500, DF1800, DF2000, HF, DF8000 and DF4000 were brought to room temperature before diluting them to 1:10 ratio (10 µl of supernatant + 90 µl of distilled water). Samples GC, AC, GI and AI, were not subjected to dilution.

- 10 µl of standards, diluted test samples RF, RPC, DF1500, DF1800, DF2000, HF, DF8000 and DF4000 and non-diluted samples GC, AC, GI and AI were pipetted into wells of the culture plate. Each solution was measured in triplicates to verify results.
- 200 µl of the diluted Bradford reagent solution was carefully added to the standards and to the test samples.
- The culture plate was incubated for 5 min at room temperature and then the absorbance was measured at 595 nm with an iMark^{TM} Microplate Absorbance Reader (Bio Rad Laboratories GmbH, Munich).
- The standard curve was created by plotting the averaged absorbance vs the protein concentration of each standard.
- The unknown protein concentration of each of the test sample were determined using polynomial interpolation with the help of the protein standard series included on each plate.

7. Determination of protein composition by SDS-PAGE

Principle

SDS-PAGE is a qualitative method used for separation of protein mixtures based on their size. In this particular gel electrophoresis, crosslinked polyacrylamide gels are formed in the presence of *N, N′* -methylene-bis-acrylamide (normally referred to as “bis-acrylamide”) causing polymerization of acrylamide monomer. Long chains of polymerized acrylamide monomer along with occasional built up of a bis- acrylamide molecule introduces a crosslinked matrix of fairly-well defined structure. This chain reaction is initiated and catalysed by the addition of ammonium persulphate (APS) and the base *N, N, N′,N′*- tetramethylenediamine (TEMED) respectively. Hence, when electrophoresis is performed, the gel serves as a size-selective sieve, in response to the applied electric field. The gel pore structure allows the movement of smaller proteins more swiftly than the larger proteins. For successful PAGE, sample preparation involves the addition of sample buffer containing β-mercaptoethanol and the anionic detergent SDS to the previously extracted protein sample that is free of contaminants. Thiol reducing agent, mercaptoethanol, aids in reducing any intramolecular and intermolecular disulphide bridges to achieve complete unfolding of proteins from their tertiary structure. This treatment allows complete denaturation of proteins making them appear as rod-like structures and when they come in contact with SDS, the intrinsic charge of protein is masked with the negative charge of SDS. The sample buffer also consists of other components such as bromophenol blue which is a tracking dye monitoring the movement of protein samples and glycerol which imparts density to the sample solution during electrophoresis. Under the applied electric field, when protein-SDS complexes moves towards the anode passing through the stacking gel, they are differentiated from one another in the separating gel based on their size and molecular weights are determined.

Chemicals:

- 1 M Tris:
  6.06 g Tris (hydroxymethyl) aminomethane (M=121.14 g/mol) (Carl Roth GmbH and co, Karlsruhe) was dissolved in ca. 25 ml distilled water, and then, was made up to 50 ml with distilled water.

- 1 M Tris pH 8.8
  6.06 g Tris (hydroxymethyl) aminomethane (M=121.14 g/mol) was dissolved in ca. 25 ml distilled water, HCl was used for adjusting to pH 8.8 and later, it was made up to 50 ml with distilled water.

- 1 M Tris pH 6.8

  6.06 g Tris (hydroxymethyl) aminomethane (M=121.14 g/mol) in ca. 25 ml distilled water, HCl is used for adjusting to pH 6.8 and finally, it is made up to 50 ml with distilled water.

- 10% SDS

  10 g of Sodium Dodecyl Sulphate was dissolved in ca. 80 ml distilled water and once the transparent solution was achieved it was made to 100 ml.

- 10% APS

  50 g of APS was dissolved in 0.5 ml of distilled water and was stored at 4°C. This solution was freshly prepared.

- 4X Sample Buffer

  8 g of SDS, 24 g of 87 % glycerol(Carl Roth GmbH and co, Karlsruhe) , 1.22 g of 100 mM Tris(hydroxymethyl)aminomethane and 10 g of β-Mercaptoethanol were weighed in a glass beaker were adjusted to pH 6.8 with 0.1 N HCl. Then, 0.02 g of Coomassie was added and mixed thoroughly and finally the resulting solution was made to 100 ml with distilled water.

- 1X Sample Buffer

  1 part of 4X Sample buffer was mixed with 3 parts of distilled water.

- Staining Solution

  This solution was prepared by adding 78.2 ml of solution A, 20 ml of Methanol (Carl Roth GmbH and co, Karlsruhe) and 1.6 ml of Coomassie Brilliant Blue.

  (Solution A: 160 g of ammonium sulphate (Carl Roth GmbH and co, Karlsruhe) was dissolved in 1536 ml of distilled water and was added to 32 ml of 85% phosphoric acid (Carl Roth GmbH and co, Karlsruhe).)

- Buffer solution for Electrophoresis

  100 ml of 10X running buffer were diluted with 900 ml of distilled water and 10 ml of 10% SDS was added and shaken thoroughly to prepare 1 L buffer solution.

  (10X running buffer: 30.3 g of Tris (hydroxymethyl) aminomethane (M=121.14 g/mol) and 144 g of Glycine (AppiChem Panreac MW Companies) were dissolved in 500 ml distilled water and then made up to 1 L).

- Sample preparation

  300 µl of all test samples, 150 µl of 1 M Tris, 150 µl of 4X Sample buffer were added to an Eppendorf tube and were centrifuged to give a thorough mix.
- Control samples preparation

  10 mg of each of the defatted test sample was dissolved in 1000 µl of 1X sample buffer.

Methodology

To assess the impact of pH on the polypeptide profile of extracted proteins Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed (SDS PAGE) according to the method suggested by Laemmli (1970), all the test samples and control samples were pooled together. They were mixed with the sample buffer in 1:2 ratio, performed according to the procedure explained in the preparation step. All the samples to be tested were brought to room temperature before mixing with sample buffer. Subsequently, denaturation of proteins was carried out at 95°C at 800 RPM for 3 min. After cooling the samples were centrifuged for quick 10 sec and 5µl of standard, control of the respective test sample and test sample adjustments from pH 1- pH 10 were carefully pippeted out into lane 1 – 12 of 15%T Tris- Glycin gels respectively. The gels were prepared with the help of vertical electrophoresis equipment supplied by biostep GmbH (Jahnsdorf, Germany). Low molecular weight calibration kit was used which was provided by GE Healthcare, Buckinghamshire, UK. For the first 10 minutes, the gel was run at 100 V, as it would improve resolution of proteins in the stacking gel by compacting them in a very thin phase before entering the separation gel. Thereafter, the gel was run approximately for 1 hr 15 min at 180 V. This step was followed in order to differentiate proteins of lower molecular weight from the higher molecular weight proteins. When the samples reached the lower front of the gels, the power was switched off and the electrophoresis process ended. The gels were removed carefully from the gel holding cassette and were immediately placed in the staining solution. The gels were stained overnight at room temperature on a shaker. The next day, the samples were de-stained for about 6- 7 hours using distilled water which was discarded time to time and replaced with fresh distilled water. After de-staining the gels were quantified using Quantity One 1-D Analysis Software, version 4.5.2 (Bio- Rad, Universal Hood II, Bio-Rad Laboratories, Segrate, Milan, Italy)

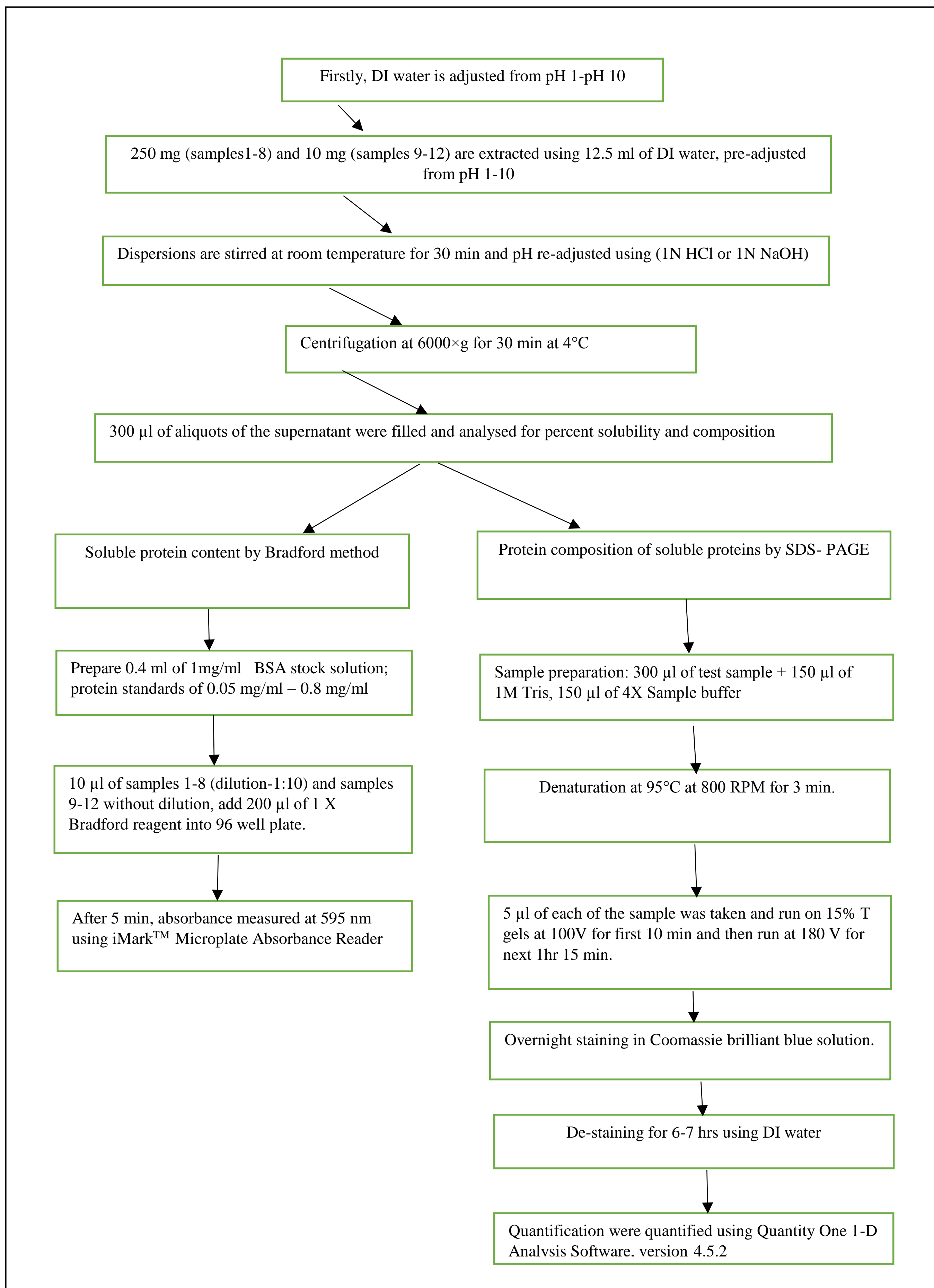


Figure 15: Summary of Extraction of proteins followed by determination of solubility and composition

8. Water binding capacity

To determine the water binding capacity, method by Smith and circle (1978), modified by Quinn and Paton (1979) was used. For this purpose 0.5 g of each of the selected air-classified sample DF2000 and DF4000, and RPC is weighed into 15ml centrifugal tubes. The required amount of water is calculated by the method of Smith and circle (1978), modified by Schwenke et al. (1981). 2.5 ml of tap water was added to each of the centrifugal tube containing 0.5 g of sample and stirred for 60 sec using overhead agitator and propeller stirrer.

Later, samples were centrifuged at 3900 × g, for 20 minutes. The supernatant is decanted and the tubes were gently inverted on the filter paper and weight of the residue in the test tube was measured after 60 min. The water binding capacity of each of the sample is calculated using the given formula:

$$\textbf{Water binding capacity [g/g]} = [(\mathbf{F} - \mathbf{I}) / \mathbf{IDM}] \times \mathbf{100}$$

Where,

F= final weight of the sample

I= initial weight of the sample

IDM= initial weight of the sample based on dry mass

9. Fat binding capacity

The method of Schwenke et al. (1981) is used for determining the fat binding capacity. 0.5 g of each of the selected air-classified sample DF2000 and DF4000, and also RPC is weighed into 15 ml centrifugation tubes and stirred with 5 ml of commercial rapeseed oil using a propeller and overhead agitator. The samples are stirred for 1 min for two times with 5 min intermission between each stirring. Following, centrifugation of samples at 3900 × g, for 20 minutes. Finally, supernatant is decanted and the tubes are gently inverted on to filter paper for 60 min. Fat binding capacity is calculated in similar manner as of water binding capacity which is as follows:

$$\textbf{Fat binding capacity [g/g]} = [(\mathbf{F} - \mathbf{I}) / \mathbf{IDM}] \times \mathbf{100}$$

Where,

F= final weight of the sample

I= initial weight of the sample

IDM= initial weight of the sample based on dry mass

II. T.molitor larvae (2nd part)

11. Rearing and processing of T.molitor larvae:

T.molitor larvae, were purchased from a commercial supplier are used as test material. As soon as the T.molitor larvae were received from the supplier, they were separated from frass by sieving, and if any dead larvae were present, they were removed by manual handpicking. Live larvae were temporarily collected in white coloured open polystyrene boxes of standard 39 L×28 W×14 H cm. The weight of the live larvae were measured and were distributed equally in six open black coloured polystyrene boxes, of standard 39 L×28 W×14 H cm, containing 300g of flour. These polystyrene boxes are widely accepted for rearing of insects as they are quiet convenient to manage and more importantly as they ensure ample space and sufficient depth for movement and prevent escaping of larvae. Hence, these boxes were chosen to support larvae during the entire process of rearing. To promote healthy and nutritious well-being of larvae, the boxes contained formulated flour which contains wheat (33%), oats (33%) and maize (33%) (Ingredients were purchased from Bako Germany). Although. T.molitor can survive on flour, but to enhance larval survival, development time and efficient conversion of food, they were also supplemented with ˜ 2.0 - 3.0g fresh organic apples and carrots to avoid interferences that may cause due to pesticides or any other chemicals. Apples and carrots were used primarily for providing water but essential nutrients such as vitamins and minerals present in these sources helped T.molitor larvae achieve a balance nutrient ratio. Proper nutrition system allows increment of protein content which slows the development time and prolongs survival **(Insects diet: Science and Technology, second edition)**. This step of rearing larvae on proper diet and favourable conditions was carried out for 1 week. Every day uneaten/dried/leftovers of carrots and apples were removed and replaced with fresh ones. Larvae were reared under dark conditions with temperature at 25.4°±1.4°C and humidity of 40.9±8.4%. Each of the six black polystyrene boxes with T.molitor larvae were provided with a unique identification number such as B79, B80, B81, B82, B83 and B84. It is ensured that all throughout the experiment these identification numbers were never changed at any point of time. Specificity of these identification numbers can be understood more clearly as we proceed further in our experiment. The weights of each group of larvae were measured carefully before inactivating them.

After 1 week, live T.molitor larvae in boxes with identification numbers B83 and B84 were separated from flour by sieving and were collected in polythene bags **P83 and P84** respectively and were killed by storing them in the freezer (LIEBHERR comfort) at -20°C. Live T.molitor larvae in boxes with identification numbers B81 and B82 were also separated from flour on the

same day and were collected in empty black polystyrene boxes of same standard but with the identification numbers, **EB81 and EB82** respectively. Larvae in these boxes were subjected to **starvation for 24 hrs**, creating stress among the larvae, followed by transferring them to polythene bags **P81 and P82** prior to killing them by storing at -20°C. Lastly, live T.molitor larvae present in last two boxes with identification numbers B79 and B80, were separated from flour, collected in polystyrene boxes of same standard with the identification number, **EB79 and EB80** respectively and were subjected to **starvation for 48 hrs**. On completion of 48 hrs, larvae present in these boxes were also killed by collecting them in polythene bags **P79 and P80** by storing them at -20°C. “P83 & P84”, “P81 & P82” and “P79 &P80” were stored at -20°C for period of 1 week to ensure that larvae in all the bags are killed and attain inactivation. The weights of each group of larvae were measured carefully before inactivating them. Point to remember is that there is no difference b/w “P83 & P84”, they both are just the duplicates, to distribute the weight of larvae of specific condition. This applies to “P81 & P82” as well as “P79 &P80”.

1 week later, once entire group of larvae in each bag is ensured dead, all bags were taken out from the freezer and brought to room temperature before grinding them by using mixer (Vorwerk thermomixer). It was ensured that temperature is not raised and maintained at 25°C throughout grinding. Initially, larvae from polythene bags with identification numbers “P83 & P84” were grinded, followed by “P81 & P82” and lastly larvae in “P79 & P80” were grinded. After grinding every sample, mixing jar was washed, and dried carefully, before proceeding with the next sample. Post grinding, samples were transferred to fresh polythene bags having same identification number as they had on polythene bags prior to grinding. Particle size reduction and homogeneity is pre-requisite for any kind of analysis and increase in surface area due to grinding or crushing ensures samples are vulnerable to analytical conditions.

11.1 Determination of fat content

Weibull-Berntrop-type acid hydrolysis method is adopted where initially, 5.0 g of each of the grinded samples from “P83 & P84”, “P81 & P82” and “P79 &P80” were weighed into 150 ml glass beaker attached with a lid and a stirrer. 160 ml of 4N HCl (CHEM SOLUTE, A BRAND OF TH. GEYER) is poured into the glass beaker and is gently placed on the heating device. The resulting mixture undergoes acid digestion for 30-40 minutes at 100°C. Secondly, once the mixture is cooled, the digest is allowed to pass through the filter paper (ALBET LabScience) and washed thoroughly, dried overnight and subjected to extraction using SOXTHERM (Gerhardt), the following day. After extraction the solvent was evaporated and recovered by condensation.

The resulting fat residue was determined gravimetrically after drying the filter paper in a pre-heated oven at 103°C for $1^{1/2}$ hr to bring the samples to constant weight.

$$\textbf{Fat content (\%)} = \{(M_2 - M_1) \times 100\} / M_0$$

Where,

M_2 = weight of the beaker after Soxtherm extraction

M_1 = weight of the empty beaker

M_0 = weight of the filter paper after drying

11.2 Determination of ash content

Dry ashing method is adopted to determine the total ash content of all the samples. Initially, about 1.0 g of each of the samples were weighed into previously dried porcelain crucibles. Post charring the samples were dried in an electrically heated muffle furnace (Heraeus instruments) at 550°C, till they attained constant weight. Percentage ash content was determined by weighing the resulting inorganic residue

$$\textbf{Ash content (\%)} = (M_{ash} \times 100) / M_{dry}$$

Where,

M_{ash} = Mass of the ashed sample

M_{dry} = Mass of the original sample

11.3 Determination of moisture content:

The moisture content of each of the samples were analysed using Sartorius moisture analyser. Moisture content can be determined with the MA 30 as soon as it is turned on, since all the parameters are pre-adjusted at the factory. Basically, its quiet convenient and time conserving to use this device for moisture analysis than using conventional method. As soon as the hood of the analyser is lowered the machine is ready for analysis. It is pre-set to temperature 130°C. It undergoes a self-test and it comes to an end on the appearance of TAR on the LCD of the analyser. Initially, the weight of the disposable aluminium dish is tared and then the sample is

placed on the dish and spread evenly all throughout. By pressing the ENTER key after lowering the hood the moisture content of each of the sample is analysed one after the other.

11.4 Determination of total protein by kjeldahl method

The protein content of the dietary samples have always been determined on the basis of total nitrogen content and therefore, the protein content of samples presented in table were determined by using the Kjeldahl method (AOAC, 2000). This method is universally accepted for determining protein content from the determined nitrogen content by multiplying with a nitrogen- to-protein conversion factor, 6.25. The principle lies in the converting N to NH_3 by digesting the sample in sulfuric acid, using $CuSO_4/TiO_2$ as catalysts, which is distilled and titrated.

% crude protein = % nitrogen × 6.25

Percent nitrogen is calculated using given formula:

$$\frac{1.4007 \times n \times K(V2 - V1)}{m}$$

Where,

n= Normality of HCl used for titration (0.1 N)

K = titration factor (1.03)

V2= volume of HCl used for titration (test sample)

V1= volume of HCl used for titration (blank sample)

12. Feed Formulation

Experiment involving development of formulation with T.molitor larvae and rapeseed press cake was being performed for the very first time. Hence, steps have been taken to avoid complexity during formula development and only basic parameters such as pH, temperature and incubation time were considered. “P83 & P84”, “P81 & P82”, “P79 & P80” which were stored at -20°C post grinding were brought to room temperature before initiating formula development. Since “P83 & P84”, “P81 & P82”, “P79 & P80” have resulted from starvation of T.molitor larvae for 0 hrs, 24 hrs and 48 hrs respectively, three different sets of formulations, **S0, S24 and S48** respectively,

were proposed keeping their starvation conditions in mind. Each set consists of a sub set with 6 different formulations, where each of the 6 formulations differs from the other in either one of these parameters (pH, temperature and incubation time). While considering pH, two different pH were taken into account namely, **pH 2 and pH 8**. **Room temperature (RT) and 45°C** were considered as temperature parameters and incubation time of **0 min and 90 min** was chosen to develop formulation.

5 kg of Rapeseed press cake which was obtained from Kroppenstedter Ölmühle GmbH was milled and the particle size of <1mm and moisture content of 10.5% was used as second test material for formula development. As shown in the fig. 16, set 'SE' is considered to be the control set containing six different formulations with 100% rapeseed press cake and 0% T.molitor larvae. Followed by second set namely 'S0' represented in fig.17 with 6 different formulations from 7-12 each of which contains 90% rapeseed cake and 10% T.molitor larval paste from "P83 and P84" (subjected to 0 hrs starvation). Similarly, set 'S24' also exhibits 6 different formulations from 13-18, each of which contains 90% rapeseed cake and 10% T.molitor from "P81 & P82" (subjected to 24 hrs starvation) showcased in fig.18. Lastly, fig.19 features set 'S48' representing 6 different formulations from 19-24 each of which contains 90% rapeseed cake and 10% T.molitor from "P79 and P80" (subjected to 48 hrs starvation). In case of sets 'S0', 'S24' and 'S48', 69ml of distilled water which is primarily adjusted with 1M HCl /1M$NaHCO_3$ to adjust the pH and moisture of the formulations. In case of control set 'SE' 84 ml of distilled water pre-adjusted with 1M HCl /1M$NaHCO_3$ is used to adjust the pH and moisture of the formulations. Variation in the quantity of water is attributed to the difference in moisture content of formulations with and without T.molitor and to attain consistent moisture among all the samples. Before incubating the samples at proposed time and temperature, proper mixing of ingredients is ensured using lab-scale mixer (VORWERK thermoxier) for about 10 minutes for each formulation to ensure thorough mixing of the ingredients. Care is taken to not raise the temperature of the mixture while blending. Formulations that required samples to be incubated at 45°C for 90 minutes were kept in incubator (WTC binder) at desired temperature.

Sample Code	Rapeseed press cake (100%)	T.molitor (0%)	pH	Incubation Time	Incubation temperature
1	300 g	-	8	0	RT
2	300 g	-	2	0	RT
3	300 g	-	8	90	RT
4	300 g	-	8	90	45
5	300 g	-	2	90	RT
6	300 g	-	2	90	45

Figure 16: Representation of 6 different formulations under set SE with 100% RPC and 0% T.molitor larvae.

Sample code	Rapeseed press cake (90%)	T.molitor (10%)	pH	Incubation time	Incubation temperature
7	270 g	30 g	8	0	RT
8	270 g	30 g	2	0	RT
9	270 g	30 g	8	90	RT
10	270 g	30 g	8	90	45
11	270 g	30 g	2	90	RT
12	270 g	30 g	2	90	45

Figure 17: Representation of 6 different formulations under set S0 with RPC and T.molitor larvae (starved for 0hrs).

No of samples	Rapeseed press cake (90%)	T.molitor (10%)	pH	Incubation time	Incubation temperature
13	270 g	30 g	8	90	RT
14	270 g	30 g	8	90	45
15	270 g	30 g	2	90	RT
16	270 g	30 g	2	90	45
17	270 g	30 g	2	0	RT
18	270 g	30 g	8	0	RT

Figure 18: Representation of 6 different formulations under set S24 with RPC and T.molitor larvae (starved for 24 hrs).

No of samples	Rapeseed press cake (90%)	T.molitor (10%)	pH	Incubation time	Incubation temperature
19	270 g	30 g	8	90	RT
20	270 g	30 g	8	90	45
21	270 g	30 g	2	90	RT
22	270 g	30 g	2	90	45
23	270 g	30 g	2	0	RT
24	270 g	30 g	8	0	RT

Figure 19: Representation of 6 different formulations under set S48 with RPC and T.molitor larvae (starved for 48 hrs).

13. Extrusion

The extruder used is a single-screw extruder from Brabender, type DNDL-44. The selected barrel length of the extruder is characterized by length- to- diameter ratio which is 20:1. The extruders are made up of 416 stainless steel. The surface of the extruder is nitrided in order to provide surface quality and ensure hardness. Heating of the extruder barrel is achieved by split ring aluminum heater-cooler-collar. Precise time is required to proportionately heat the barrel and solenoid valves are called for cooling the system. Before running the feed formulations, operation is tared by running rapeseed press cake of desired moisture and mass flow, shape of the extrudate and extrusion parameters are checked. Moisture of the feed formulation while extrusion ranged about 29% for each run. During extrusion the temperature of the feed within the extruder barrel ranged between 126-128°C. The screw speed was adjusted at 120 rpm. For all runs, temperature of the feed while exiting the nozzle ranged from 60°C on entering the metering zone , 130°C while shaping of extrudate within the die and 130°C while exiting the metering section. The raw feed was funneled in small amounts in order to avoid jamming at the receiving end. This step is manually performed. The residence time of the feed was 1minute and 45 seconds. Output of the extrudate was determined to be 1.8 kg/h. Rod dies were used to provide shape to the extrudate. Detailed description of extrusion parameters, such as temperature profile, torque, screw speed, pressure are tabulated underneath.

Sample number	1	2	3	4	5	6	7	8	9	10	11	12
Extrusion Moisture (%)	29.55	29.47	29.53	29.67	29.65	29.77	29.87	29.67	29.36	29.22	29.96	29.49
Torque (Nm) Mw	6.4	7.4	6.5	5.5	5.4	6.6	5.8	6.4	8.9	5.2	4.1	5.3
Mass temp. (° C) Mw	126.7	128.1	129.7	123.5	128.4	131.7	127.9	127.9	129.8	129.1	128.2	128.1
Mass pressure (bar)	4.8	3.7	4.8	5.5	2	2.2	2.9	3.1	3.6	4.3	1.9	3.2
Exit from the nozzle												
Temperature (° C)	60 / 130 / 130											
Screw speed (rpm)	120											
Residence time (min)	01:45											
Throughput (kg / h)	1.8											

	Rapeseed cake+ T. Molitor (24 hrs diet)						Rapessed cake + T. Molitor (48 hrs diet)					
Sample number	13	14	15	16	17	18	19	20	21	22	23	24
Extrusion Moisture (%)	29.46	29.74	29.65	29.55	29.43	29.74	29.33	29.54	29.68	29.71	29.91	29.68
Torque (Nm) Mw	3.6	4.2	4.0	3.5	3.5	4.0	3.8	3.9	3.4	3.4	3.4	3.6
Mass temp. (° C) Mw	127.5	128.3	128.3	128.0	127.2	127.4	128.0	128.0	127.1	127.6	128.0	128.0
Mass pressure (bar)	2.3	3.7	1.9	2.4	1.8	1.6	2.3	3.4	1.6	2.1	2.2	2.1
Exit from the nozzle												
Temperature (° C)	60 / 130 / 130											
Screw speed (rpm)	120											
Residence time (min)	01:45											
Throughput (kg / h)	1.8											

Samples were collected post extruding of feed formulations and were dried for 48 hrs at room temperature on large racks. Later the samples were sent for defatting prior to quantitative and qualitative analysis of protein of extruded feed formulations.

14. Defatting of Extrudates

5 g of each of the grounded 24 samples were weighed into cellulose thimbles. Prior to this step, empty cellulose thimbles and 250 ml glass beakers were weighed. 130ml of petroleum benzene, used as the extracting solvent, was poured into 250 ml glass beakers. The desirable conditions were set and the system was allowed for extraction of fat. The extraction was carried out approximately for $1^{1/2}$ hr. Later, the glass beakers were kept overnight at room temperature and following morning, samples were subjected to drying in pre-heated oven at 110°C for approximately 1 hr in order to remove excess solvent. Then the glass beakers were taken out of the oven and placed in the desiccator. The weight of the glass beakers were measured and fat

content was determined and defatted sample from the thimble was collected and sampling was done carefully and stored in cool and dry place till further use.

Samples	Raps.cake	T. molitor		Diet (hrs)			pH		Incbtn. Time (min)	Temp °C	After. Extrusion		Tot. prot.cont
		with	without	S0	S24	S48	BE	AE			Defatted	Non- defat	
1	✓		✓				8	8.01	0	RT	✓		31.1
2	✓		✓				2	4.58	0	RT	✓		30.2
3	✓		✓				8	7.91	90	RT	✓		31.1
4	✓		✓				8	7.51	90	45	✓		31.4
5	✓		✓				2	4.58	90	RT	✓		30.1
6	✓		✓				2	4.57	90	45	✓		29.8
7	✓	✓		✓			8	7.92	0	RT	✓		31.4
8	✓	✓		✓			2	4.66	0	RT	✓		30.7
9	✓	✓		✓			8	7.76	90	RT	✓		31.4
10	✓	✓		✓			8	7.59	90	45	✓		31
11	✓	✓		✓			2	4.65	90	RT	✓		30.4
12	✓	✓		✓			2	4.65	90	45	✓		30.9
13	✓	✓			✓		8	7.73	90	RT	✓		31.6
14	✓	✓			✓		8	7.44	90	45	✓		31.5
15	✓	✓			✓		2	4.66	90	RT	✓		30.7
16	✓	✓			✓		2	4.65	90	45	✓		30.6
17	✓	✓			✓		2	4.66	0	RT	✓		30.6
18	✓	✓			✓		8	7.55	0	RT	✓		31.3
19	✓	✓				✓	8	7.62	90	RT	✓		31.6
20	✓	✓				✓	8	7.57	90	45	✓		31.4
21	✓	✓				✓	2	4.64	90	RT	✓		30.1
22	✓	✓				✓	2	4.63	90	45	✓		30.5
23	✓	✓				✓	2	4.65	0	RT	✓		30.8
24	✓	✓				✓	8	7.62	0	RT	✓		31.3

Figure 20: Overall view of different extrudates post defatting (DES)

Samples	Raps. Cake	T. molitor		Diet (hrs)			pH		Incubtn time	Temp	After. Extrusion	
		with	without	S0	S24	S48	BE	AE			Defat	Non-defatted
1	✓		✓				8	7.97	0	RT		✓
2	✓		✓				2	4.56	0	RT		✓
3	✓		✓				8	7.97	90	RT		✓
4	✓		✓				8	7.57	90	45		✓
5	✓		✓				2	4.57	90	RT		✓
6	✓		✓				2	4.56	90	45		✓
7	✓			✓			8	7.93	0	RT		✓
8	✓	✓		✓			2	4.81	0	RT		✓
9	✓	✓		✓			8	7.24	90	RT		✓
10	✓	✓		✓			8	7.48	90	45		✓
11	✓	✓		✓			2	4.72	90	RT		✓
12	✓	✓		✓			2	4.63	90	45		✓
13	✓	✓			✓		8	7.69	90	RT		✓
14	✓	✓			✓		8	7.61	90	45		✓
15	✓	✓			✓		2	4.64	90	RT		✓
16	✓	✓			✓		2	4.63	90	45		✓
17	✓	✓			✓		2	4.61	0	RT		✓
18	✓	✓			✓		8	7.63	0	RT		✓
19	✓	✓				✓	8	7.64	90	RT		✓
20	✓	✓				✓	8	7.58	90	45		✓
21	✓	✓				✓	2	4.61	90	RT		✓
22	✓	✓				✓	2	4.60	90	45		✓
23	✓	✓				✓	2	4.61	0	RT		✓
24	✓	✓				✓	8	7.75	0	RT		✓

Figure 21: Overall view of non-defatted extrudates (NDES)

15. Extraction of proteins

The solubility profile of the proteins for the samples described in fig. 20 and 21 are determined under varying pH values. For this purpose, the protein content of the samples were determined according to Bradford method. 250 mg of both defatted (DES) and non-defatted (NDES) extruded samples described in the table were weighed into 15 ml falcon tubes, and were dissolved/extracted by adding 12.5 ml of distilled water respectively. The stirring was continued thereafter for 30 min at room temperature. The pH of solution is noted and the suspensions were centrifuged at 6,000 × g for 30 min at 4°C. To determine the soluble protein content and composition of the dissolved proteins, aliquots of the supernatant (3×100 µl), containing the dissolved proteins, are filled in 1.5 ml centrifuge tubes (n=3) and stored at -4°C till further use.

16. Determination of protein concentration of Extrudates (DES and NDES)

The standard curve is prepared according to the dilution table given underneath using the BSA standard solution (1mg/ml).

Dilution Table:

	Water/ buffer (µl)	BSA- Sol (µl)	End conc (µg/ml)
1	400	0	0
2	380	20	50
3	360	40	100
4	320	80	200
5	240	160	400
6	80	320	800

Chemicals:

Bio-Rad Bradford Reagent (conc):- Dilution: 1 part reagent + 4 parts water

Sample preparation:

One of the previously stored 300 µl aliquots of each of DES and NDES (total of 48 samples) were brought to room temperature prior to analysis of protein content.

Analysis:

- Pipette 10 µl of standard solutions/ DES/ NDES into wells of the culture plate. Each solution is measured in triplicates to verify results.
- 200 µl of the diluted Bradford reagent solution was added to the standards, DES and NDES on the plate.
- The 96-well culture plate was incubated for 5 min at room temperature and then the OD was measured at 595 nm with an iMark^{TM} Microplate Absorbance Reader (Bio Rad Laboratories GmbH, Munich).
- The standard curve was created by plotting the averaged absorbance vs the protein concentration of each of the standard.
- The unknown protein concentration of each of the test sample was determined using polynomial interpolation with the help of the protein standard series included on each plate.

17. Determination of Protein composition of Extrudates by SDS-PAGE

Chemicals:

- 1 M Tris:
  6.06 g Tris (hydroxymethyl) aminomethane (M=121.14 g/mol) (Carl Roth GmbH and co, Karlsruhe) was dissolved in ca. 25 ml distilled water, and then, was made up to 50 ml with distilled water.

- 1 M Tris pH 8.8
  6.06 g Tris (hydroxymethyl) aminomethane (M=121.14 g/mol) was dissolved in ca. 25 ml distilled water, HCl was used for adjusting to pH 8.8 and later, it was made up to 50 ml with distilled water.

- 1 M Tris pH 6.8
  6.06 g Tris (hydroxymethyl) aminomethane (M=121.14 g/mol) in ca. 25 ml distilled water, HCl is used for adjusting to pH 6.8 and finally, it is made up to 50 ml with distilled water.

- 10% SDS
  10 g of Sodium Dodecyl Sulphate was dissolved in ca. 80 ml distilled water and once the transparent solution was achieved it was made to 100 ml.

- 10% APS
  51 g of APS was dissolved in 0.5 ml of distilled water and was stored at 4°C. This solution was freshly prepared.

- 4X Sample Buffer
  8 g of SDS, 24 g of 87 % glycerol(Carl Roth GmbH and co, Karlsruhe) , 1.22 g of 100 mM Tris(hydroxymethyl)aminomethane and 10 g of β-Mercaptoethanol were weighed in a glass beaker were adjusted to pH 6.8 with 0.1 N HCl. Then, 0.02 g of Coomassie was added and mixed thoroughly and finally the resulting solution was made to 100 ml with distilled water.

- 1X Sample Buffer
  1 part of 4X Sample buffer was mixed with 3 parts of distilled water.

- Staining Solution

  This solution was prepared by adding 78.2 ml of solution A, 20 ml of Methanol (Carl Roth GmbH and co, Karlsruhe) and 1.6 ml of Coomassie Brilliant Blue.

  (Solution A: 160 g of ammonium sulphate (Carl Roth GmbH and co, Karlsruhe) was dissolved in 1536 ml of distilled water and was added to 32 ml of 85% phosphoric acid (Carl Roth GmbH and co, Karlsruhe).)

- Buffer solution for Electrophoresis

  100 ml of 10X running buffer were diluted with 900 ml of distilled water and 10 ml of 10% SDS was added and shaken thoroughly to prepare 1 L buffer solution.

  (10X running buffer: 30.3 g of Tris (hydroxymethyl) aminomethane (M=121.14 g/mol) and 144 g of Glycine (AppiChem Panreac MW Companies) were dissolved in 500 ml distilled water and then made up to 1 L).

- Sample preparation

  300 µl of all test samples, 150 µl of 1 M Tris, 150 µl of 4X Sample buffer were added to an Eppendorf tube and were centrifuged to give a thorough mix.

- Control samples preparation

  10 mg of each of the DES were dissolved in 1000 µl of 1X sample buffer.

Methodology

To assess the impact of pH on the polypeptide profile of extracted proteins of DES and NDES, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed (SDS PAGE) according to the method suggested by Laemmli (1970), DES, NDES were pooled together. They were mixed with the sample buffer in 1:2 ratio, performed according to the procedure explained in the preparation step. Aliquots of DES/NDES were brought to room temperature before performing mixing with sample buffer. Subsequently, denaturation of proteins was carried out at 95°C at 800 RPM for 3 min. After cooling the samples were centrifuged for quick 10 sec and 5µl of standard, DES and NDES were carefully pipetted out into lane 1 – 12 of 15%T Tris- Glycin gels respectively. The gels were prepared with the help of vertical electrophoresis equipment supplied by biostep GmbH (Jahnsdorf, Germany). Low molecular weight calibration kit was used which was provided by GE Healthcare, Buckinghamshire, UK. For the first 10 minutes, the

gel was run at 100 V, as it will improve resolution of proteins in the stacking gel by allowing them to compact in a very thin phase before entering the separation gel. Thereafter, the gel was run approximately for 1 hr 15 min at 180 V. This step was followed in order to differentiate proteins of lower molecular weight from the higher molecular weight proteins. When the samples reached the lower front of the gels, the power was switched off and the electrophoresis process ended. The gels were removed carefully from the gel holding cassette and were immediately placed in the staining solution. The gels were stained overnight at room temperature on a shaker. The next day, the samples were de-stained for about 6- 7 hours using distilled water which was discarded time to time and replaced with fresh distilled water. After de-staining the gels were quantified using Quantity One 1-D Analysis Software, version 4.5.2 (Bio- Rad, Universal Hood II, Bio-Rad Laboratories, Segrate, Milan, Italy)[27].

RESULTS AND DISCUSSIONS

I. Air classification (1st part)

Sample codes	Codes of the sample	Ash content (%)	Moisture content (%)	Fat content (%)	Protein content by (%)
1	**RPF**	7.17 ± 0.01	1.5 ± 0.14	45 ± 0.01	34.82 ± 0.23
2	**RPC**	7.87 ± 0.02	3.42 ± 0.28	11.6 ± 0.49	36.45 ± 0.56
3	**DF1500**	6.63 ± 0.08	7.33 ± 0.62	2.79 ± 0.18	29.86 ± 0.42
4	**DF1800**	7.21 ± 0.03	7.91 ± 0.54	3.88 ± 0.78	33.84 ± 0.63
5	**DF2000**	8.13 ± 0.05	8.45 ± 0.03	1.52 ± 0.00	33.97 ± 0.17
6	**HF**	7.04 ± 0.21	8.45 ± 0.37	1.77 ± 0.11	26.53 ± 0.22
7	**DF8000**	6.83 ± 0.14	7.96 ± 0.54	0.65 ± 0.23	28.31 ± 0.67
8	**DF4000**	7.63 ± 0.07	8.82 ± 0.23	1.44 ± 0.01	31.97 ± 0.32
9	**GC**	2.8 ± 0.20	7.28 ± 0.14	-	83.25
10	**AC**	6.9 ± 0.14	7.81 ± 0.32	-	76.89
11	**GI**	-	-	-	-
12	**AI**	-	-	-	-

Figure 22: Representation of percent ash, moisture, fat and protein content of test samples.

1. Changes in physio-chemical properties of test samples as result of air-classification

1.1 Total ash Content

The ash content for test samples (1-10) ranged between 3% - 8%. (figure 22) GC had the lowest ash content (2.8%) and sample DF2000 was found to have the highest with 8.13%. it is surprising to observe that ash content of RPF (7.17%) is lower than RPC (7.87%). Ash content of RPF and RPC are in correlation with results presented in [1]. There is no considerable difference has been reported b/w air fractionated samples. The low level of ash content indicates less amount of impurities and allows the oil to be good source of minerals. Results obtained are more or less in accordance with the ash content reported in Air Classification of Rapeseed Meal by (R.D.KING and H.M.DIETZ) [17]. This also suggests that it can be recommended for animal and human consumption and used for microbial growth without the addition of mineral supplement.

1.2 Total moisture content

Determining moisture content is an important aspect as it allows to determine the other important components of the sample. For instance, protein content is reported as a percentage of the defatted meal at 10% moisture. The amount of moisture is dependent on various factors such as relative humidity, temperature during each stage of processing and storage. In the present case, moisture content of test samples (1-10) has covered a range of 1.5% lowest to 8.82% (figure 22). The least was recorded in case of RPF (1.5%) and highest in case of DF4000 (8.82%). It is quite evident from the results exhibited by air fractionated samples (3-8) and concentrates (AC and GC), that processing and storage conditions have a significant effect on the moisture content of the samples. However, the results of moisture content reported are < 10%. Low moisture content helps in prolonging shelf life and prevent product spoilage by reduced activity of microorganisms [18].

1.3 Total fat content

Among all the test samples analysed for fat content, RPF (45%) exhibited the highest fat content followed by RPC (11.6%). As expected, the fat content of fractionated samples (3-8), ranged b/w 3%-4%, which was quiet low when compared to RPF and RPC. The results from total fat content by acid hydrolysis were quiet similar to the amount of oil content obtained from defatting the samples using Soxhlet (results not shown). In case of RPF there is an approximate increase of 6% fat content analysed by acid hydrolysis than the amount of oil content by Soxhlet.

RPC, showed no variation in fat content by both the methods. But, there is a slight difference in the fat content observed in samples 3-8. This can be due to extraction of non-lipid moieties in the solvent adding weight to the fat content analysed by Soxhlet.

1.4 Total protein content

As mentioned earlier, rapeseed characteristically contains high amount of protein among most of the oil seeds, besides soybean. Defatted RPC is determined to have higher protein content (36.45%) than RPF (34.82%) (figure 22). In case of air-fractionated samples, upward trend has been noticed, for instance, protein percentage has increased from sample DF1500 (29.86%), DF1800 (33.84%) and finally DF2000 (33.97%) and then a gradual decrease in the protein content has been noticed in case of samples DF 4000 (31.97%) and DF 8000 (28.31%). Hulls fraction (HF) ended up being the least scoring sample with protein percentage of (26.53%). Tyler et al. (1984) has examined the effect of cut size on separation and reported that increasing the cut size improves the protein recovery and yield of the fine fraction, however, it reduces the concentration of protein in the fine fraction.

The protein content of resulting air-fractionated sample might result in higher yield but there is no guarantee to achieve high protein content. For example, according to citations, on air-fractionation, high yield was recorded in case of lupine, however, the protein content of the fractionated portion was lower than the original meal. Similarly, protein content exhibited by the air-fractionated samples (3-8) presented in the figure 22 show lower values than RPC and RPF. Anti-nutritional factors such as oligosaccharides (Vose et al., 1976), phytic acid, trypsin inhibitors (Elkowicz and Sosulski, 1982) could be recovered in the protein fractions and reduce the protein content.

2. Influence of pH over protein solubility of extracted proteins

Protein solubility is one of the most important functional properties of proteins, as it determines the amount of soluble nitrogen present in a sample when dissolved in aqueous systems under defined parameters. It is considered as one of the preliminary steps to determine the protein solubility because solubilisation of proteins strongly determines the quality and composition during the development of new protein ingredients.

Solubility is a direct manifestation of functional properties of a food system, like emulsion stability, foaming capacity, water and fat binding capacity etc. (Vaclavik and Christian, 2003).

Protein solubility is largely influenced by processing treatments, storage and extraction conditions such as pH value, temperature and ionic strength (Khalid, Babiker, & EL Tinay, 2003; Lawal, 2004; Ragab, Babiker & Eltinay, 2004). Variation in processing conditions of samples is most likely to exhibit different protein content (Boye, Zare, et al., 2010). In addition to this, it has been outlined that the process of extraction of oil from seeds is generally considered to reduce the overall protein solubility ((Pedroche and others 2004). 12 Different rapeseed samples RPF, RPC, DF1500, DF1800, DF2000, HF, DF8000 and DF4000, GC, AC AI and GI were mixed with water, which were pre-adjusted from pH 1-10, using 1M HCl and 1M NaOH were analysed for protein solubility. The percent protein solubility of the described rapeseed samples in aqueous solution is depicted as function of pH.

pH of the solvent	RPF	RPC	DF1500	DF1800	DF2000	HF
1	13.87 ± 0.67	24.60 ± 0.45	24.97 ± 0.83	18.93 ± 0.90	20.69 ± 0.34	28.29 ± 0.92
2	5.69 ± 0.09	15.41 ± 0.68	11.40 ± 0.36	13.83 ± 1.02	10.17 ± 0.06	15.94 ± 0.88
3	1.52 ± 0.04	13.01 ± 0.11	2.99 ± 0.04	4.23 ± 0.12	6.82 ± 0.13	12.32 ± 0.26
4	0.34 ± 0.00	9.07 ± 0.32	4.16 ± 0.24	8.81 ± 0.77	7.78 ± 0.15	10.38 ± 0.13
5	8.13 ± 0.26	15.38 ± 0.32	4.54 ± 0.06	6.00 ± 0.25	5.09 ± 0.25	9.96 ± 0.28
6	9.71 ± 0.09	19.73 ± 0.24	8.14 ± 0.24	16.19 ± 1.97	7.23 ± 0.55	15.38 ± 0.17
7	18.79 ± 0.51	23.78 ± 0.15	15.55 ± 0.88	14.72 ± 0.71	12.42 ± 1.10	27.72 ± 0.46
8	11.61 ± 0.17	30.53 ± 0.38	21.58 ± 1.96	29.90 ± 3.84	13.41 ± 1.60	45.67 ± 1.95
9	17.78 ± 0.16	31.91 ± 1.20	27.35 ± 1.17	27.20 ± 1.86	26.73 ± 1.35	42.28 ± 1.48
10	23.53 ± 0.45	40.27 ± 1.46	37.40 ± 1.31	47.04 ± 3.49	38.95 ± 0.99	52.37 ± 0.39

Figure 23: Percent protein solubility of extracted proteins over a pH range of 1-10 (a)

pH of the solvent	DF8000	DF4000	AC	GC	AI	GI
1	21.43 ± 0.38	19.45 ± 0.42	34.56 ± 0.77	42.32 ± 1.03	38.03 ± 0.84	31.07 ± 0.51
2	10.57 ± 0.10	13.82 ± 0.15	37.34 ± 1.45	47.31 ± 0.35	40.75 ± 1.34	33.66 ± 0.55
3	7.04 ± 0.02	6.12 ± 0.08	37.58 ± 0.39	23.44 ± 0.19	44.47 ± 0.79	8.22 ± 0.11
4	6.24 ± 0.25	6.43 ± 0.10	28.17 ± 0.18	6.18 ± 0.14	44.33 ± 0.58	3.66 ± 0.01
5	7.61 ± 0.16	6.08 ± 0.14	23.87 ± 0.20	9.45 ± 0.21	43.65 ± 1.25	4.43 ± 0.05
6	15.07 ± 0.41	8.15 ± 0.19	28.40 ± 0.58	7.73 ± 0.22	44.65 ± 0.72	3.51 ± 0.04
7	19.89 ± 0.83	13.04 ± 0.30	8.08 ± 0.02	14.54 ± 0.36	39.62 ± 0.44	3.08 ± 0.04
8	28.67 ± 0.92	19.67 ± 0.78	30.84 ± 0.19	20.82 ± 0.53	41.57 ± 0.85	6.15 ± 0.07
9	18.16 ± 0.19	18.35 ± 0.30	32.00 ± 0.32	35.56 ± 0.56	41.71 ± 0.45	19.98 ± 0.31
10	29.16 ± 0.37	22.85 ± 0.73	37.05 ± 0.47	52.26 ± 0.64	42.20 ± 1.54	19.69 ± 0.11

Figure 24: percent protein solubility of extracted proteins over a pH range of 1-10 (b)

Basically, protein products that are derived from Rapeseed proteins are primarily seed storage proteins. The albumin and globulin fractions of Rapeseed proteins, namely cruciferin and napin, exhibit a distinct solubility behaviour. This dissimilarity is attributed to various conditions such pH, temperature, ionic strength and salt concentration (Wanasundara et al., 2012). The pH of the medium is one of the factors having highest impact on Solubility. At the first glance, the protein solubility of RF, RPC, DF1500, DF1800, DF2000, HF, DF8000 & DF4000 resembles to a larger extent with the report presented by M. N RADWAN (solubility of Rapeseed proteins in aqueous solutions) which shows that the solubility increases gradually when it approaches higher basic and acidic values away from the isoelectric point. This gives the solubility curve a U-shaped form when plotted against pH on the graph. It's clearly evident that apart from concentrates i.e., AC, GC, and isolates AI, GI, the protein solubility curve of rest of the processed samples exhibits the minimum solubility b/w pH 4-5 (Sosulski and Bakal) and holds maximum solubility at pH 10. In other words, it is evident from the table shown above isoelectric points of most of the samples is observed at pH values between 4 and 5, which can be justified with previous works of Ghodsvali et al.,2005; Tan et al., 2011b. Nevertheless, this is not the case with samples DF1500 and DF1800, which have isoelectric point or display the lowest protein solubility at pH 3, which is in close agreement with the works of authors Pedroche et al. (2004) whose reports indicated that the lowest solubility is at pH 3.5 and 5. This can be justified by the work done by (Wanasundara et al., 2012; Wanasundara and McIntosh, 2013) who suggested that rapeseed proteins that are insoluble at pH 3 and 4, namely cruciferin, forms complexes with entities that impart neutrality by altering the overall charge. The maximum or highest solubility of all the processed samples can be attributed to the fact that in alkaline medium the repulsive forces being predominant as the net charge of the particles is same, they restrict the formation of aggregates. Therefore, there is no precipitation of suspended particles at pH 10. Also, minimum solubility is at pH 3-5 is due to the effect of pH being willingly adjusted. It is notable from the results that buffering action plays an important role in the solubility especially at minimum solubility pH. Also, protein extracts showed higher buffer capacities on moving away from pH 4 towards pH 10.

Work done by Alina Gerzhova, Martin Mondor, Marzouk Benali, Mohammed Aider which states that usually proteins carry a net negative charge above the isoelectric point and at below the isoelectric point, the protein in the solution possess a net positive charge but when salt is added to the solution, the salt ions tend to counter the charge surrounding the protein. This leads to the deposition of counter ions around the protein molecule, decreasing the nitrogen solubility.

Hence, shifting the isoelectric point to a more acidic pH as a result of specific ion binding effects. The protein molecule pulls the inorganic anions more strongly than the inorganic cations due to smaller hydrated radii, anions can screen the charged group of proteins more effectively than cations This can be explained more clearly, by the binding of cl^- ions to the protein molecule changes the overall charge on the protein molecule by neutralising the positive charges and exposing the excess of negative charge at the pH of original isoelectric point consequently changing the protein confirmation. The resulting confirmation is the reason for shifting to a new isoelectric point. .

Moreover, in case of RPF and DF4000 at pH 8 and HF and DF8000 at pH 9, a slight dip in the solubility curve resonates with the data by Quinn and Jones (1976) who found that minimum solubility for canola proteins at pH values of 3.7-4.0 and 7.7-8.0. Gillberg*, L., and* Törnell*, B.* 1976 has reported that the multiple isoelectric points of rapeseed proteins, covering a relatively wide range of pH 4.5-8 is due to the formation of undesirable complexes formed due to interaction b/w phytates and proteins,that are extracted along with the proteins. Phytic acid is strongly negatively charged over most of the pH scale, at acid pH, the protein possess a net positive charge while phytic acid is negatively charged; thus a binary-phytate complex forms. On the other hand, at alkaline pH, both protein and phytic acid are negatively charged; thus the interactions mediated by multivalent cations to form a ternary protein-mineral- phytate complex Additionally, there was no considerable colour difference was observed at pH with minimum solubility. However, the colour changed gradually from yellow to dark green in the alkaline conditions, whereas the colour turned from yellow to grey in acidic environment similar to observations reported by M. N RADWAN. Phenolic compounds identified in rapeseed are basically flavonoid- based polymeric phenolic compounds which hold 1% (w/w) overall concentration of the meal. These compounds are highly reactive and undergo enzymatic as well as non-enzymatic oxidation to form quinones, which thereafter react with proteins and exhibit dark green or brown colouring to rapeseed protein solutions [18][15][13].

Excluding GC, AC, GI and AI, all samples have exhibited a trend that depicts a sharp rise when the pH is moved either to more acidic (pH 1 and pH 2) and alkaline environments (pH 10) In case of AC, GC, AI and GI, completely different solubility profile has been exhibited from the rest of the tested samples. GC has shown the highest solubility at basic (52.26% at pH 10) and acidic (47.31% at pH 2) media, but has shown the least solubility at pH 4 and 6. This could be due to dominance of 11S globulins. Similar kind of solubility profile has been exhibited by GI at acidic and mildly alkaline media but at pH 10, contrary to GC and rest of the samples, it has exhibited the lowest protein percent solubility. This can be explained by the fact that proteins

soluble at low pH were lost during the preparation of GI, which is frequently prepared from defatted meals by solubilisation of proteins in alkaline media and precipitation at the acidic pI. The soluble fraction of AI, likely to contain 2S albumins, has almost a wide range of solubility over the entire pH range, thus having a great potential in the beverage industry [4].

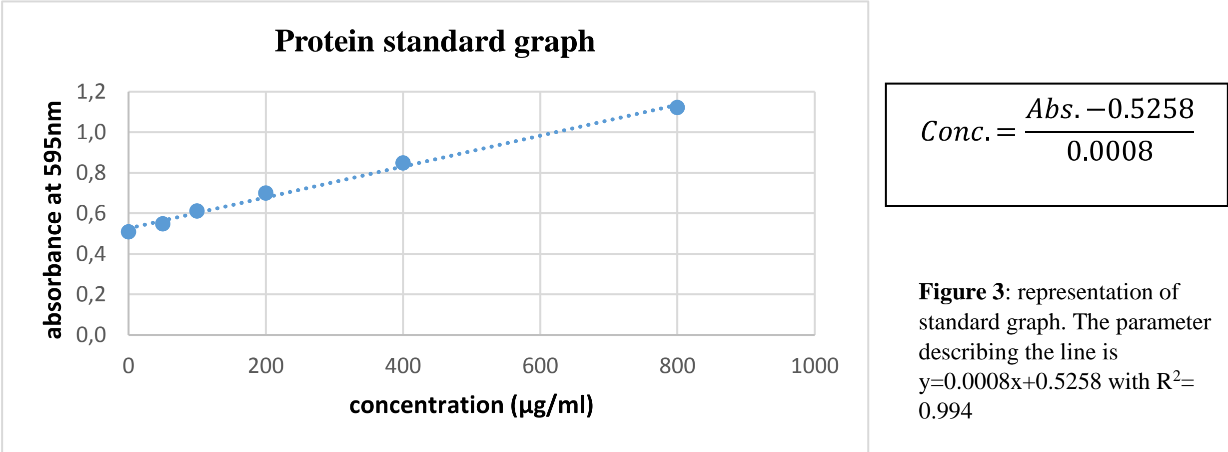


Figure 3: representation of standard graph. The parameter describing the line is y=0.0008x+0.5258 with R^2= 0.994

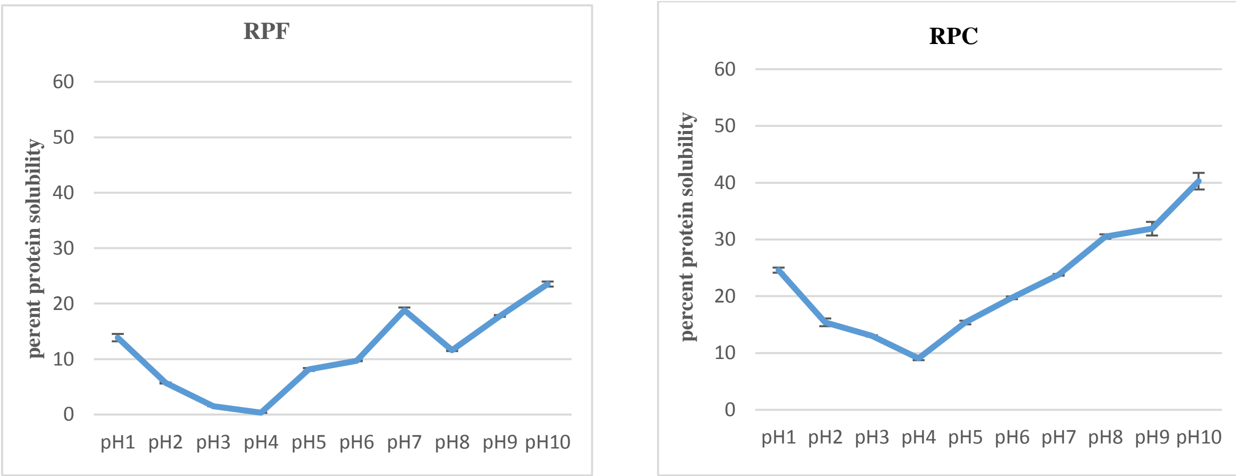
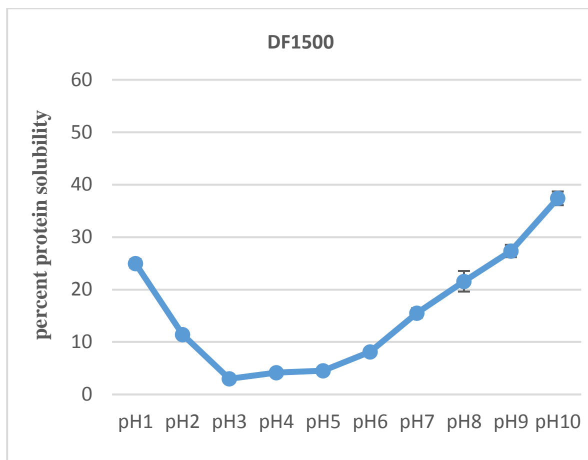


Figure 25: Graphical representation of protein solubility of rapeseed flour (RPF) and rapeseed press cake (RPC) on a pH scale of 1-10 determined using Bradford method



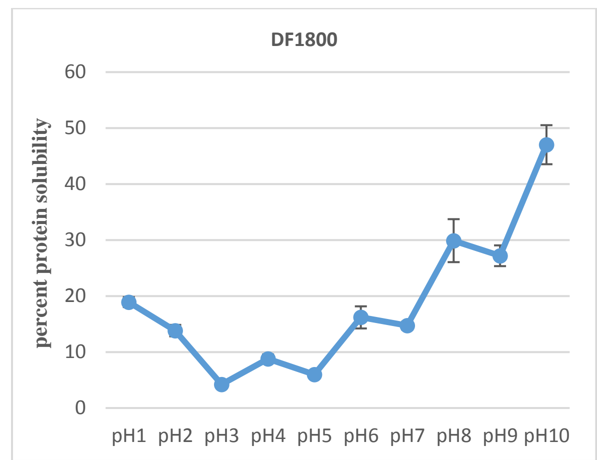
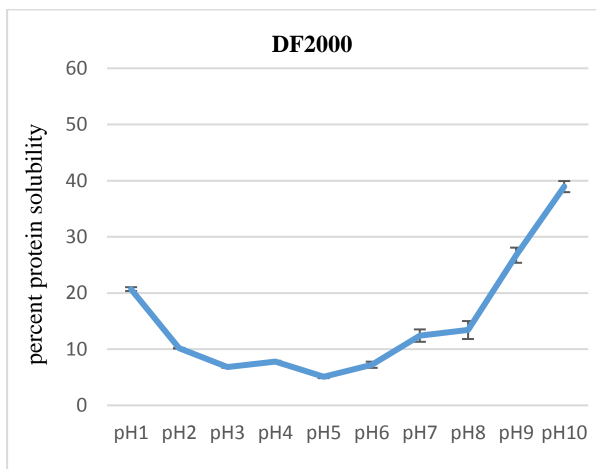


Figure 26: Graphical representation of protein solubility of air-classified samples-DF1500 and DF1800 on pH scale of 1-10.



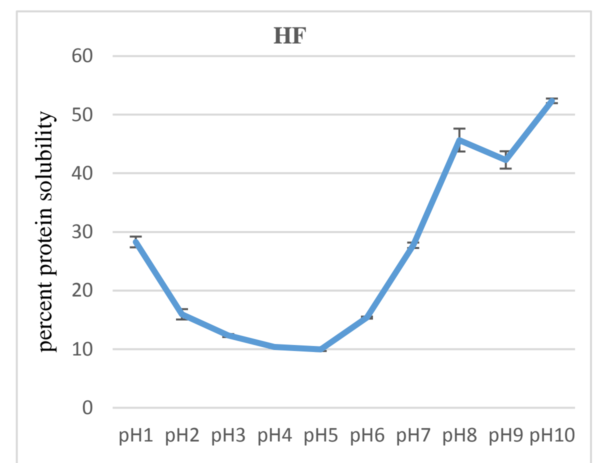
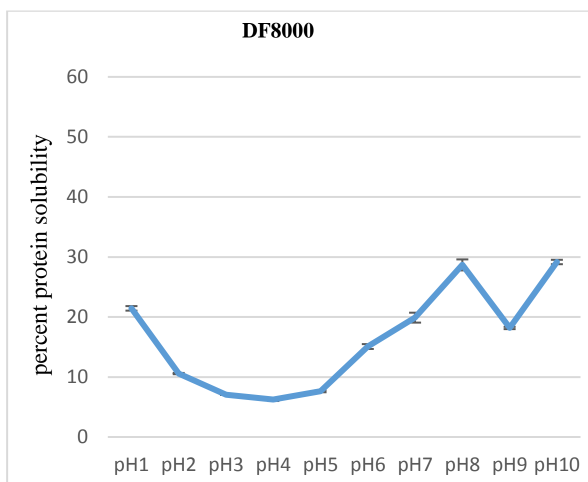


Figure 27: Graphical representation of variation in protein solubility of air classified samples –DF2000 and HF (rich in hulls) when extracted over a pH range of 1-10.



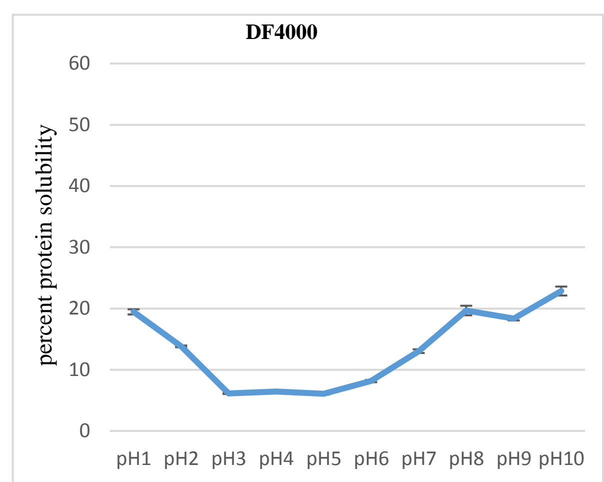


Figure 28: Graphical representation of percent protein solubility over a pH range of 1-10 for air-classified samples DF8000 and DF4000.

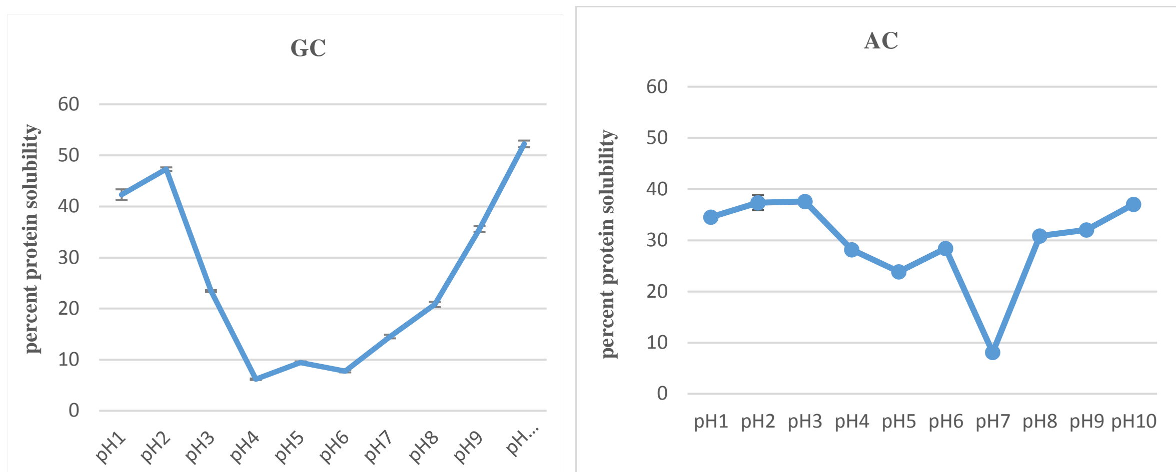


Figure 29: Graphical representation of influence of pH (1-10) on protein solubility trend of concentrates (AC and GC).

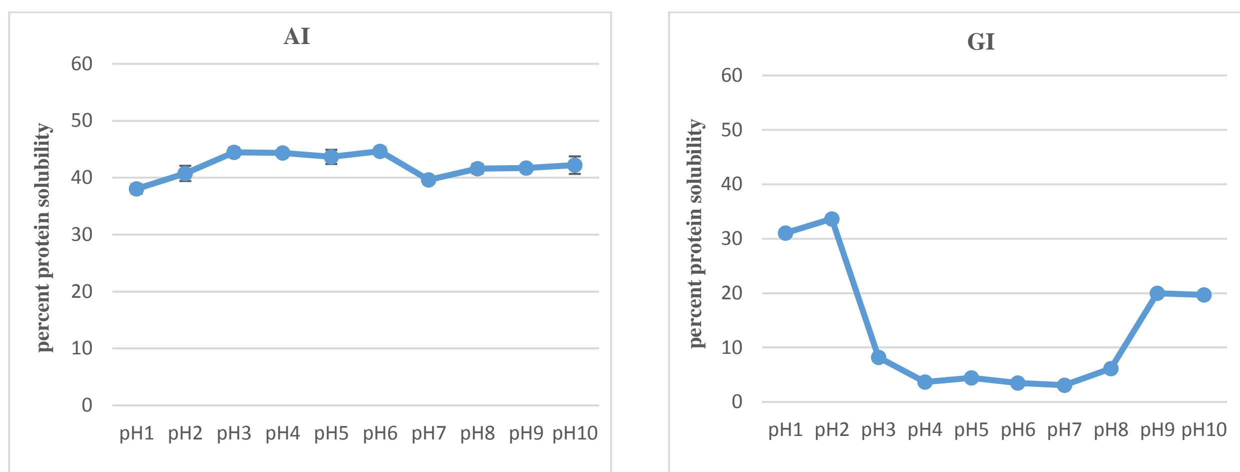


Figure 30: Graphical representation of protein solubility on a pH scale of 1-10 for rapeseed albumin isolate (AI) and globulin isolate (GI) [reference samples].

3. Protein composition as related to extraction of proteins over a pH range of 1-10

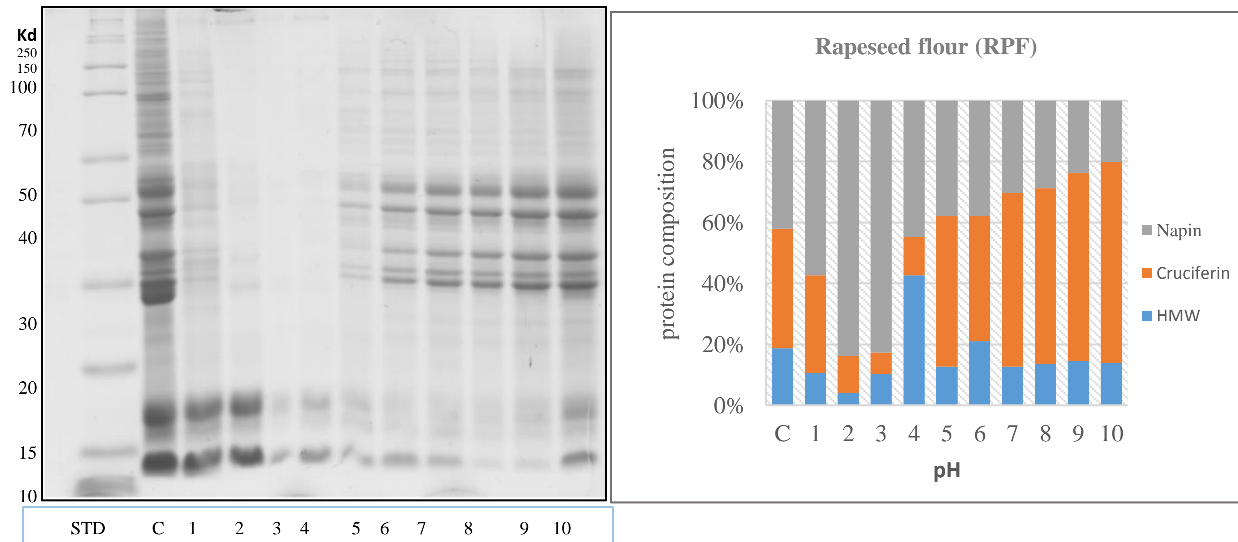
In order to study the variability of the protein composition during the extraction /solubilisation process, it is necessary to allocate the major protein fractions as analysed by SDS-PAGE for different processed air classified rapeseed samples. The objective was to observe and study the impact of air classification technique on protein compositions of test samples. Percent protein composition is determined as the function of pH ranging from 1-10. Change in composition and structural profile of proteins is mainly influenced by the method of extraction which effects the functional properties.

It is evident from citations that Rapeseed main proteins are Cruciferin, is salt soluble 12S globulin, and napin which is 2S water soluble albumin. They both differ from each other by their molecular weights, sedimentation coefficients and functionalities .

Based on the allocation, the composition of individual fraction distribution in the extracted proteins for the different processed rapeseed samples. In case of RPF, RPC, DF1500, DF1800,DF2000, HF, DF8000 & DF4000, gels of each of the samples portray justification with the results obtained by Bradford, where the protein solubility forms a U-shaped curve with isoelectric points ranging b/w 3-5 and increase in solubility is observed on either side of Isoelectric point. This condition is evident with the pattern of distribution of cruciferin over the entire pH range. As expected, percentage of globulin composition becomes more dominant in the pH range above the isoelectric point.

Similarly, in context with cruciferin, the distribution of cruciferin over the pH range 1-10, exhibits that solubility increases on moving away from the isoelectric points and becomes more profound in alkaline conditions, as expected. The band intensity of molecular weights 20-40 Kd intensifies on moving away from isoelectric points and shows maximum intensity at pH 10. This shows that cruciferin has better solubility in alkaline region rather than acidic region. This could be substantiated by formation of new polypeptides apart from disulfide linking. Although, it is slightly different from the works reported by others in terms of bands with 45-50 kDa. There is little or no appearance of the band with molecular weight 45-50 kDa in the gels observed underneath. Napin bands with 10 kDa appeared under reducing conditions due to the formation of new polypeptides. They are better visible in lower alkaline conditions suggesting they are water soluble proteins linked by disulphide bonds. However, there is prominence of aggregated proteins (HMW fraction) was observed except at pH 2, whereas in case of HF, solubility trend was contrary to the above mentioned samples at pH2. In case of concentrates (AC and GC), both show a similar trend in solubility of extracted proteins which is slightly contrary to the solubility trend exhibited by previously described samples in their pH range 1-7. There is a significant drop in the solubility at the neutral point (pH6 and pH7), followed by an increase at the basic points, similar to the other samples. One of the prominent reasons for insolubility is the development of aggregated proteins (HMW) in the pH range 4-7, where pH4 exhibiting strong presence of HMW molecules. In case of isolates (AI &GI), there is significant difference in the solubility profile. In case of AI, greater degree of solubility is observed in the pH range 3-7 against the trend exhibited by GI, which shows the lowest solubility in the described range. Formation of aggregate proteins could be due to ionic interactions that are responsible for maintaining protein-

protein interactions resulting in minimum solubility or due to hydrophobicity that has been reported for different proteins e.g. milk and soy proteins (Hayakawa & Nakai, 1985) [22][3][4]



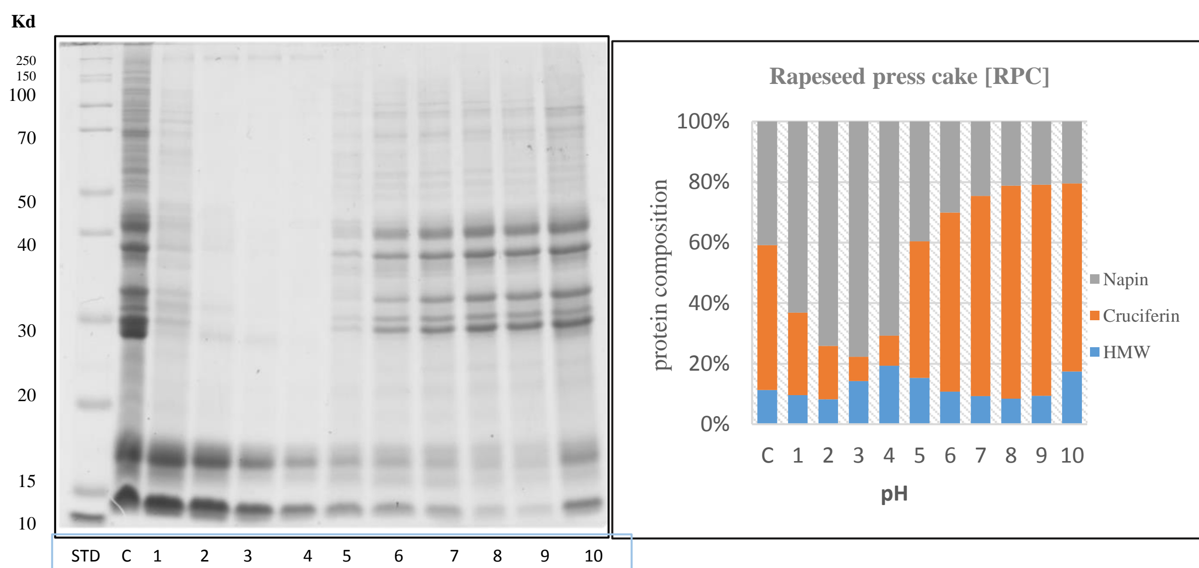
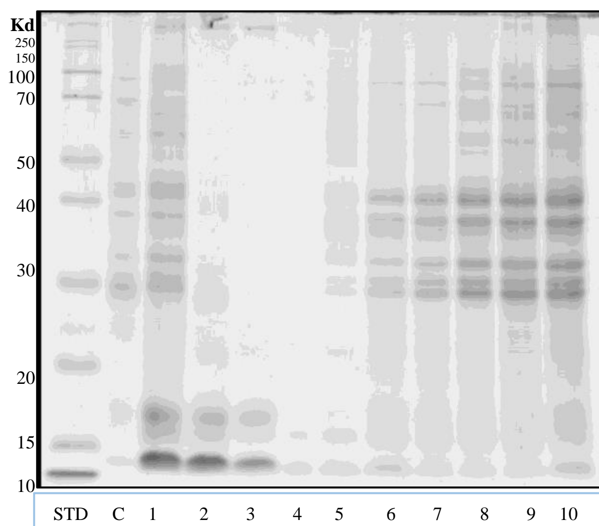
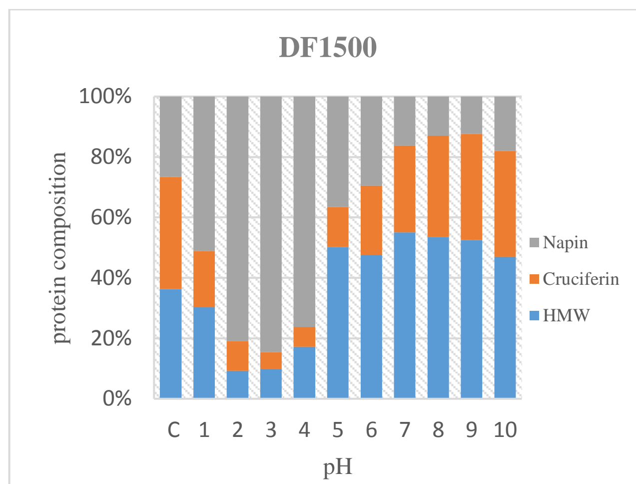
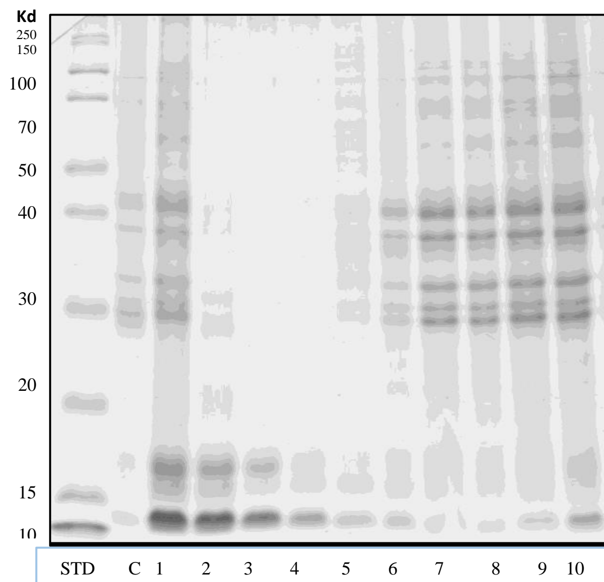


Fig. 31 represents protein composition of RPF and RPC respectively for their corresponding native gels (15%T). 10-15 KDa represent napin; 20-40 KDa represent cruciferin and 40KDa represent High molecular weight compounds (HMW). On The x-axis, STD- standard; C- control; 1-10 represents the pH range







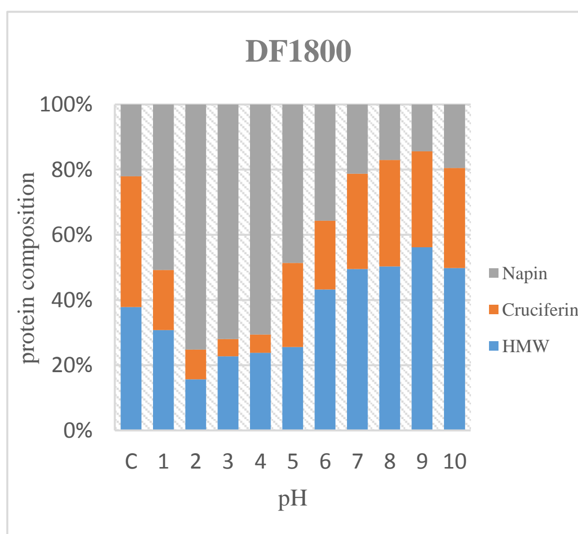
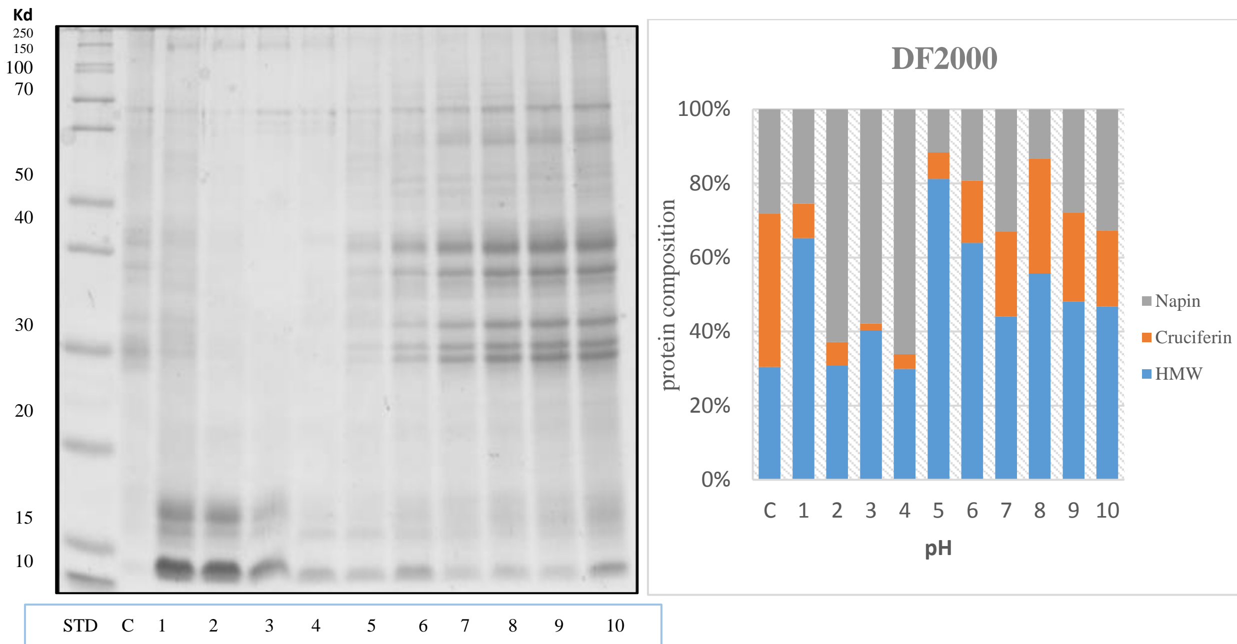


Fig. 32 represents protein composition of DF1500 and DF1800 respectively for their corresponding native gels (15%T). 10-15 KDa represent napin; 20-40 KDa represent cruciferin and 40KDa represent High molecular weight compounds (HMW). On The x-axis, STD- standard; C- control; 1-10 represents the pH range



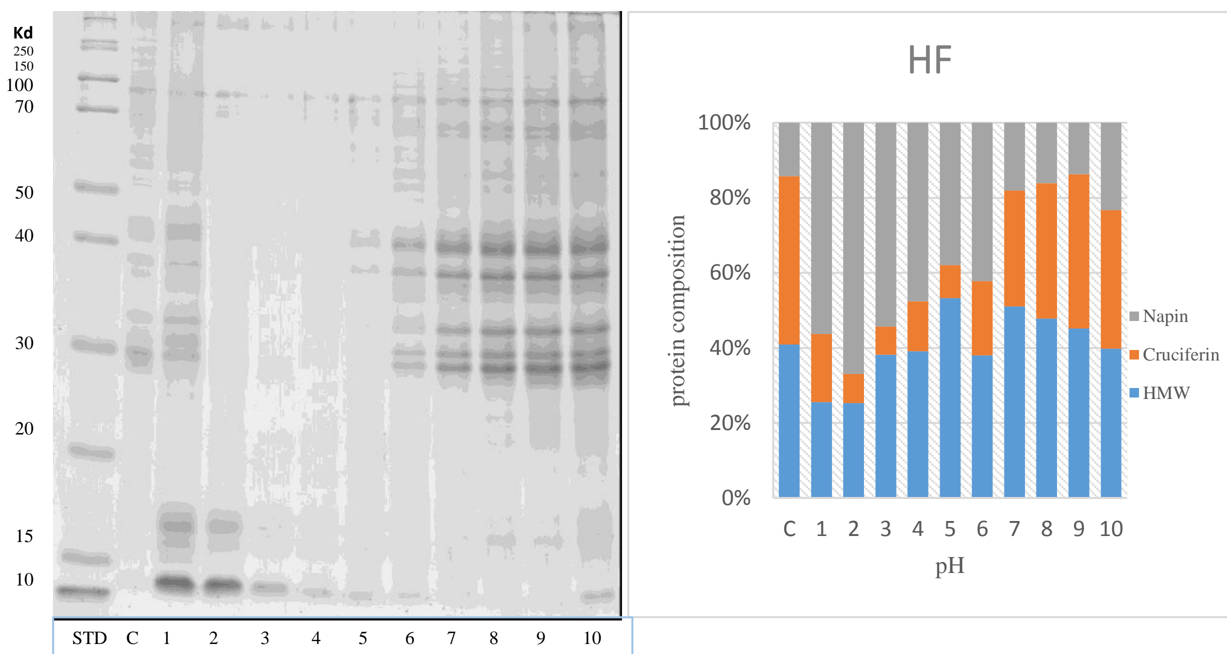
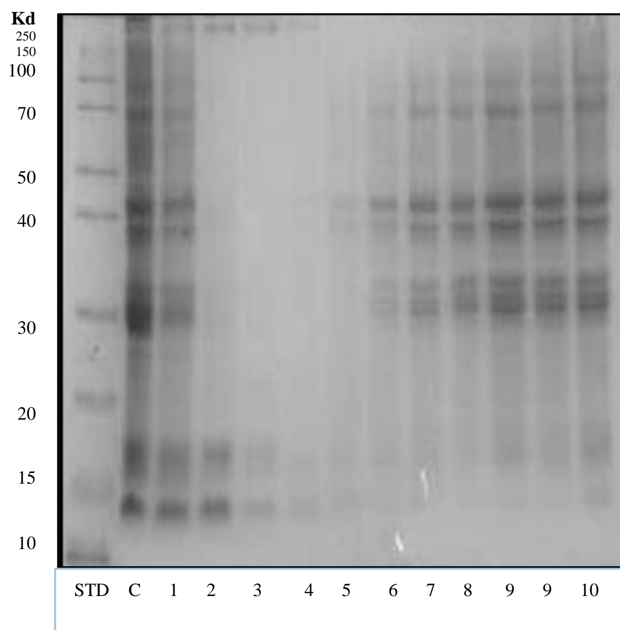
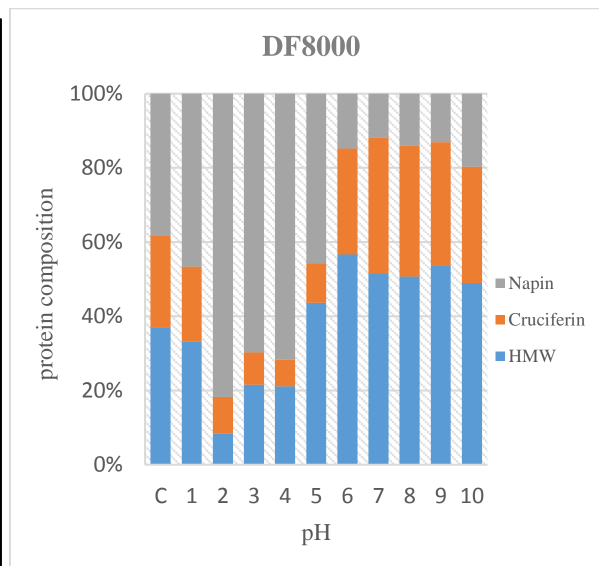
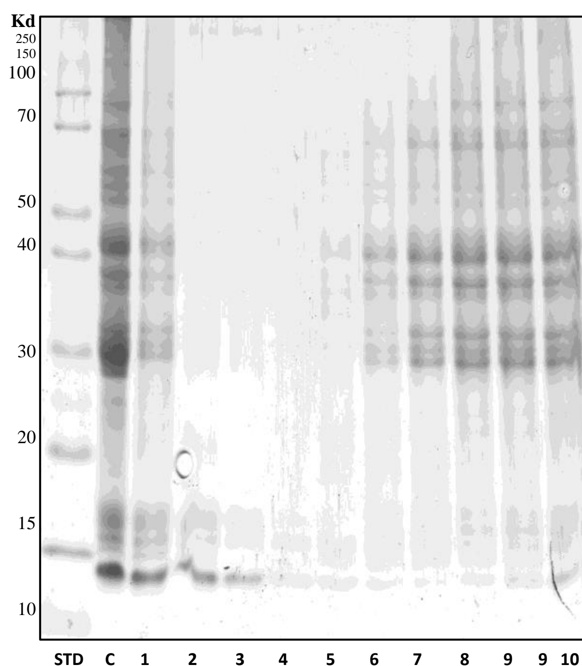


Fig. 33 represents protein composition of DF2000 and HF respectively for their corresponding native gels (15%T). 10-15 KDa represent napin; 20-40 KDa represent cruciferin and 40KDa represent High molecular weight compounds (HMW). On The x-axis, STD- standard; C- control; 1-10 represents the pH range







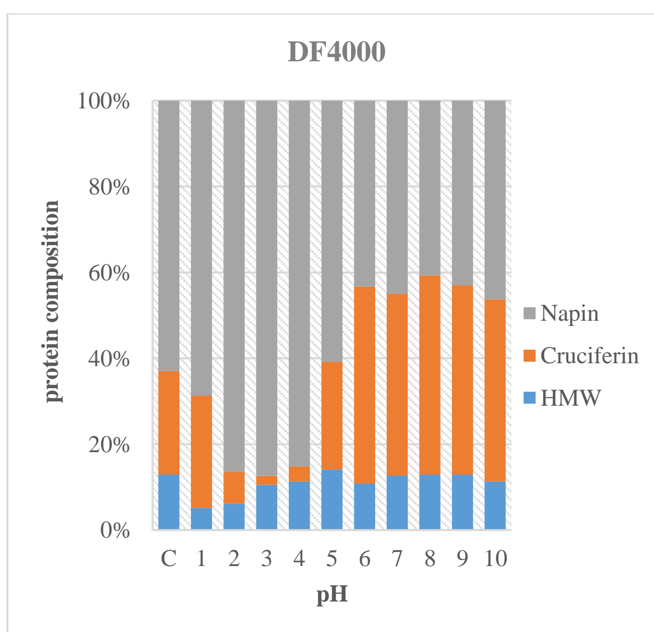
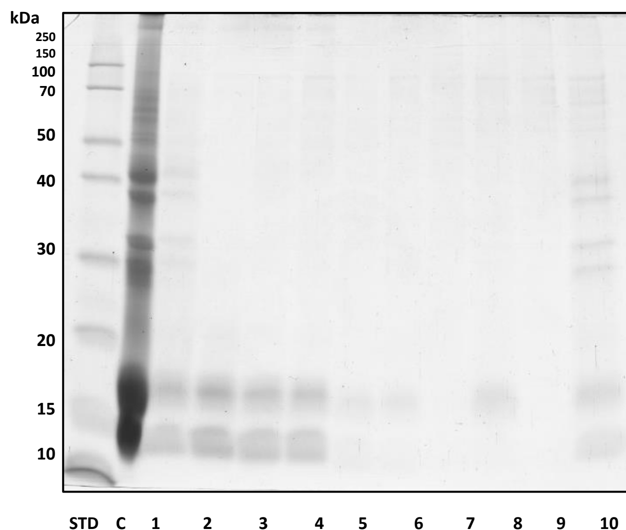
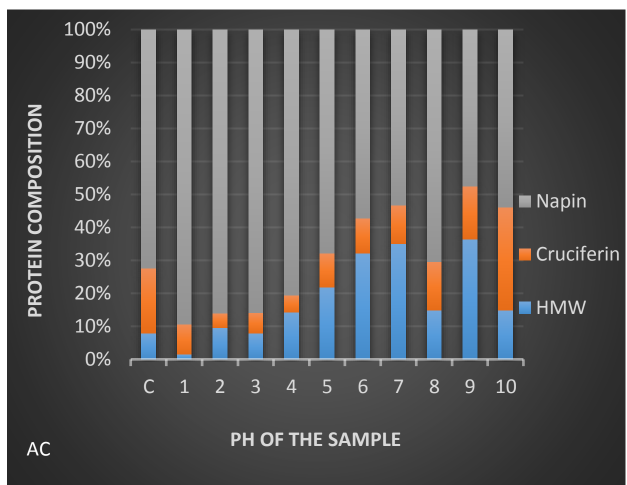
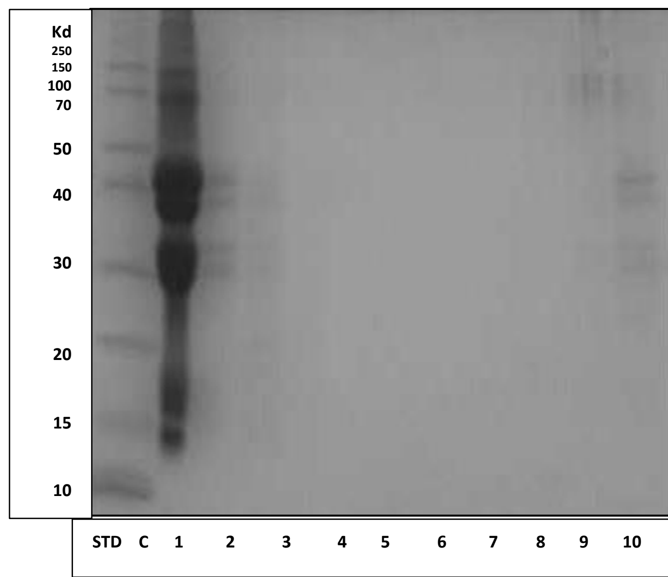


Fig. 34 represents protein composition of DF8000 and DF4000 respectively for their corresponding native gels (15%T). 10-15 KDa represent napin; 20-40 KDa represent cruciferin and 40KDa represent High molecular weight compounds (HMW). On The x-axis, STD- standard; C- control; 1-10 represents the pH range

X







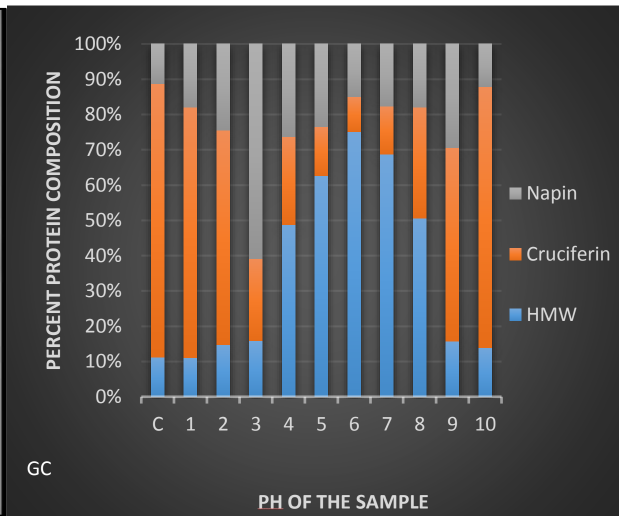
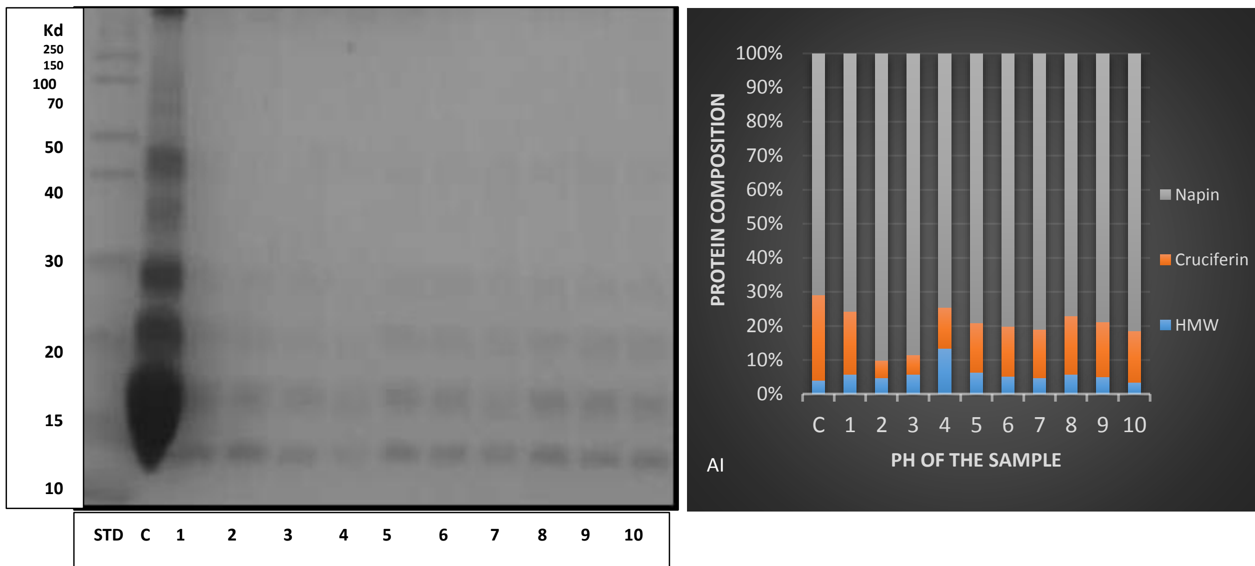


Fig. 35 represents protein composition of AC and GC respectively for their corresponding native gels (15%T). 10-15 KDa represent napin; 20-40 KDa represent cruciferin and 40KDa represent High molecular weight compounds (HMW). On The x-axis, STD- standard; C- control; 1-10 represents the pH range



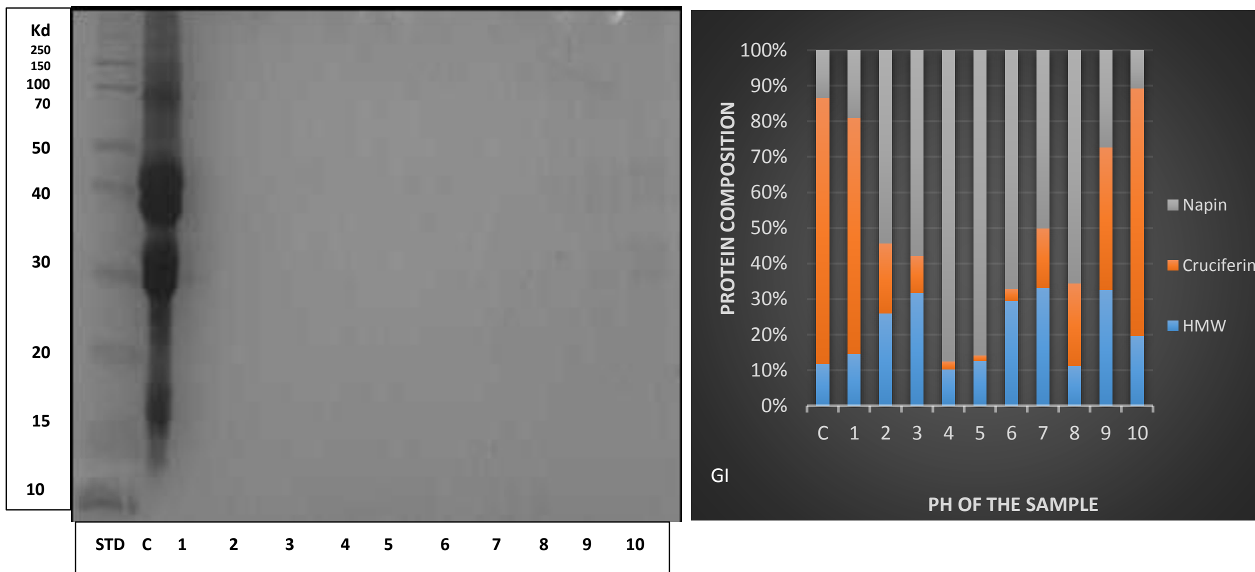


Fig. 36 represents protein composition of AI and GI respectively for their corresponding native gels (15%T). 10-15 KDa represent napin; 20-40 KDa represent cruciferin and 40KDa represent High molecular weight compounds (HMW). On The x-axis, STD- standard; C- control; 1-10 represents the pH range

4. Water Binding Capacity (WBC) as related to air-classification

Samples	Water Binding Capacity [g/g]
RF	3.25 ± 0.14
RPC	4.47 ± 0.07
DF1500	4.22 ± 0.07
DF1800	3.52 ± 0.06
DF2000	4.06 ± 0.05
HF	4.40 ± 0.05
DF8000	4.64 ± 0.01
DF4000	3.06 ± 0.01
GC	3.96 ± 0.29
AC	5.58 ± 0.23

Figure 37: WBC of the test samples as related to air-classification

Water Binding capacity is the ability of meal to bind and retain water. WBC enhances the flavour and integrity of the food product, improves mouthfeel and reduces loosing of moisture and fat from food products (Sreerama, Sasikala, & Pratape, 2008).

Water binding capacity (WBC) was determined for the samples given in fig 37. Among all the samples, the lowest was determined in case of sample DF4000 (3.06 g/g) and highest was seen in case of sample AC (5.58 g/g). This result was not expected, as AC and GC possessed similar protein content, however, GC (3.96 g/g) exhibited significantly lower WBC than AC (5.58 g/g). These results are in correlation with the results of WBC, in case of pea flour and commercially available pea flour, where both the flours had similar protein content but showed significant variation in values of WBC. Various factors, such as ionic strength, concentration, pH, solubility of proteins can cater to such variations. Globulin fractions are better soluble at alkaline conditions rather than acidic and neutral conditions. WBC of GC could possibly be affected by improper balance between protein-protein and protein-solvent interactions.

Among the air-fractionated samples highest was observed in case of sample DF8000 (4.64 g/g), which also stands next to AC. Moreover, DF1500 followed by DF1800 also had similar WBC results. This suggests that WBC can be favourably manipulated but with more detailed research. Interestingly, in case of RPC (4.47 g/g) and HF (4.40 g/g), WBC’s of both the samples are quite better than most of their counterparts. This could be attributed to presence of considerable amount of hull polysaccharides, which enhances the overall WBC. This is in agreement with the

reports suggested by (Wanasundara and Shahidi, 1994). Apart from swelling of crude fibres, WBC is also affected by gelation of carbohydrates (Narayana & Narasinga Rao, 1982)

5. Fat Binding Capacity (FBC) as related to air classification

Samples	Fat Binding Capacity [g/g]
RF	2.57 ± 0.27
RPC	1.97 ± 0.20
DF1500	2.40 ± 0.26
DF1800	2.57 ± 0.18
DF2000	2.66 ± 0.05
HF	2.26 ± 0.18
DF8000	2.34 ± 0.18
DF4000	2.51 ± 0.09
GC	3.75 ± 0.42
AC	4.11 ± 0.72

Figure 38: FBC of test samples as related to air-classification

The fat binding mechanism mostly relies on physically entrapping the oil molecules by the meal with a complex capillary action. In food industry, FBC is one of the most studied techno-functional characteristic. Better FBC enhances organoleptic properties such as flavour, mouthfeel and exhibits better consistency of food matrix.

FBC of the samples given in figure 38 was determined using commercially available rapeseed oil. FBC results of above tabulated samples, did not exhibit wide variation as it was witnessed in case of WBC. The highest FBC was noticed in case of AC (4.11 g/g) and lowest by RPC (1.97 g/g).Among all the air-fractionated samples, highest and lowest FBC is seen in case of DF2000 (2.66 g/g) and DF8000 (2.34 g/g) respectively. These results display a relationship between FBC and classifier speed. FBC of air-fractionated samples gradually increases with increasing classifier speed, for instance, among all the air-fractionated samples, DF2000 (2.66 g/g) exhibited highest FBC. However, on further increasing the classifier speed, FBC gradually decreases and exhibited minimum value in case of sample DF8000 (2.34g/g). This shows that processing and extraction of proteins can prove significant in improving techno-functional properties. Additionally, among air-fractionated samples, this trend can also be justified by the increasing protein content. Similar results were seen in case of soy and sunflower, where flours,

concentrates and isolates revealed higher fat absorption capacities with increasing protein content (Hutton and Campbell, Water and Fat absorption).

In case of Concentrates AC and GC, determined FBC values are not similar in both cases, though both the fractions contain similar protein content. It can be suggested from these results that FBC is primarily attributed to protein content of these two fractions but carbohydrates present among these fractions might have not absorbed oil as much as the protein [20].

Additionally, FBC and WBC of the given samples are negatively correlated as reported by Naczk *et al*. (1985) and Ghodsvali *et al.* (2005). For instance, WBC values of RPC and HF were significantly higher than FBC values. Similarly it is witnessed in case of all the samples but with lesser variation. AC has ended up showing best results for both WBC and FBC. However, FBC of AC is considerably lower than the value of WBC of AC. Additionally, variation between WBC of GC and AC was higher than compared to variation between FBC of GC and AC. Better FBC of samples compared to WBC reveal that proteins structure might be more lipophilic in nature and they tend to bind with hydrocarbon chains, contributing to enhanced FBC.

Results and discussions:

II. T.molitor larvae (2nd part)

Weight measurement of T.molitor larvae post starvation

	P83	P84	P81	P82	P79	P80
wt. of insects prior to feeding (g)	603.9	517.3	500	513	542.2	526.1
Wt. of T.molitor larvae before grinding (g)	692.9	610.5	576.3	566.6	532.9	581
Wt. of T.molitor larvae post grinding (g)	687.95	610	575.97	566.36	532.30	580.98

Figure 39: Table representing weight of insects before and after starvation

Weight measurement of T.molitor larvae post starvation was one the primary steps in analysing the effect of varying lengths of starvation on T.molitor weight. The results are represented in the figure 39. The results clearly exhibit that weight of T.molitor larvae under S0 has the maximum weight and gradually, weight of T.molitor larvae decreases with increase in starvation period and lowest was recorded in case of S48, since P79 and P80 were subjected to starvation for 48 hrs.

II. T.molitor (2nd part)

6. Determination of physiochemical properties

(%)	P83	P84	P81	P82	P79	P80
Fat content	19.4	21.5	20.8	20.1	19.3	19
Ash content	1.26	0.99	1.24	1.26	1.02	1.07
Moisture content	55.09	55.68	55.21	56.16	55.65	57.32

Figure 40: tabulation of fat, ash and moisture content of T.molitor larvae post starvation

6.1 Total moisture content

The Total moisture content of "P0", "P24" and "P48" were determined using Sartorius moisture analyser after grinding.

The moisture content of "P0", "P24" and "P48" was determined to be 55.38%, 55.68% and 56.48% respectively. Values exhibit that there is no significant impact of starvation conditions on the moisture content. Otherwise, values presented here are in close proximity with moisture content determined from fresh T.molitor larvae by (Ewa Siemianowska[1]*, Agnieszka Kosewska[2], Marek Aljewicz[3], Krystyna A. Skibniewska[1], Lucyna Polak-Juszczak[4], Adrian Jarocki[5], Marta Jędras[5] (2013). Interestingly, moisture content determined in the present work is higher than the T.molitor larvae grown on diet consisting of wheat flour and soybean flour (51.91g/100g) and diet consisting of 50% wheat flour and soybean flour and 50% bocaiuva pulp flour (52.78g/100g). It would be justified to say that feeding with fruits and vegetables such as apples and carrots, is reflected in the moisture content. However, it is worth noticing that the moisture content has gradually increased in the direction of maximum starvation period that is 48 hrs (P0=55.38% < P24= 55.68% < P=56.48%). Since, for arthropods like T.molitor, water percentage dominates the composition of tissues and hence for survival and biological importance adequate amount of water is necessary. Moreover, presence of waxy layer of epicuticle which is highly differentiated in these groups promote higher degree of resistance to water loss. This could be a possible explanation of higher moisture content showed by "P24" and "P48" as they were subjected to starvation for 24 and 48 hours respectively, to overcome stress development and continue the biological activity they could have absorbed water from the environment [35].

6.2 Total ash content

The ash content was determined using muffle furnace at 550°C. The ash content was determined to study the impact of different starvation period on the total mineral composition of "P0", "P24" and "P48". The ash content of "P0", "P24" and "P48" were recorded as 1.125%, 1.25% and 1.04 % respectively. Categorically, there is no symbolic difference in the ash contents of "P0", "P24" and "P48". Moreover, the values determined are lower than the values presented by *(Ewa Siemianowska[1]*, Agnieszka Kosewska[2], Marek Aljewicz[3], Krystyna A. Skibniewska[1], Lucyna Polak-Juszczak[4], Adrian Jarocki[5], Marta Jędras[5] (2013)* and *(Nergui Ravzanaadii, Seong-Hyun Kim, Won Ho Choi, Seong-Jin Hong, and Nam Jung Kim 2012).* When compared with the results presented in the work, *A Study on the Potential of Insect Protein and Lipid as a Food Source* by **Liya Yi**, ash content is significantly higher (7.5±2.2%) than the values presented fig 39. Ash content of T.molitor larvae is directly dependent on feeds ash content. In other words, it depends

on the diet ingredients on which they are fed. Lower mineral content of diet would reflect in lower ash content of T.molitor larvae. Despite starvation, ash content of “P0”, “P24” and “P48” is similar or little more than the conventional animal proteins such as chicken (1%), egg (1%), beef (1%), and pork (1%) and fish rainbow trout (1.2%) as tabulated in http://dx.doi.org/10.4236/as.2013.46041. Sustainable provision of minerals such as calcium, iron, magnesium, zinc and iodine, from edible sources support proper muscle movement, tissue formation, enables nerve impulse transmissions, balances acid-base ratio. Hence, minerals such as calcium, iron, magnesium, zinc and iodine However, ash contents in beetles are lower compared to ash content of T.molitor larvae because beetles require lower mineral levels for metabolism. This could be possible the reasons for ‘P48’ to exhibit lowest ash content (1.04 %), as under stress they tend to reduce their body size to conserve energy loss.

6.3 Total fat content

The fat content was determined by acid hydrolysis method. Total fat content of “P0”, “P24” is 20.45% which is exactly equal to the results described in [39]. However, the difference between the total fat content of “P48” (19.15%) and the former two is quiet less, approximately 1%. Whole insects that are high in total fat content tend to exhibit low moisture content. This could be one of the possible reasons for “P0”, “P24” to exhibit slightly more fat than “P48” [35].

7. Percent protein solubility of rapeseed protein extrudates and their composition

7.1 Protein concentration of DES/NDES

As known to us, Extrusion has gained lot of appreciation and has become a well-established industrial process in producing baby food, pet food, snacks, cornflakes etc. Extrusion brings change in rheology, functionality and morphology of extrudate. Further, it leads to starch gelatinization, inactivation of secondary plant metabolites such as anti-nutritional factors, enzymes and also micro-organisms. Similarly, when proteins are subjected to extrusion, they undergo complex bioconversions resulting in plasticization of texturized vegetal proteins by forming various networks.

Generally, extrusion of proteinaceous substrates attracts researchers to focus on texture and functional properties with respect to extrusion parameters and handling conditions. However, in the present experiment, focus is primarily on the influence of Extrusion on protein solubility of various formulations of rapeseed press cake with T.molitor larvae subjected to starvation. Hence percent protein solubility of all the extrudates were determined by Bradford method.

Initially, the results obtained for total protein content by Kjeldahl (N× 6.25) of all the DES and NDES (fig. 20) had expressed slightly lower values than the total protein content of Rapeseed press cake (36%). This condition is not similar as stated in. Lowering of total protein content in all the DES and NDES could be attributed to the proteolytic activity of serine and cysteine proteinases present in the digestive tract of T.molitor larvae.

The results obtained by performing Bradford analysis of DES and NDES are represented in fig.42. At very first glance, the percent protein solubility of all the samples analysed at pH 2 and pH 8 has reduced to as low as 2% post extrusion. However, among all DES, maximum protein solubility is seen in case of SE 2_90_45 followed by S48 2_90_RT and S48 8_90_45. Quantitative analysis of DES reveals that among all the parameters analysed, combination of pH2, incubation time of 90 minutes and room temperature (RT) have proven to exhibit better

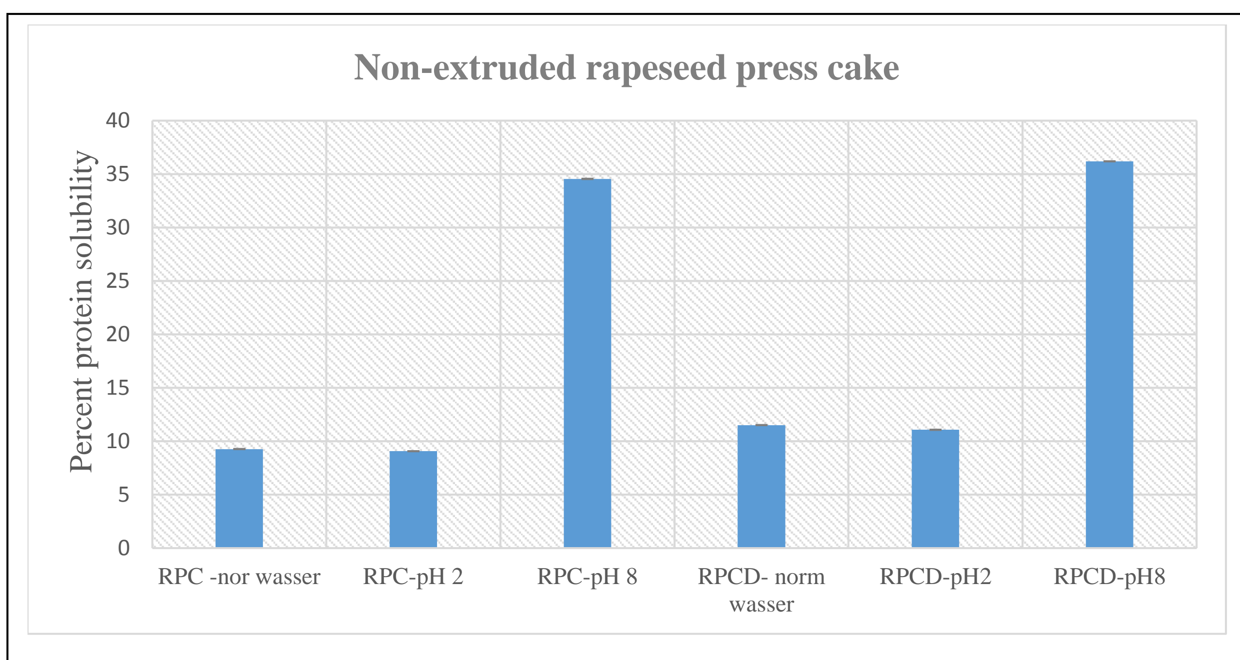


Fig 41 represents variation in protein solubility of non-defatted (RPC) and defatted (RPCD) rapeseed press cake extracted with normal water and with water pre-adjusted to pH2 and pH8.

solubility results than other combinations. Contrastingly, comparing the percent solubility of DES with defatted non-extruded rapeseed press cake (RPCD_pH2) the solubility of RPCD_pH8 is significantly higher at pH 8 than pH 2. These contrasting results reveal that protein solubility has decreased post extrusion. This could be due to strong prevalence of intermolecular interactions that has occurred because of change in protein confirmation at alkaline pH while extrusion and consequently forming aggregates, contributing to insolubility [38].

Decreased solubility of extrudates could also be attributed to extraction of proteins with distilled water, where the impact of ionic strength leads to instability in the intermolecular electrostatic interactions in opposite charges. Moreover, defatting has not played any significant role in improving the solubility. This is evident by comparing the results of defatted non-extruded rapeseed press cake (RPCD_W, RPCD_2 and RPCD_8) and non-defatted non-extruded rapeseed press cake (RPC_W, RPC_2 and RPC_8). For instance, the maximum solubility in both the conditions, is analysed to be at pH 8. Surprisingly, there is no significant difference in percentage solubility of proteins extracted with normal water and pH 2 at both the conditions (defatted and non-defatted)

The maximum protein solubility (8.27%) is exhibited by NDES **8_0_RT** belonging to set **S48** and least is reported in case of NDES **2_90_RT** belonging to set **S0**. In case of all NDES, solubility of S48 has outdone in each and every condition with considerable margin from samples of sets SE, S0 and S24. Also, there is correlation among the solubility patterns of S24 and S48. The solubility trend line of both the sets (S24 and S48) project similarity. For instance, the maximum solubility in S48 was seen in case of 8_0_RT and minimum was seen in case of 8_90_45. Similarly, maximum solubility in S24 was observed in case of 8_0_RT and minimum in case of 8_90_45. NDES represented in table have protein solubility better that DES. One of the possible reasons could be presence of solvent in the defatted meal causing protein aggregation while defatting (petroleum benzene). There could be possibility of various interaction among lipids, solvent and proteins adding to insolubility.

Overall reduction in protein solubility could be due to increased exposure of hydrophobic residues in aqueous systems at pH 2 and pH 8, contributing to protein-protein/peptide interactions.

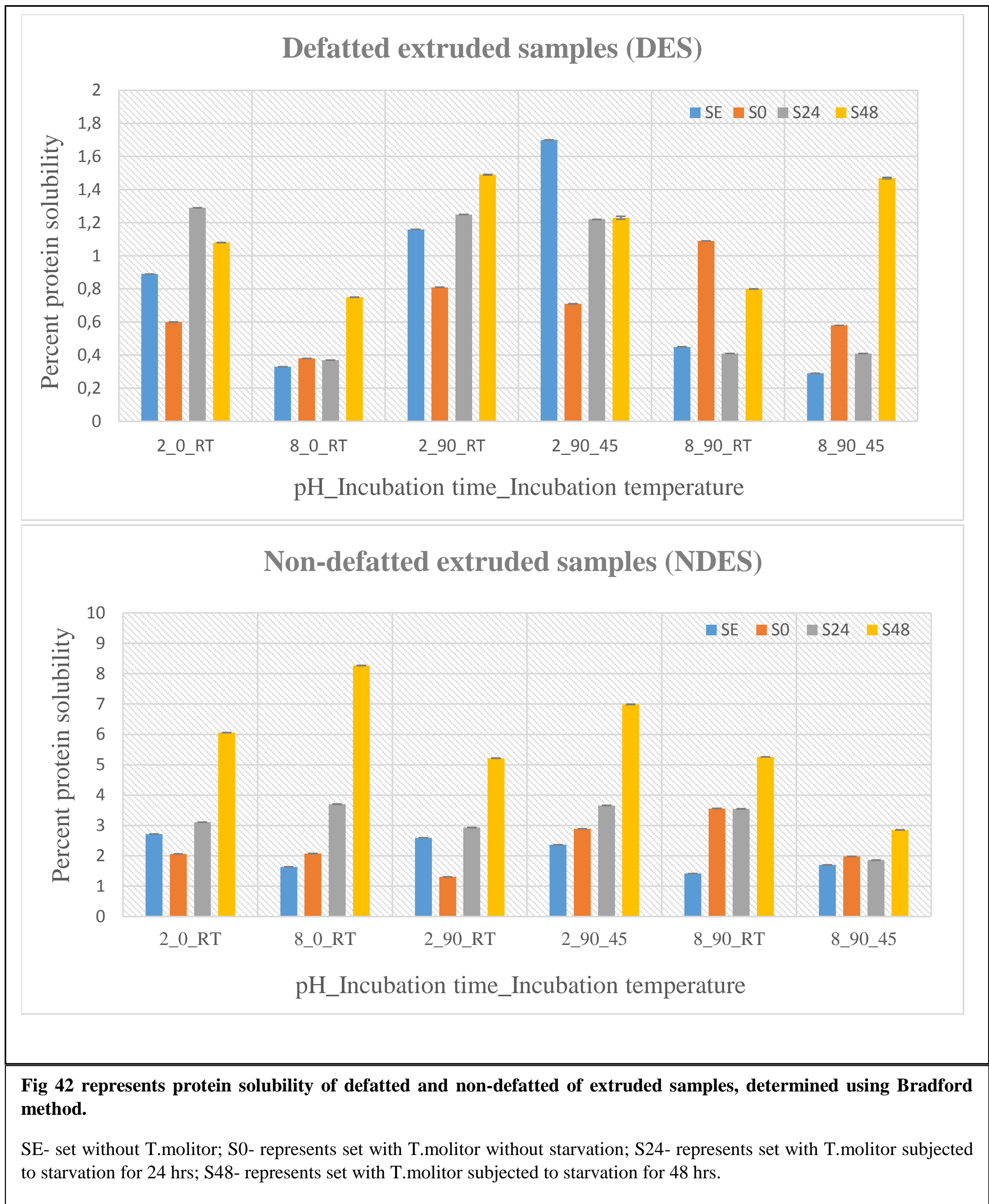


Fig 42 represents protein solubility of defatted and non-defatted of extruded samples, determined using Bradford method.

SE- set without T.molitor; S0- represents set with T.molitor without starvation; S24- represents set with T.molitor subjected to starvation for 24 hrs; S48- represents set with T.molitor subjected to starvation for 48 hrs.

To understand the major factors responsible for variation in solubility can be studied in more detail by analysing the allocation of major protein fractions using SDS-PAGE.

7.2 SDS-PAGE

Protein composition of each of DES and NDES is determined by allocating individual fractions of proteins based on their molecular weight. In case of control samples, solubility and composition of proteins are quite related. The relatability can be confirmed from the figure.42 where, proteins are more soluble in alkaline conditions (pH8) than acidic conditions (pH2) in both the cases. Solubility of RPC and RPCD at pH 2 is mostly because of napin and cruciferin, although in RPCD_pH2 presence of small amounts of HMW (>40 kDa) compounds are also responsible. Similarly, when RPC and RPCD at pH 8 are considered, major percentage of napin, considerable percentage of HMW (>40 kDa) compounds and lowest percentage of cruciferin are responsible for solubility.

In case of DES gels of SE, S0, S24 and S48, solubility behaviour is very low compared to the standards. Firstly, SE and S0 gels are considered and their solubility behaviour is being discussed. From the figure 43, the solubility can be witnessed only for samples set at pH2 and fractions with molecular weight 10-15 kDa are prominent. This proves that napin is major contributor to solubility of these samples. However, in case of SE, especially in case of samples 2, 5, 6 represented in the graph, exhibit fractions of molecular weight 20-40 kDa, apart from 10-15 kDa. This suggests that solubility of samples 2, 5, 6 of set SE is also due to presence of cruciferin. Importantly, it can be observed that presence of T.molitor has some effect on solubility of globular fractions. Another reason could be insolubility of cruciferin in acidic conditions could also be result of lowering of overall protein solubility. One of the interesting revelations from the graph is S0 (8_90_45), which exhibits solubility in the graph, however, there is no visual appearance of protein fractions in the gel. This shows that there are certain fractions available on the gel which are not visible to human eye but can be recognized by Quantity One 1-D analysis software while quantifying the gels.

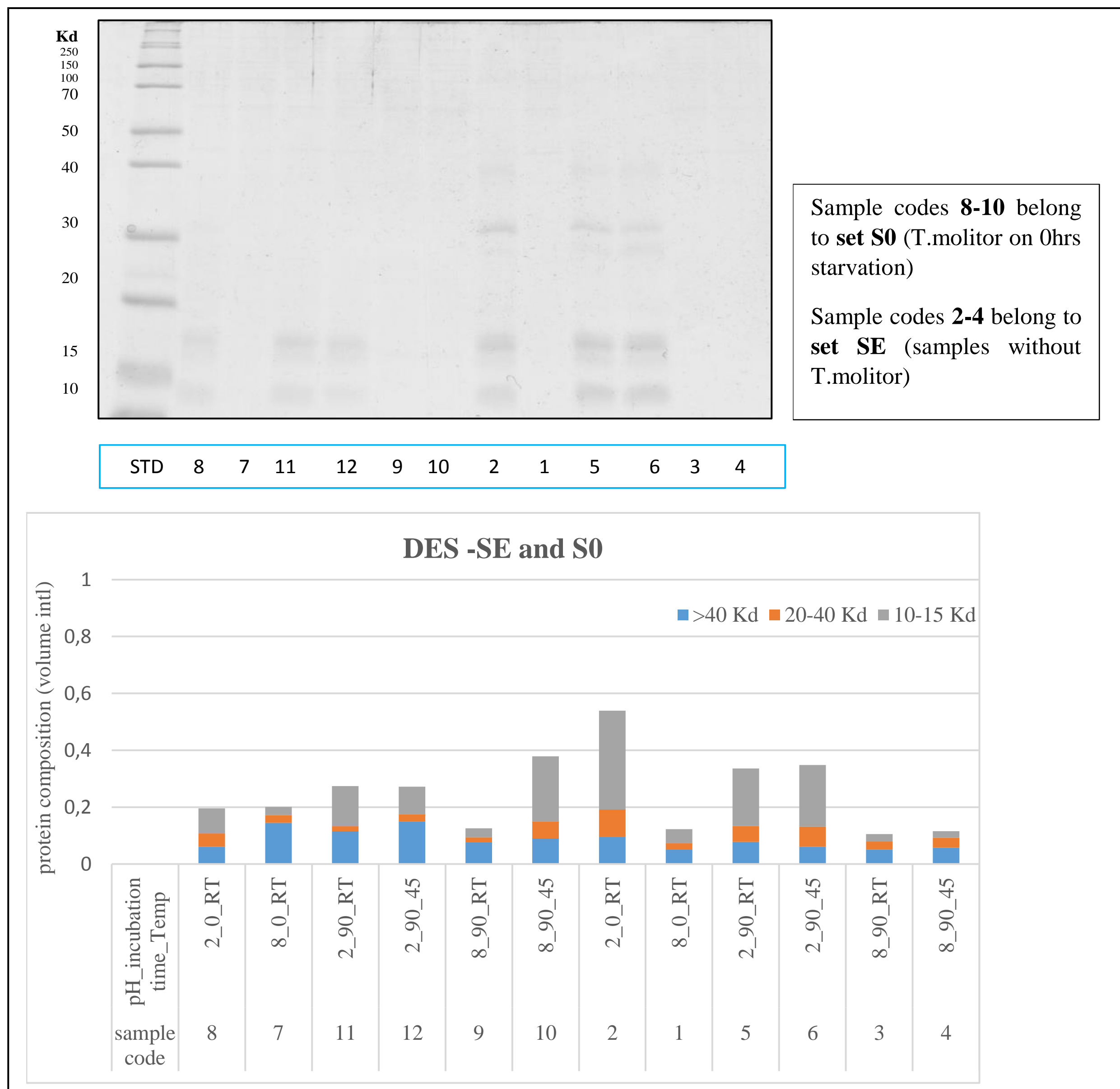


Figure 43: graphical representation of protein composition of defatted extruded samples (DES) [set SE (without T.molitor) and S0 (0 hrs starvation)] depending on the pH of the extraction solution. >40 Kd (high molecular weight compounds); 10-15 Kd (napin) and 20-30 Kd (cruciferin).

Similar results are can be witnessed in case of S24 and S48 (fig.44), where the solubility is mainly at pH 2 as it was witnessed in case of S0 and SE. Solubility is majorly distributed around molecular weight 10-15 kDa, proving that napin is the major contributor to overall solubility. However, there are also certain deviations from S0 and SE, for instance, protein fractions with molecular weights 30-40 kDa is quiet visible on the gels. This can suggest that on increasing starvation period enzyme activity of T.molitor on rapeseed proteins reduces and hence appearance of cruciferin fractions are witnessed on the gels. This point can be more strengthened

by observing composition of S48 samples on the gels, where presence of HMW compounds can be witnessed which later adds to the solubility of overall proteins. Reasons behind such instances can be attributed to effect of starvation on T.molitor larvae prior to extrusion.

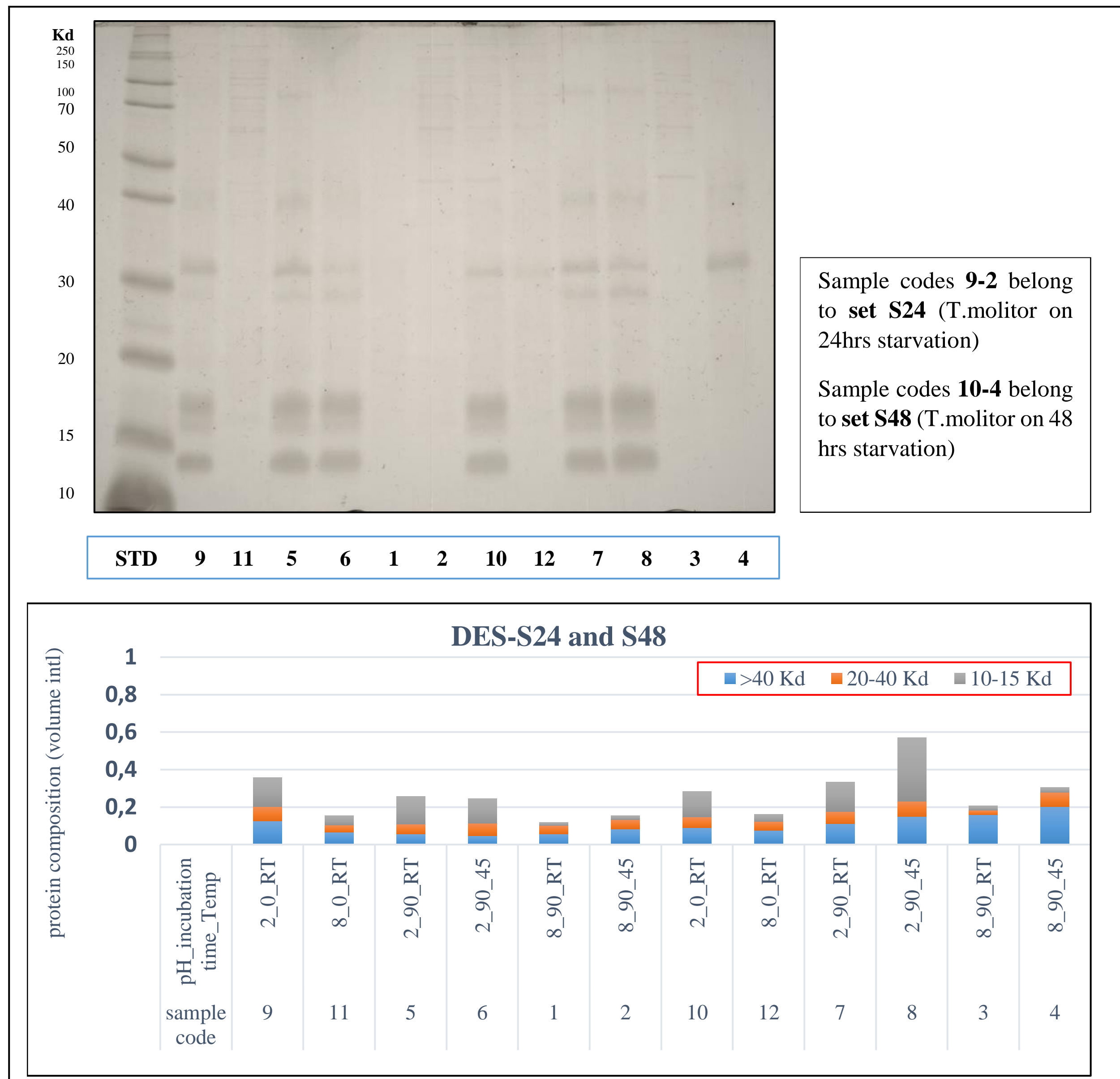


Figure 44: graphical representation of protein composition of defatted extruded samples (DES) [set S24 (24 hrs starvation) and S48 (48 hrs starvation)] depending on the pH of the extraction solution. >40 Kd (high molecular weight compounds); 10-15 Kd (napin) and 20-30 Kd (cruciferin).

Protein composition of each of NDSE is analysed by allocating the major protein fractions. Overall, on observing gels of NDSE, there is no sharp appearance of protein fractions. However, from the fig 45, protein solubility of NDSE is mainly because of HMW compounds (> 40kDa). Higher solubility is witnessed in case of SE (2_90_RT), which is majorly because of HMW compounds followed by cruciferin (Mw. of 20-40 kDa). In set S0, 3b and 3c have same pH and incubation time but their compositions are differentiated by incubation temperature. At room temperature (RT) napin portion (almost negligible) is comparatively smaller than cruciferin in overall solubility but at 45°C the napin portion increases at the expense of cruciferin. The same trend is observed with samples 4c and 4b, where both the samples are adjusted at pH 8 with incubation time of 90 minutes but change in incubation temperature from RT to 45°C has napin portion more prevalent than cruciferin. Moreover, as witnessed in DES, presence of T.molitor has reduced the portion of HMW compounds in S0 when compared to SE. Also, in S0, on increasing incubation time and temperature at either of the pH's the cruciferin portion reduces considerably when compared to its counterparts.

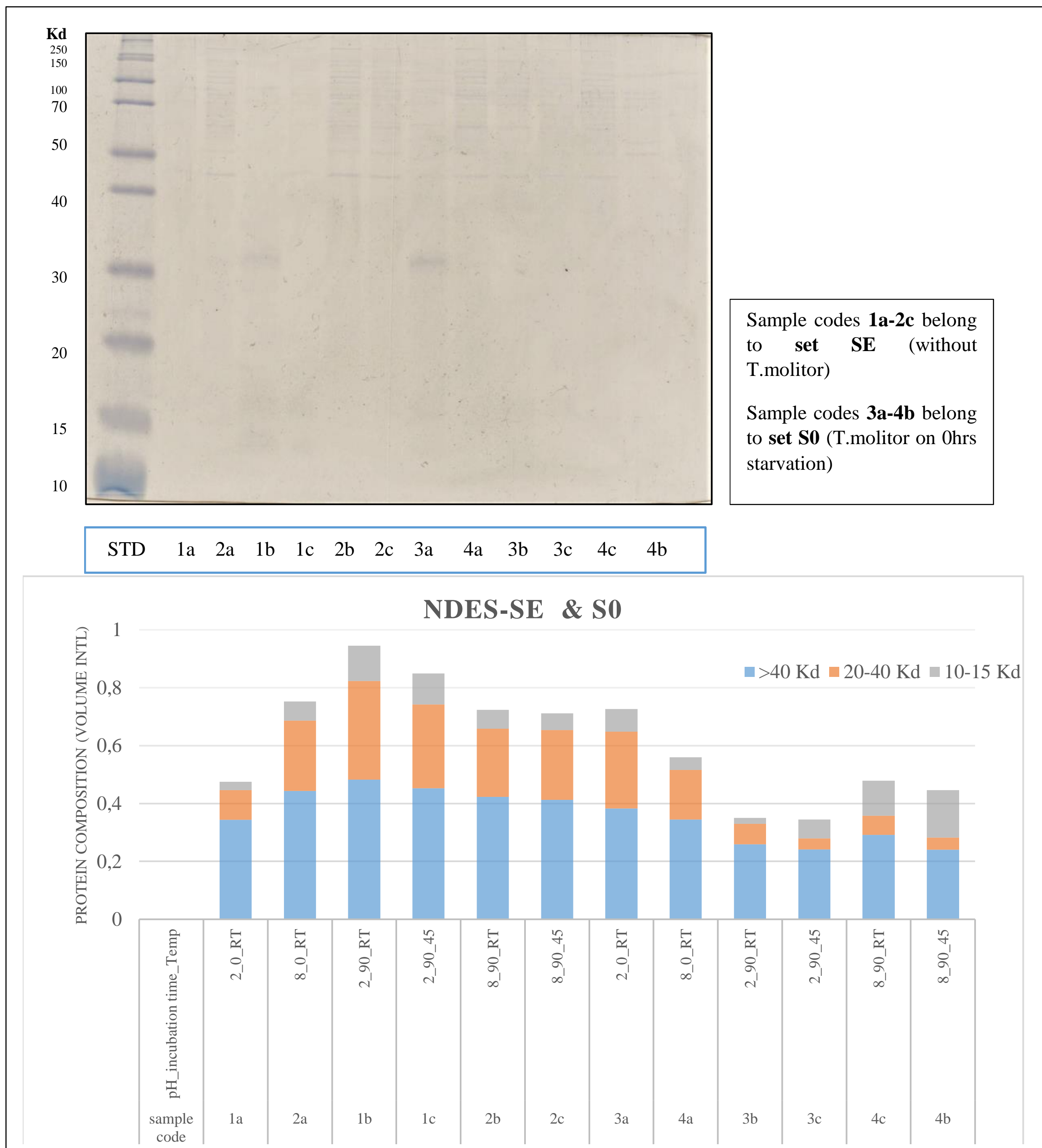


Figure 45: graphical representation of protein composition of non-defatted extruded samples (NDES) [set SE (without T.molitor) and S0 (0 hrs starvation)] depending on the pH of the extraction solution. >40 Kd (high molecular weight compounds); 10-15 Kd (napin) and 20-30 Kd (cruciferin).

Lastly, composition of NDES of sets S24 and S48 are analysed and the most common factor that prevails all throughout is the reduction of HMW compounds when compared to SE. Solubility is mostly attributed to albumin fraction (napin) of the samples. In all the cases reduction in the portion of HMW has contributed majorly to increment in napin fraction. This is pretty evident in the case of sample 2_90_45 of set S48, where the HMW fraction has increased, leading to decrease of napin and cruciferin fractions respectively.

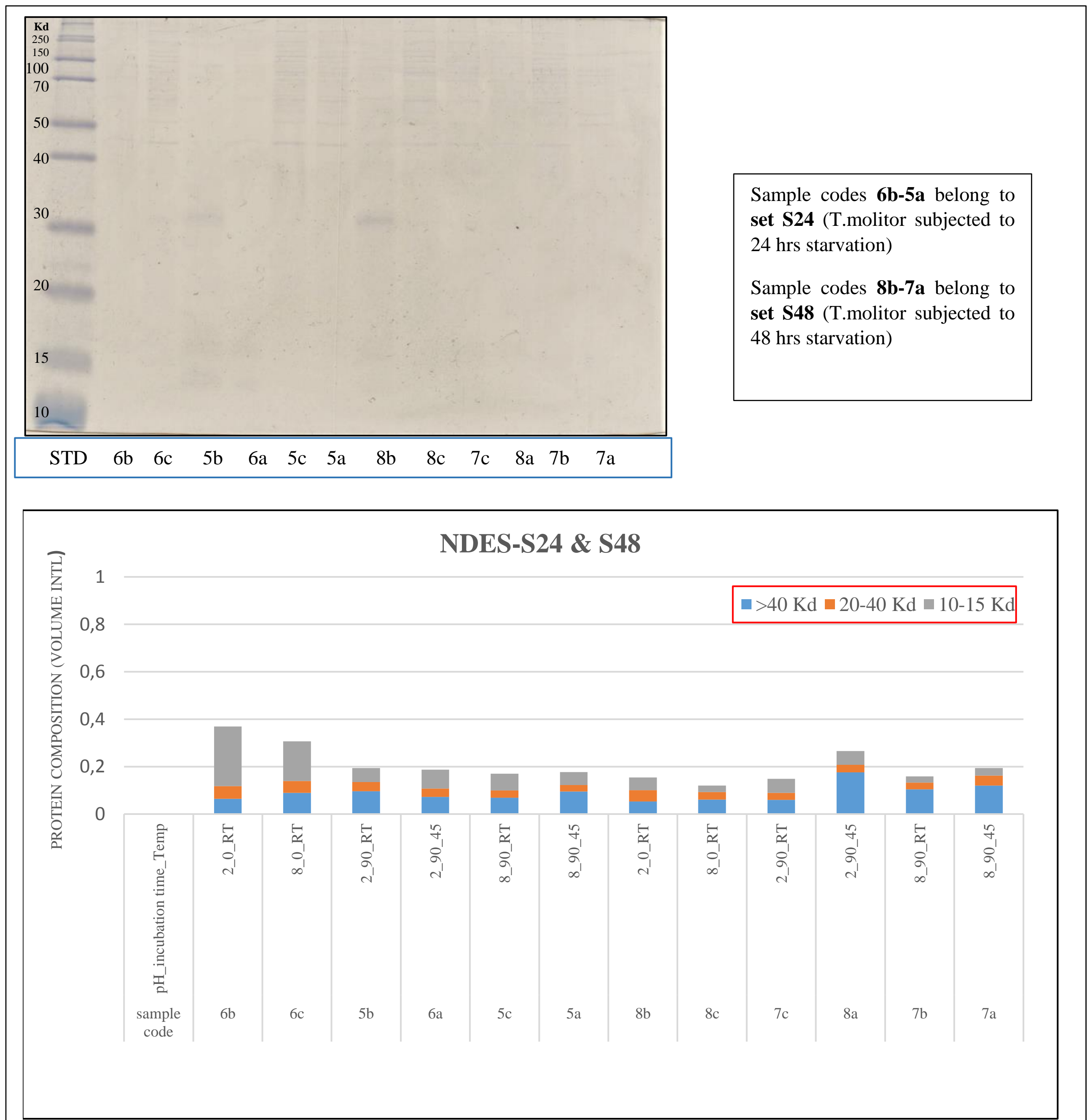


Figure 46: graphical representation of protein composition of non-defatted extruded samples (NDES) [set S24 (24 hrs starvation) and S48 (48 hrs starvation)] depending on the pH of the extraction solution. >40 Kd (high molecular weight compounds); 10-15 Kd (napin) and 20-30 Kd (cruciferin).

There are various ways that are cited in the literature that could be used to study the structural changes post extrusion. Few of such techniques are SDS-PAGE, solubilizing protein in various buffers, gel filtration and ion exchange chromatography, electron-microscopy, scanning calorimetry and spectroscopy etc. were used by researchers to study the physical and chemical changes in protein confirmation when they were governed under high moisture and high temperature conditions. Hence, protein solubility and its related composition in various buffer systems have been analysed to study the important interactions between protein-protein and other molecules.

Figure 47 depicts the differences in electrophoretic mobility and solubility of RPC_C, RPCD_2 followed by DES of SE (2_0_RT), S0 (2_0_RT), S24 (2_0_RT) and S48 (2_0_RT) in 1X sample buffer and aqueous buffer respectively. It exhibits the variation in electrophoretic migration of samples containing SDS and thiol reducing agent β-Mercaptoethanol. Electrophoretic mobility and ban intensity of proteins extracted in aqueous systems is significantly lighter and invisible to be detected by human eye. In all the samples that were dissolved in sample buffer, three different protein sub-units were detected. One subunit with Mw. of 10-15kDa representing napin, second subunit with Mw. of 20-30kDa confirming presence of cruciferin and lastly protein fractions with Mw. >40 kDa which are characterized as HMW compounds. Above mentioned protein fractions are clearly displayed on the gels in presence of reducing agent β-Mercaptoethanol. Hence, these results indicate that while extrusion, formation of disulphide and non-covalent bonds has encouraged protein-protein interactions, leading to aggregation and resulting in insolubility in the absence of reducing agents such as β-Mercaptoethanol. Similar results were seen in case of soybean proteins (Marsman and others 1998), peanut proteins. However, there is significant presence of HMW fractions, which indicates that there are fractions which are not soluble even in presence of reducing agents such as β-Mercaptoethanol. This suggests that these fractions could have been linked by stronger linkages such as iso-peptide bonds (Stanley 1989) [38].

Similar to fig 47, fig 48 depicts the differences in electrophoretic mobility and solubility of RPC_C, RPCD_8 followed by DES of SE (8_0_RT), S0 (8_0_RT), S24 (8_0_RT) and S48 (8_0_RT) in 1X sample buffer and aqueous buffer respectively. Percent protein composition of samples dissolved in sample buffer depicts that protein solubility at pH8 is higher than protein solubility witnessed at pH2 (fig.12). This could be due to the fact that at alkaline conditions, both cruciferin and napin, have better solubility. With increase in protein solubility at pH8, there is considerable increase in the percentage of HMW compounds. Higher percentage of HMW in the solubility profile has decreased percent solubility of cruciferin. For instance, in case of S48

(8_0_RT) cruciferin percentage (26.5%) has decreased with increase in percentage of HMW compounds (42.05%). Interestingly, in case of samples that are aqueous extracted, with increase in solubility, solubility of HMW compounds also increases. This suggests that apart from disulphide and non-covalent bonds, there is presence of other stronger linkages that are not reduced with the help of thiol reducing agents such as β-Mercaptoethanol[38].

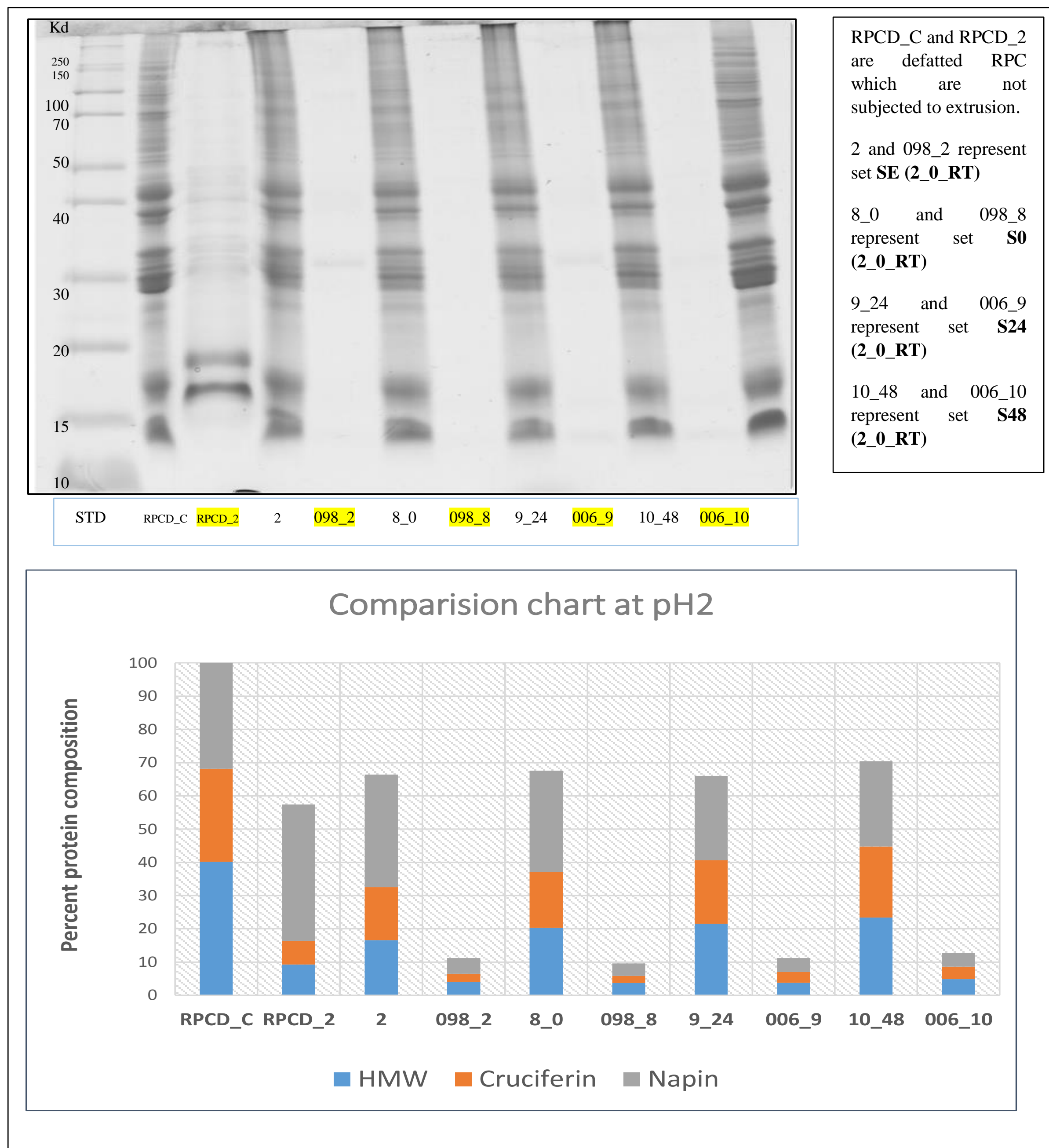
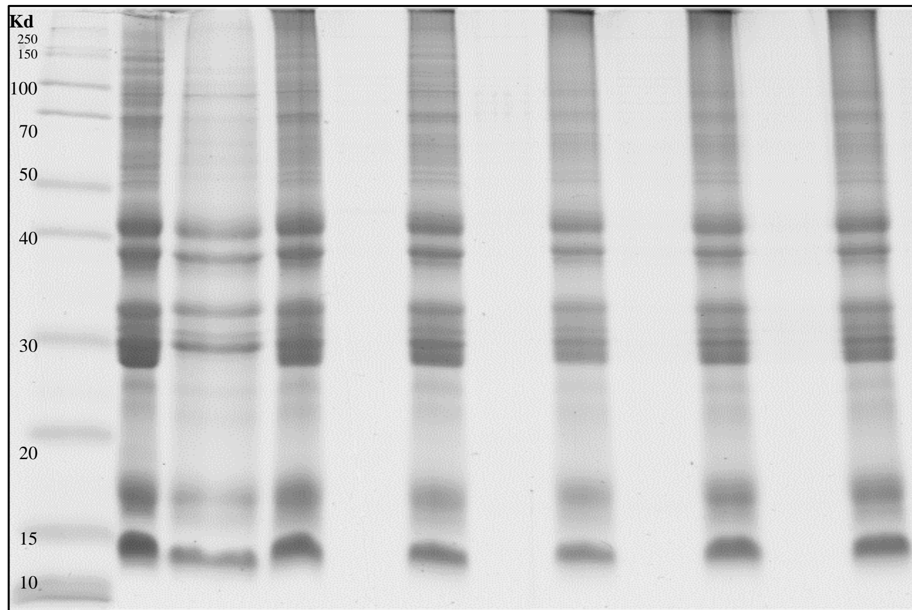


Fig 47 represents comparison of variation in protein composition of samples when extracted with sample buffer and distilled water at pH 2.Samples highlighted in yellow represent extraction with distilled water.



STD	RPCD_C	RPCD_8	1	098_1	7_0	098_7	11_24	006_11	12_48	006_12

RPCD_C and RPCD_8 are defatted RPC which are not subjected to extrusion.

1 and 098_1 represent set **SE (2_0_RT)**

7_0 and 098_7 represent set **S0 (2_0_RT)**

11_24 and 006_11 represent set **S24 (2_0_RT)**

12_48 and 006_12 represent set **S48 (2_0_RT)**

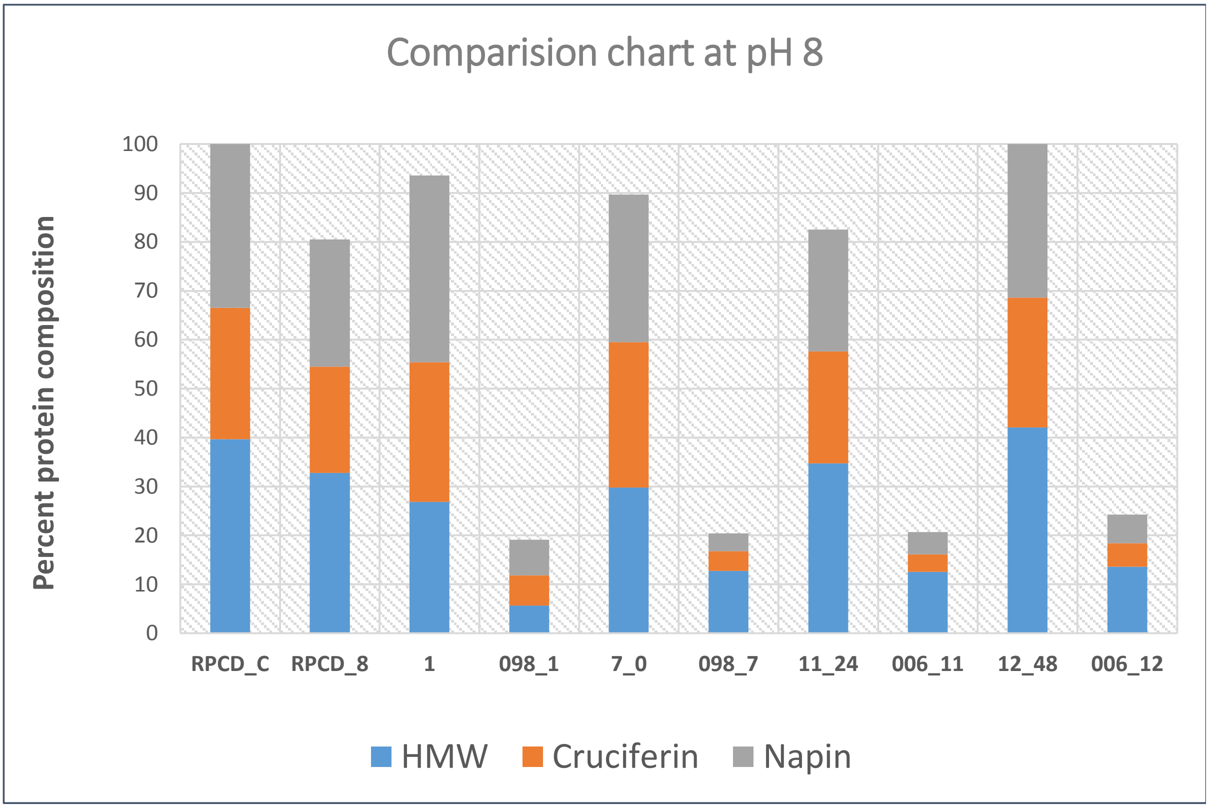


Fig 48 represents comparison of variation in protein composition of samples when extracted with sample buffer and distilled water at pH 8. Samples highlighted in yellow represent extraction with distilled water.

CONCLUSIONS

1. Determination of physiochemical properties of air-classified has revealed that air classification increases protein yield however, it reduces the protein content of the fractions. This is proved by comparing results of RPC and air-classified samples.
2. Protein solubility of rapeseed proteins in this work is able to verify various aspects of previously published work. Protein solubility increases on moving away from the isoelectric point. Highest solubility was observed at pH 10 and lowest was noted at pH 3-5 (pI) which is also the isoelectric points of most proteins.
3. Protein composition by SDS-PAGE has revealed that protein solubility is inhibited due to formation of aggregates that are formed due to protein-protein interactions appear in HMW (represented by fractions>40 Kd). Also, napin (10-15 Kd) has a wider solubility range than cruciferin (20-40 Kd).
4. WBC and FBC of air-classified samples reveal that techno-functional properties can be effectively manipulated and can be used in various food products as food product enhancers.
5. Combination of RPC with T.molitor larvae has not been improved protein solubility, however, further research and effective manipulation of process parameters might produce better results.
6. High moisture and temperature while extrusion, are the major drawback supporting formation of disulphide linkages leading to aggregation and reducing solubility in aqueous systems considerably.
7. Finally, the potential of RPC and its by-products needs to be investigated further, for identifying RPC as functionalized value-added products.

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