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# Study on the valorization of rapeseed press-cake with special emphasis on meal protein

*A thesis submitted in partial fulfillment of the requirements for the degree*

*of*

## Doctor of Philosophy

**By**

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***Dedicated to my parents, beloved brother and late grandparents***

# Study on the valorization of rapeseed press-cake with special emphasis on meal protein


## ABSTRACT

The thesis includes a methodical study to remove the anti-nutritional factors from rapeseed meal, and their subsequent effect on physicochemical and functional properties of protein in the treated meal. Optimal condition for protein extraction from treated meal was derived by Response Surface Methodology and the recuperated protein isolate was chemically treated with maleic anhydride for improving its functional properties. The fibrous meal residue left after protein extraction, was then treated by hydrothermal carbonization for the formation of carbon nanoparticles (≈10 nm), the applicability of which was assessed in making rapeseed protein-based nanocomposite film.

The thesis is divided into six chapters which are briefly discussed below:

**Chapter 1** presents the general introduction about rapeseed press-cake and its under-utilized role as an important industrial waste. Different techno-functional properties of the meal and its protein have been detailed. It reviews the important works or techniques proposed for removing the anti-nutritional components from rapeseed meal. It also appraises the chemical modifications performed on rapeseed protein and their useful outcomes in terms of functional features. A brief note on processing and usability of rapeseed protein film has also been added. It highlights the gap of study and background behind the selection and planning of current investigation. The scope and objectives of the thesis are included at the end.

**Chapter 2** deals with the testing of different solvent mixtures containing acetone or methanol pure or combined with water or an acid (hydrochloric, acetic, perchloric, trichloroacetic, phosphoric) for their efficiency in extraction of the antinutritive compounds from rapeseed press-cake. Acidified extraction mixtures (non-aqueous) were found to be superior to the non-acidified ones ($p<0.05$). The characteristic differences in the efficacy of these wide varieties of solvents were studied by principal components analysis, based on which the mixture of 0.2% perchloric acid in methanol-acetone (1:1 v/v) was deemed as 'the best' for detoxification of rapeseed meal. Despite its high reductive (antioxidant) potential, hemolytic activity of the extract from this

solvent mixture clearly indicated the toxicity of the above-mentioned compounds on mammalian erythrocytes.

In **Chapter 3**, effect of anti-nutrients removal from rapeseed meal with organic solvent mixture (methanol-acetone, 1:1 v/v, combined with an acid (hydrochloric, acetic, perchloric, trichloroacetic, phosphoric)) on the physico-chemical and functional properties of rapeseed protein isolate (RPI) was investigated. The extraction resulted in substantial reduction of anti-nutrients from RPI, especially polyphenols and phytates, with concomitant decrease in protein yield and solubility. Treatment harbored significant improvement in their Degree of Whiteness, which was highest in the perchloric acid case. Surface hydrophobicity and free sulfhydryl group of RPI changed considerably, with perchloric acid-treated samples showing higher values; while the disulfide content remarkably increased in trichloroacetic and phosphoric acid-treated samples, signifying aggregation. Intrinsic emission fluorescence and FTIR spectra showed significant changes in proteins' tertiary and secondary conformations, and the changes were more pronounced in samples treated with higher concentration of acids. No appreciable alteration appeared among the electrophoretic profile of proteins from pristine meal and those treated with lower level of acids. Interfacial surface properties of proteins were variably improved by the solvent extraction, whereas the converse was true for their extent of denaturation. The results suggest close relation between the physicochemical and functional properties of rapeseed protein isolate.

In **Chapter 4**, optimization using Response Surface Methodology (RSM), for producing high yield of protein, having superior whiteness and emulsion properties, and reduced level of residual phytate content, has been presented.

In **Chapter 5**, influence of maleylation on the physicochemical and functional properties of rapeseed protein isolate was studied. Acylation increased whiteness value and dissociation of proteins, but reduced free sulfhydryl and disulfide content ($p<0.05$). Intrinsic fluorescence emission and FTIR spectra revealed distinct perturbations in maleylated proteins' tertiary and secondary conformations. Increase in surface hydrophobicity, foaming capacity, emulsion stability, protein surface load at oil-water interface and decrease in surface tension at air-water interface, occurred till moderate level of modification. While maleylation impaired foam stability, protein solubility and emulsion capacity were markedly ameliorated ($p<0.05$), which

are concomitant with decreased droplet size distribution ($d_{32}$). *In-vitro* digestibility and cytotoxicity tests suggested no severe ill-effects of modified proteins, especially up to low degrees of maleylation. The study shows good potential of maleylated proteins as functional food ingredient.

**Chapter 6** describes the hydrothermal carbonization of oil-and-protein spent rapeseed meal to form antioxidative, hemocompatible, fluorescent carbonaceous nanoparticles (FCD). In the present investigation, an interesting application of FCD in fabricating low-cost rapeseed protein-based fluorescent film, with improved antioxidant potential (17.5-19.3 fold) and thermal stability has been demonstrated. The nano-composite film could also be used as forgery-proof packaging due to its photoluminescence property. For assessing the feasibility of antioxidative FCD in real food system, a comparative investigation was further undertaken to examine the effect of such nano-carbon loaded composite film on the oxidative shelf-life of rapeseed oil. Oil samples packed in nano-composite film sachets showed significant delay in oxidative rancidity compared to that packed in pristine protein-film sachet (free fatty acids, peroxide value and thiobarbituric acid-reactive substances reduced upto 1.4, 2 and 1.2 fold, respectively). The work presents a new concept of bio-based fluorescent packaging and avenues for harnessing this potent waste.

**Chapter 7** presents the salient findings and future scope of the current investigation. It concludes that the major anti-nutritive compounds of rapeseed press-cake can be reduced substantially using acidified organic solvents (0.2% perchloric acid in methanol:acetone (1:1 v/v)). Maleylation can be a key to improve the functional properties of rapeseed protein. These treatments in-turn result in amelioration of colour and surface activities of the protein, together with marked changes in its secondary and tertiary conformations. Oil-and-protein spent meal has unequivocally been shown as a raw material for synthesis of beneficial CNP. Further studies should be undertaken to analyze their effect on organoleptic properties, gastro-intestinal tract, and toxicological assessment using *in-vitro* and *in-vivo* models.

# DECLARATION BY THE CANDIDATE

The thesis entitled "**Study on the valorization of rapeseed press-cake with special emphasis on meal protein**" being submitted to School of Engineering, Tezpur University, in partial fulfillment for the award of the degree of Doctor of Philosophy in the Department of Food Engineering and Technology is a record of bonafide research work accomplished by me under the supervision of **Prof. Charu Lata Mahanta**.

All helps from various sources have been duly acknowledged.

No part of the thesis has been submitted elsewhere for the award of any other degree.

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# CERTIFICATE OF THE SUPERVISOR

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All helps received by her from various sources have been duly acknowledged.

No part of the thesis has been submitted elsewhere for the award of any other degree.

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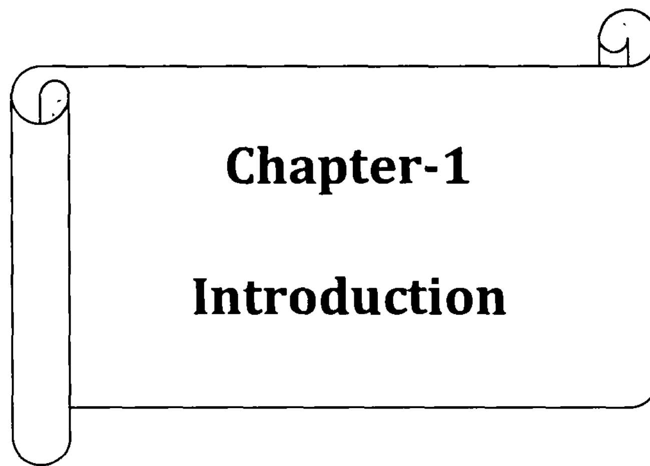
## Abbreviations

| | | |
|---|---|---|
| AITC | : | Allyl isothiocyanate |
| ANOVA | : | Analysis of variance |
| AOAC | : | Association of Official Analytical Chemists |
| ASTM | : | American Society for Testing and Materials |
| CCD | : | Central composite design |
| C-dots | : | Carbon-dots |
| CD | : | Conjugated diene |
| CPI | : | Canola protein isolate |
| CNP | : | carbon nanoparticle |
| DPPH | : | 2,2-diphenyl-1-picrylhydrazyl |
| DSC | : | Differential Scanning Calorimetry |
| EAI | : | Emulsification activity index |
| EB | : | Elongation at break |
| ES | : | Emulsion stability |
| FCD | : | Fluorescent carbon-dots |
| FC | : | Foaming capacity |
| FFA | : | Free fatty acid |
| FS | : | Foam stability |
| FTIR | : | Fourier Transform Infrared spectroscopy |
| GPC | : | Gel Permeation Chromatography |
| HEK | : | Human Embryonic Kidney |
| HPLC | : | High performance liquid chromatography |
| HRTEM | : | High resolution transmission electron microscopy |
| HTC | : | Hydrothermal carbonization |

| | | |
|---|---|---|
| IR | : | Infra-red |
| kDa | : | kilodalton |
| LGC | : | Least gelling concentration |
| MA | : | Maleic anhydride |
| Mayo | : | Mayonnaise |
| MEF | : | Mouse embryonic fibroblasts |
| MPa | : | Mega Pascal |
| MW | : | Molecular weight |
| NMR | : | Nuclear Magnetic Resonance |
| NP | : | Nanoparticle |
| PER | : | Protein Efficiency Ratio |
| PMM | : | Protein micellar mass |
| OAC | : | Oil absorption capacity |
| PBS | : | Phosphate buffer solution |
| PPP | : | Protein precipitable phenols |
| PC | : | Principal Components |
| PL | : | Photoluminescence |
| PS | : | Protein solubility |
| PV | : | Peroxide value |
| QD | : | Quantum dots |
| QY | : | Quantum yield |
| RBC | : | Red blood corpuscle |
| RH | : | Relative humidity |
| RPI | : | Rapeseed protein isolate |
| RSEE | : | Relative standard error of the estimate |

| | | |
|---|---|---|
| RSM | : | Response surface methodology |
| SAED | : | Selected-area electron diffraction |
| SEM | : | Scanning Electron Microscope |
| SH | : | sulfhydryl |
| SPE | : | Sodium phytate equivalent |
| $S_o$ | : | Surface hydrophobicity |
| SS | : | disulfide |
| TAE | : | Tannic acid equivalent |
| TBARS | : | Thiobarbituric acid-reactive substances |
| TEM | : | Transmission electron microscopy |
| TGA | : | Thermogravimetric analysis |
| TNBS | : | Trinitrobenzene sulphonic acid |
| TP | : | Total phenolics |
| Trp | : | Tryptophan |
| TS | : | Tensile strength |
| $\Gamma_s$ | : | Protein surface load |
| TT | : | Total tannins |
| UV | : | Ultraviolet |
| UV-vis | : | Ultraviolet-visible spectrum |
| v/v | : | volume by volume |
| v/w | : | volume by weight |
| WAC | : | Water absorption capacity |
| WVP | : | Water vapour permeability |
| WVTR | : | water vapour transmission rate |
| XRD | : | X-ray Diffraction |


Chapter-1
Introduction

## Chapter-1: Introduction

### 1.1 Preface

Rapeseed and mustard (*Brassica napus*, *B. campestris* and *B. juncea*) rank number five among the oilseeds.[1] Rapeseed is a herbaceous plant belonging to the *Cruciferae* family, and is farmed in many countries mainly for its high-quality edible oil.[2] Rapeseeds contain high level of oil (between 45-50%), approximately 25–30% proteins, and 25–30% carbohydrates (dry basis).[2] The scientific and economic interest for the cultivation and the processing of rapeseed is increasing, which is reflected by a 65% increase of rapeseed production from 38 million tons in 2000 to 62.5 million tons in 2011.[3] China, Canada and India are the main producers of rapeseed, and together are responsible for more than half of worldwide production in 2007 (56.3%).[2] India is one of the largest rapeseed-mustard growing countries in the world, occupying the second position in area and third position in production after China and Canada. Rapeseed-mustard (*Brassica*) is the second most important edible oilseed crop in India after groundnut and accounts for nearly 30% of the total oilseeds produced in the country. Rapeseed is cultivated in the states of Assam, Bihar, Gujarat, Haryana, Himachal Pradesh, Jammu & Kashmir, Madhya Pradesh, Orissa, Punjab, Rajasthan, Uttar Pradesh and West Bengal.[4] The projected demand for oilseeds in India is around 34 million tonnes by 2020, of which about 14 million tonnes (41%) is to be met by rapeseed-mustard.[4] Unfortunately, the Indian *Brassica* cultivars do not fetch a premium price in the international market, due to the presence of high levels of erucic acid (cis-13-docosenoic acid; 22:1n-9) (≈50%) and glucosinolates (≥100 mol/g defatted seed meal). However, the very intensive breeding programs in several countries produced high quality varieties that are significantly lower in these two toxicants. Canola is the rapeseed variety which was developed in Canada. The name 'canola' was introduced in Canada in 1979 that specifically denotes rapeseed varieties that produce oil having less than 2% erucic acid and less than 30μmol/g meal of total glucosinolates.[5] As indicated, rapeseed and canola seed are different only in regard to their erucic acid content in the extracted oil and total glucosinolates level in the meal. Thus, in the following text, due to its focus on protein, the term "rapeseed" and "canola seed" are used interchangeably.[5] The images of rapeseed plant and its mature seed are shown in **Fig. 1.1.**

Rapeseed oil, the most important product, is of vegetable origin and is extensively used in human food and biodiesel production.[2] Oil is extracted by rolling the seed to fracture the seed coat and rupture the oil cells (**Fig. 1.2**). It is a light yellow to brownish yellow oil. Rapeseed oil is obtained from the seeds of several species of *Brassica*, and the oil from different species is usually not distinguished on the market, since all have similar properties.[4] After the oil is extracted from the seed, the resultant byproduct is a defatted meal (**Fig. 1.2**). De-oiled cake, formed essentially of proteins and carbohydrates, has a high nutritional level. Rapeseed meal comprises about 40-50% protein (dry basis), with a well-balanced amino acid composition and protein efficiency ratio (PER) higher than that of soybean (2.64 vs 2.19).[2] In addition, some functional properties of canola meal proteins were shown to be comparable to those of casein and better than those of other plant proteins, such as soybean, pea, and wheat.[6] However, the usefulness of rapeseed protein is limited by the presence of some undesirable compounds, such as glucosinolates, phytates, and polyphenols, which are responsible for the toxic (antinutritional) and undesirable coloration of rapeseed protein products. For this reason commercial value of rapeseed meal is significantly low and is primarily used as animal feed and fertilizer.[4]

### 1.2. Edible products derived from rapeseed meal

The primary edible products derived from canola meal/flakes are flour/grits (at 50% protein), protein concentrates (containing 65–75% protein) and protein isolates (85-90% protein or more).[7] The nutritional quality, availability, price, and functionality of these products make them suitable for use in a wide variety of food and feed products.

**Fig. 1.1** Different stages in the development of rapeseed plant and mature seeds in the pod.

**Fig. 1.2** Extraction of rapeseed oil using an expeller and the resulting press-cake.

### 1.2.1. Canola proteins

Canola proteins of interest are mainly storage proteins, located in the cotyledons. They represent ≈80% of total protein in the developing embryo.[8] Canola proteins can be classified into four groups: albumin, which constitute the water soluble fraction; globulin, which is soluble in salt solution; prolamin, which is the ethanol soluble fraction; and glutelin, which is the fraction that is insoluble in all of the solvents mentioned above. Canola proteins can be also divided into various fractions according to the corresponding sedimentation coefficient in Svedberg units (S). This coefficient indicates the speed of sedimentation of a macromolecule in a centrifugal field. For canola proteins, the following fractions have been reported: 12S, 7S and a split 2S, 1.7S or 1.8S. Cruciferin and napin are the two major families of storage proteins found in canola/rapeseed. Napin is a 2S albumin, and cruciferin is a 12S globulin. They constitute 20% and 60% of the total protein content of mature seeds, respectively.[8] Napins are low molecular weight proteins (12.5-14.5 kDa) characterized by strong alkalinity that is mainly due to a high level of amidation of amino acids. Napin possesses good foaming properties.[9] Cruciferin is a neutral protein with a high molecular weight (300-310 kDa) and comprises of several subunits. In its native form, cruciferin acts as a gelling agent. Data on the structural composition of canola proteins are often controversial mainly due to the varied extraction and purification methods.[10] Cruciferin is stabilized mainly by non-covalent bonds and napin is stabilized primarily by disulphide bonds. Compared to the 2S globulin, the polypeptide composition of cruciferin is much more complex.

Cruciferin is an oligomeric protein that consists of at least 6 subunits, each composed of 2 polypeptide chains, whereas napin contains 2 subunits.[10] Beside these two reserve proteins, there is another structural protein called oleosin (19-20 kDa), which is associated with oil bodies[6] and represents about 2-8% of the total canola seed proteins.

The amino acid compositions of the canola protein isolates have been studied and reported by several researchers;[6,8,9,11] these proteins are well balanced and show high glutamine, glutamic acid, arginine and leucine contents and low amounts of sulfur-containing amino acids (**Table 1.1**). The amino acid composition depends on the mechanical pressing applied during oil extraction, heat used for de-solventization of the meal and also on the process used for protein extraction from the meal residue.[11,12] Considering the promising techno-functional properties of rapeseed/canola proteins, a structured approach could easily rectify this problem; a combination of canola proteins with other proteins would compensate for the deficiency or lack of certain amino acids.[12]

**Table 1.1** Amino acids composition expressed as mass percent of the globulin and albumin isolates from rapeseed.[12]

| Amino acid | Albumin isolate | Globulin isolate |
|---|---|---|
| Asx | 5.1 | 9.5 |
| Glx | 30.4 | 20.2 |
| Ser | 4.1 | 4.4 |
| His | 4.7 | 5.1 |
| Gly | 1.9 | 1.7 |
| Thr | 4.4 | 4.7 |
| Ala | 3.4 | 3.5 |
| Arg | 8.6 | 9.8 |
| Tyr | 3.7 | 4.5 |
| Cys | 0.1 | 0.0 |
| Val | 4.3 | 3.3 |
| Met | 0.5 | 1.2 |
| Phe | 3.7 | 5.7 |
| Ile | 4.3 | 5.3 |
| Leu | 8.5 | 9.1 |
| Lys | 6.2 | 4.7 |
| Pro | 6.4 | 6.8 |
| Trp | n.d. | n.d. |

Asx: aspartic acid+asparagines; Glx: glutamic acid+glutamine; n.d.: Not determined.

In comparison to other plant proteins, information on physicochemical properties of canola/rapeseed proteins, such as molecular structure, pI, hydrophobicity, and studies relating these findings to protein functionalities, are still limited, and thus more research is necessary in this domain.[5]

### 1.2.2. Canola protein extraction

Continuous efforts are being made to improve extraction technologies or to modify canola proteins to obtain desirable functionalities for its potential applications. As noted, in majority of the studies, canola protein was extracted as a whole rather than as specific protein fraction, because transferring the protein fractionation process onto an industrial scale will not be economically attractive.[5]

#### 1.2.2a Preparation of seed meals

Canola seeds are typically crushed or ground to aid the separation and defatting process. Removal of fat from the crushed canola seed is normally carried out using hexane, ether, alkanol or their mixtures as solvent.[13-15] The defatted meal is usually dried at room temperature in a fume hood[6] or under vacuum in an oven at 40-50°C.[16] The dried and defatted meal may then be ground to pass through 40-mesh or 60-mesh screen in order to assure thorough interaction of the meal with chemicals during the protein extraction process.[15] It has been suggested that hexane may not be the best solvent for subsequent use of the protein.[17] Yellow mustard seed, extracted with diethyl ether and ethyl acetate (1:1 v/v), rather than hexane, have lower sinapic acid level concomitant with high oil yield.[18]

#### 1.2.2b Solubilization and precipitation/recovery of protein

The most frequently used solvent for the extraction of oilseed protein is water with sodium or potassium hydroxide to modify pH. Sodium chloride has also been proposed at different concentrations for the extraction of protein from canola. Alkaline extraction with sodium hydroxide solution followed by precipitation with dilute acid is the most typical procedure used in preparation of canola protein isolates (CPIs).[19,20] The use of alkali has been popular because of the high degree of solubility that can be obtained. The pH of the solution is varied from 9 to 12.5, with higher yields associated

with higher pH values.[17] High pH values (especially pH 12 and above) are associated with increased lysinoalanine formation and do not always produce the best quality isolates.[17] For this reason, a number of researchers have preferred to solubilize proteins with lower pH alkaline solutions. Generally, the alkaline solution is first added to the defatted canola meal and stirred or shaken for a given period of time to solubilize the proteins. The mixture is then centrifuged, and the pH of supernatant is adjusted by dilute acid to precipitate the proteins. Precipitated protein is then separated by centrifugation and the precipitate is freeze-dried (**Fig. 1.3**).

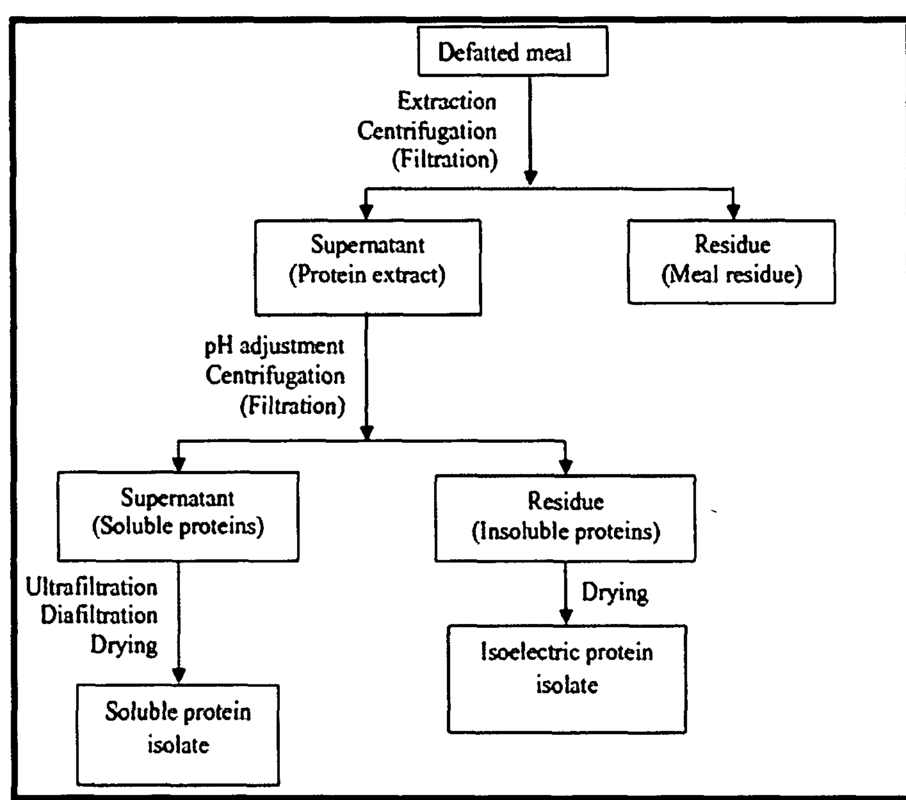



**Fig. 1.3** Schematic representation for alkaline extraction of oilseed meal protein isolates.[5]

Canola protein has been more of a challenge in terms of protein recovery. The adjustment of the pH of the extract's supernatant to the pI is normally carried out by using dilute acid solutions. Canola proteins have a very complicated protein composition. Extraction of canola proteins from canola meal often results in a low protein extraction

yield due to the widely differing isoelectric points (pH 4 to 11) and molecular weights (13000 to 320000 Da).[17] Aluko & McIntosh[21] suggested adjusting the pH to 4 using 0.1 M HCl. Adjustment of pH to 3.5 has also been reported by using acetic acid, phosphoric acid or hydrochloric acid.[19] Ghodsvali et al.[6] in their study on extraction of protein from 3 different canola varieties, adjusted the extracted proteins from pH 3.5 to 7.5 in increments of 0.5 pH units and found the range of pH 4.5 to 5.5 as the optimum pH for protein precipitation. In some studies, such as work carried out by Pedroche et al.,[20] more than one pI was reported. At both pH 3.5 and 5, different protein fractions extracted from defatted *Brassica carinata* meal achieved their lowest solubility.[20]

All of the non-protein components of rapeseed meal may be removed with a protein solubilization operation at alkaline pH, elimination of the solid residue, and re-precipitation of the proteins when the solution is brought to an acid pH. This procedure, when applied to meal or flour that have not been dephenolized, gives rise to the undesirable oxidation of polyphenols, with the subsequent formation of undesirable coloration and reduction of the bioavailability of some amino acids. For this reason, the isolation of protein is preceded by or combined with dephenolizing operations.[22]

**1.2.2c Protein extraction by micellization**

Protein micellar mass (PMM) method has been developed as an alternative process for extracting canola meal proteins. This method reduced the concentration of problematic antinutritional or toxic factors, including the glucosinolates and their degradation products.[23,24] PMM method is made up of 4 main steps that consist of extraction, ultrafiltration, dilution, and precipitation. Accordingly, the defatted meal is first extracted by sodium chloride solution, followed by ultrafiltration process to concentrate and purify the proteins. This ultrafiltration step has been proven to be efficient in removing glucosinolates with minimal loss of proteins.[6] The retentate is then diluted with cold water to reduce the ionic strength of the concentrated protein and promote precipitation effectively through the formation of protein micelles. The protein micelles are then separated from the water through centrifugation. Precipitates are collected and freeze-dried. However, in comparison to the alkaline extraction method, there is not much literature on PMM for protein extraction. This could possibly be due to

the poorer overall protein yield (71.3-78.5%) as reported by Yoshie-Stark et al.,[25] and Ismond and Welsh[26] in comparison to protein yield from alkaline extracts.

Although numerous studies have been carried out on canola/rapeseed protein extraction, there is limited literature on mathematical modeling of the extraction processes. It has been observed that designing of process conditions is essentially a way of optimizing key ingredients to generate the best formulation. The main objective of optimization is to determine the levels of independent variables that lead to the best characteristics of a particular product, such as physicochemical, colorimetric, sensory and nutritional properties, without extending excessively the experiment time with a large number of assays. So, technological unit operations involved in protein extraction protocol should be optimized for a product with enhanced features and, therefore, more likely to be accepted by the target public. Various parameters were found to play critical roles in the experimental conditions required for the optimization of the extraction efficiency of rapeseed protein.[27] Dong et al.[27] studied the effects of the three factors (ultrasonic power, ultrasonic treatment time and solid/liquid ratio) on the extraction efficiency of rapeseed protein, using Response Surface Methodology. A central composite design was used to model 2nd-order response surfaces for the protein yield and the functional properties of canola protein isolates, where the solubilization pH and precipitation pH were used as design factors.[28] A full factorial design for canola protein–κ-carrageenan mixtures at varied conditions (CPI, salt and hydrocolloid concentrations; pH, denaturants) was studied by turbidimetric testing for improving the emulsifying activity index and emulsion stability of the mixture.[29]

### 1.3. Factors affecting the utilization of canola meal protein in human food manufacture

Oilseeds have a range of chemical compounds which aid their growth and serve as defense factors against insects and microorganisms. These compounds generally end up in the meal component that is left after oil extraction.[17] Antinutritional factors in canola meal are the major obstacle for its use in human food manufacture. Canola meal contains glucosinolates, phenolics, phytates, and a high amount of fiber that make it problematic for food use. The impact of these components leads to unacceptable

properties of canola proteins that include relatively inferior physicochemical properties, poor digestibility, objectionable color, and bad taste.[30-32]

### 1.3.1. Phenolic compounds

Polyphenols have received a lot of attention in relation to their negative impact on the proteins from oil-producing plants. Rapeseed meal is rich in phenolic compounds (**Table 1.2**), which are usually present either as phenolic acids or condensed tannins.[31] The phenolic acids, including caffeic, vanillic, syringic and coumaric can be found in free, esterified and bound forms, and are responsible for astringency, flavours, dark colours and decrease in mineral availability.[31] The total content of phenolic acids varies between 6400 and 18400 μg/g depending on the variety of the plant and oil processing method. In addition, growing conditions and the degree of maturation affect the phenolic composition.[33] The main phenolic acid, sinapic acid, is unique to canola/rapeseed, where it represents 70-85% of the free phenolics and 71-97% of the esterified phenolic acids.[17] Majority of the phenolic acids (≈80% in meal) exist in the esterified form as sinapine (sinapoylcholine), followed by sinapoylglucose (**Fig. 1.4**). Sinapate esters cause a dark color and bitter taste in rapeseed meal and extracted protein products.[31] Besides, sinapate esters have negative effects on the digestibility of rapeseed meal. During rapeseed oil processing, sinapine may form complexes with protein through oxidation that then decrease the digestibility of rapeseed meal.[32] The formation of the dimer of sinapic acid, which has been identified as thomasidioic acid, can negatively impact the functional properties of the isolated protein. Insoluble bound phenolics represent a relatively low proportion of the phenolic acids, which can be released free on hydrolysis.[17]

**Table 1.2** Phenolic acid content of selected oilseed flours.[33]

| Flour | Phenolic acid (μg/g) |
|---|---|
| Soybean | 234 |
| Cottonseed | 567 |
| Peanut | 636 |
| Rapeseed | 6399 |

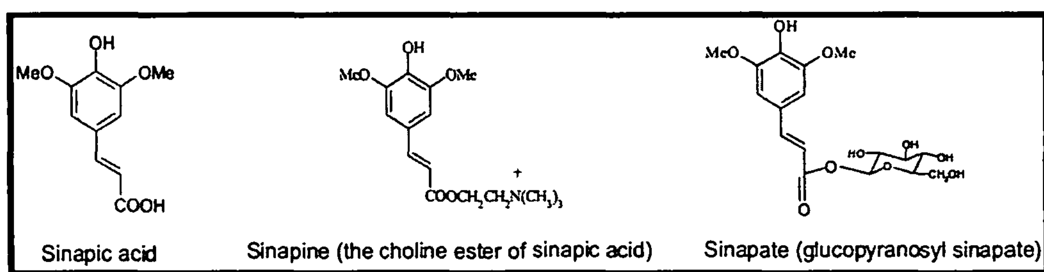



**Fig. 1.4** The main phenolics in rapeseed meal.[33]

Food tannins are polyphenolic compounds with molecular weights ranging from 500-3000 gallic acid esters equivalent, and can be hydrolyzed to produce phenolic acids. They can be classified as condensed or hydrolysable tannins. Most of the tannins in rapeseed are condensed tannins (**Fig. 1.5**), formed by polymerization of flavan-3-ols or flavan-3,4-diols.[17] Amount of tannins in rapeseed depends on the variety, the degree of maturation and extraction method, and varies from 0.2-3% of defatted meal,[33] though values around 0.7% seem to be a reasonable estimate.[17] Some of the tannins in rapeseed exist in insoluble forms and their insolubility may be due to polymerization as-well-as to the formation of insoluble complexes with the fiber and protein fractions of the seed.[17] The concern with having tannins in the meal relates to the ability of these compounds to precipitate protein[31] and form complexes with minerals and amino acids thereby reducing the nutritional value of the isolated protein.[17] It has been shown that the extent to which protein precipitation is induced increases with the amount and molecular weight of the tannin as-well-as the type and concentration of the protein.[17]

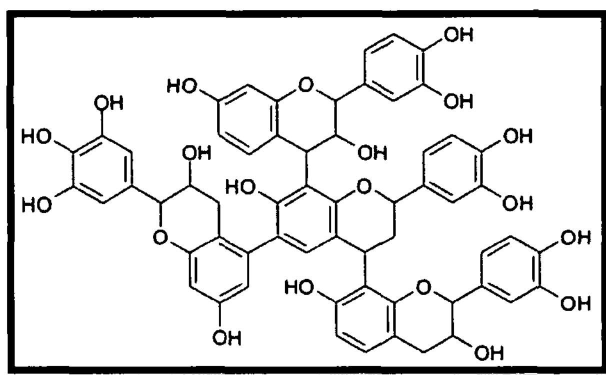


**Fig. 1.5** Structure of a condensed tannin[33]

### 1.3.2. Glucosinolates

The most commonly found glucosinolates in *Brassica* species are shown in **Table 1.3**. The concern with glucosinolates is not the glucosinolates themselves, but their degradation products.[17] Glucosinolates present in the meal release goitrogenic factors, such as isothiocyanates, thiocyanates, oxazolidinethione, cyanides and nitriles when hydrolyzed by the enzyme, myrosinase (thioglucoside glucohydrolase), also present in the seed/meal. These compounds interfere with iodine uptake and thyroxine synthesis by the thyroid gland,[34,35] and also create problems for kidney and pancreas.[17] Actually, industrial rapeseed extraction meal is used as ruminant feed because the hydrolysis products of the thioglucosides are destroyed in the rumen, the only negative factor is its nasty flavour. The use of rapeseed protein products for monogastric animals, or human consumption, however, requires the removal of thioglucosides and/or the inactivation of the myrosinase enzyme. Thermal treatment of the seed/meal is not sufficient to detoxify rapeseed meal because even though myrosinase is completely inactivated, it is not possible to exclude possible hydrolysis of the thioglucosides through other nonenzymatic methods. Interestingly, a lower level of glucosinolates content has been reported to have positive effect on health. Song and Thornalley[36] found that glucosinolates level of 0.61$\mu$mol/g or lower in broccoli can be linked to a reduced cancer risk. Also, the original use of rapeseed-mustard was for its spiciness, which in-turn is caused by isothiocyanates (allyl isothiocyanate of brown mustard, and 4-hydroxybenzyl isothiocyanate of yellow mustard). Nevertheless, it is a fundamental task to reduce the level of glucosinolates and/or its degraded products so that the proteins extracted from canola meal are fit for human consumption.

### 1.3.3. Phytic acid

Phytic acid is another antinutritional factor in canola meal, typically existing as mixed salts (phytates) of calcium, magnesium, and potassium. This is mainly due to the fact that the molecule is negatively charged at normal pH; therefore, it is very reactive with cations such as minerals.[37,38] The formation of phytic acid-mineral complexes thus decreases the availability of minerals. Phytate levels of 2.0-5.0% have been reported for the defatted meal, and up to 9.8% for the protein isolates and concentrates depending on the method of protein isolation.[38] Other than binding with minerals, phytic acid also

binds to proteins, reducing the protein solubility, digestibility, and amino acid availability.[38,39] Despite serious concerns about phytate consumption, studies indicating that low concentration of phytic acid may play a role in reducing blood glucose and plasma cholesterol[17] and aid in cancer prevention have been reported.[40]

Although the above mentioned compounds have been acknowledged as antinutritional factors and their presence in large quantity make it almost impractical to use canola/rapeseed protein in any meaningful way for human food, it is equally important to note that these factors at very low level may prove to be beneficial antioxidant or anti-carcinogen, as indicated by some reports.

**Table 1.3** Structures of glucosinolates from *Brassica* species[17]

| Type of glucosinolate | Common name | R group* |
|---|---|---|
| Alkyl | Sinigrin | Allyl |
| | Gluconapin | 3-butenyl |
| | Glucobrassicanapin | 4-pentenyl |
| | Progroitrin | 2-hydroxy-3-butenyl |
| | Gluconapoleiferin | 2-hydroxy-4-pentenyl |
| | Gluconasturtiin | Phenylethyl |
| | Glucoerucin | 4-methyl thiobutyl |
| | Glucosinalbin | 4-hydroxybenzyl |
| Indolyl | Glucobrassicin | 3-indolymethyl |
| | 4-hydroxyglucobrassicin | 4-hydroxy 3-idolymethyl |
| | Neoglucobrassicin | 1-methoxy-3-indolymethyl |
| Sulphinyl | Glucoalyssin | 5-methylsulphinylpentyl |

*Basic structure Glucose-S-C(-R)=$NSO_4$

### 1.4. Removal of antinutritional factors

Many studies have been carried out with the objective of removing or reducing antinutritional factors in rapeseed and canola, so that the extracted protein isolates can be incorporated into human foods without significant effects on sensory and nutritional qualities.

Two approaches have been used to address the concerns about phytic acid in oilseed protein products. One approach is to pre-extract or remove the phytates from the protein source. An alternate approach is to use the enzyme phytase to break the phytic acid molecule to a point where it is no longer able to bind to minerals in the diet.[17] Mahajan and Dua[41] reported the use of enzymes, such as pepsin, papain, trypsin, ficin and hemicellulase, in reducing the antinutritional factors and improving functional properties. Blaicher et al.[42] and El Nockrashy et al.[43] studied the countercurrent extractions of defatted rapeseed meal in the successive stages, which ensured highest dissolution of meal protein (92%) and phytic acid (70%).

Despite the fact that glucosinolates level has been significantly reduced in canola varieties, the presence of glucosinolates continues to be an impediment to the utilization of canola protein for human consumption. As such, a number of treatments have been considered to minimize this problem, including inactivation of myrosinase by dry heat or steam, and extraction of glucosinolates or its breakdown products (by charcoal or organic solvents).[17] Embaby et al.[44] reported a reduction of glucosinolates and other antinutritive compounds in the canola meal when subjected to heat treatment. Mansour et al.[45] have reported similar findings that glucosinolates were destroyed by high temperature, thus improving the *in-vitro* digestibility of rapeseed proteins. The reductions in antinutritional factors by heat processing ranged from 47-94% for glucosinolates, from 9-43% for phytic acid and from 41-67% for tannic acid after toasting and autoclaving treatments. Sadeghi et al.[46] prepared protein isolate by steam injection heating for reduction of antinutritional factors. However, the drastic thermal treatment of oilseeds reduces the quality of extracted oil and proteinaceous materials.[47] Pastuszewska et al.[48] stated that increasing the heating temperature of rapeseed cakes from 90-140 °C decreased protein solubility, protein digestibility and biological value. The excessive heating in the conventional process decreased the nutritional availability of some essential amino acids (mainly lysine) that complex with reducing sugars.

The dark colour which develops in rapeseed proteins, particularly under alkaline conditions, has been a concern of almost all researchers working on the preparation of rapeseed protein isolates. Methods to reduce the phenolic compounds have focused on minimizing the interaction between the phenolics and proteins, thus creating a situation

where the phenolic compounds can be removed from the protein due to a difference in solubility (organic solvent) or size (ultrafiltration or nanofiltration).[17] In fact, problems due to interaction between canola proteins and canola phenolics are much more prevalent and complex than is generally recognized. None of the reported methods is able to completely remove polyphenols from proteins.[32] So abolition of phenolics from rapeseed protein products poses a major obstacle. Elimination of tannins is often done in conjunction with the removal of phenolic acids and the same approaches apply.[17]

Defatted meal is usually extracted with organic solvents, most commonly aqueous methanol, ethanol, or acetone as described in **Table 1.4**. For production of high quality canola meal with low glucosinolate and phenolic content and improved quality of the oil with lower phosphatidyl content (gum), two-phase solvent extraction method was developed.[13] Naczk et al.[14] reported a 2-phase solvent extraction system (methanol-ammonia-water, hexane) to produce canola meal with trace levels of glucosinolate. The canola meals produced by this method have superior functional properties.[14] It has 25% more crude protein than the meal produced by hexane extraction, but the protein extractability was greatly reduced. Use of sodium sulphite and polyvinyl pyrolidone (PVP) has been shown to be effective at significantly reducing the levels of phenolic compounds associated with canola proteins.[32] The color of the protein isolates could also be improved considerably by pre-extracting the meal at isoelectric pH, however, it leads to considerable loss in protein content.[42] Hydrogen peroxide ($H_2O_2$) and sulfur dioxide ($SO_2$) treatments were found to produce rapeseed isolates with almost bland flavour and light colour **(Fig. 1.6)**.[49]

A protein extraction method, which is based on the formation of protein micellar mass (PMM), has proven to be efficient in removing glucosinolates with minimal loss of proteins, with the reduction in glucosinolate level being associated with the ultrafiltration step, as the toxic compounds have significantly lower molecular weights than rapeseed proteins.[6,67] An ultrafiltration-diafiltration-based technique was developed by Marnoch and Diosady,[68] and Das et al.[69] for simultaneous preparation of protein isolate and recovery of phenolic rich fraction from seed meal. Nanofiltration based rapeseed protein extraction was developed by Bérot et al.[70] While a high purity of protein isolate (96%) with improved functional properties was obtained,[25,69] the yield of such membrane-based

processes was found lesser (31.4% on dry basis) compared to precipitation process (42% on dry basis), and its use is limited by the cost of the filtration set-up, flux decline of membrane, and large consumption of water.

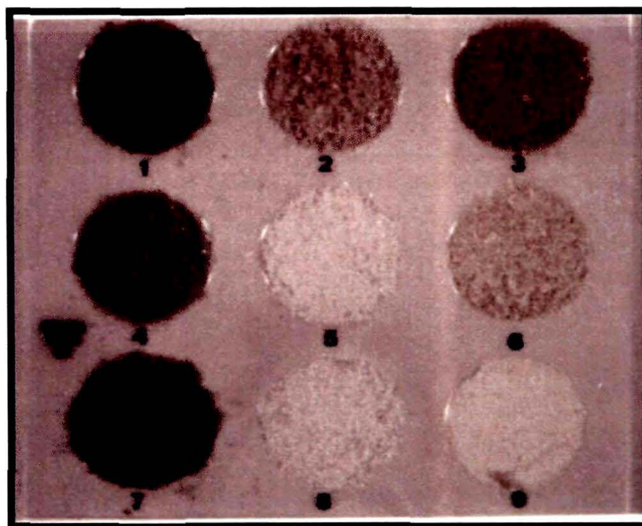


**Fig. 1.6** Colour improvement of rapeseed protein isolates. 1- base extracted fraction (adjusted at pH 4), before decolourization (control); 2- base extracted fraction (adjusted at pH 4), after $H_2O_2$ treatment; 3- base extracted fraction (adjusted at pH 4), after $SO_2$ treatment; 4- second acid extracted fraction (adjusted at pH 7), before decolourization (control); 5- second acid extracted fraction (adjusted at pH 7), after $H_2O_2$ treatment; 6- second acid extracted fraction (adjusted at pH 7), after $SO_2$ treatment; 7- acid extracted acidic fraction (adjusted at pH 3.8), before decolourization (control); 8- acid extracted acidic fraction (adjusted at pH 3.8), after $H_2O_2$ treatment; 9- acid extracted acidic fraction (adjusted at pH 3.8), after $SO_2$ treatment.[49]

**Table 1.4** Methods of extraction of rapeseed phenolics for analysis.[33]

| Extracted compounds | Extraction solvent | Reference |
|---|---|---|
| Free, esterified and insoluble bound phenolics | Methanol/Acetone/water (7:7:6) | Pink et al.[50] |
| Free, esterified and insoluble bound phenolics | 80% methanol | Zadernowski and Kozlowska[51] |
| Free, esterified and insoluble bound phenolics | 80% methanol | Kozlowska et al.[52] |
| Free, esterified and insoluble bound phenolics | Hot 80% methanol | Kozlowska et al.[53] |
| Free, esterified and insoluble bound phenolics | 70% methanol/70% acetone (1:1) | Krygier et al.[54] |
| Free, esterified and insoluble bound phenolics | Methanol/Acetone/water (7:7:6) | Naczk and Shahidi[55] |
| Free, esterified and insoluble bound phenolics | Several such as 70% methanol, 70% ethanol, 70% acetone, 70% isopropanol, Methanol/Acetone/water (7:7:6) | Thiyam et al.[56] |
| Phenolic compounds | 95% ethanol | Wanasundara et al.[57] |
| Total phenolic acids | 60% acetone, pH 3 | Xu and Diosady[58] |
| Phenolic acids | 70% acetone | Fenton et al.[59] |
| Sinapic acid and vinylsyringol from crude rapeseed oil | 80% methanol | Koski et al.[60] |
| Sinapic acid | Methanol/Acetone/water (7:7:6) | Pink et al.[50] |
| Sinapic acid | Methanol/Acetone/water (7:7:6) | Naczk et al.[61] |
| Sinapine | 70% methanol (75°C, 20 min) | Wang et al.[62] |
| Sinapine | 100% methanol | Li and Rassi[63] |
| Sinapate | 95% ethanol | Amarowicz and Shahidi[64] |
| Soluble and insoluble tannins | Soluble tannins: 70% acetone<br>Insoluble tannins: Methanol/butanol/HCl | Naczk et al.[65] |
| Tannins | 70% acetone, N,N-dimethyl formamide or methanol | Naczk et al.[66] |

## 1.5. Functional properties of protein

Protein extraction is only the first step towards incorporating proteins from oil-producing plants into food products. To reap the benefits of these nutritious proteins, it is critical that their incorporation into food products be such that the food remains appealing to the consumer. The functional properties that seem to receive the most attention when characterizing isolated oilseed proteins are solubility, water absorption capacity (WAC), oil absorption capacity (OAC), emulsification activity index (EAI), emulsion stability (ES), foaming capacity (FC), foam stability (FS) and gelation. In many cases, different methodology is employed to determine the same functional property. This creates an important problem to compare data from various authors because the operational conditions of each assay (protein concentration, solvent, time and measurement) and its units are different. The diversity in the methodology used for determining the functional properties makes it difficult for data comparison.[17,71]

### 1.5.1. Water absorption capacity (WAC) and oil/fat absorption capacity (OAC)

In food applications the water retention capacity is related with the ability to retain water against gravity, and includes bound water, hydrodynamic water, capillary water and physically entrapped water. The amount of water associated to proteins is closely related with its amino acids profile and increases with defatting, germination, fermentation, soaking or thermal treatments (toasting/autoclaving).[71] The fat-adsorption capacity of any food compound is important for food applications because it relies mainly on its capacity to physically entrap oil by a complex capillary-attraction process. In many food applications, such as emulsion-type meat products, the ability of a food component to entrap oil is an important characteristic because fat acts as a flavor retainer, a consistency trait and an enhancer of mouthfeel.[72] The values of WAC, OAC and protein solubility (or Nitrogen Solubility) are summarized in **Table 1.5** for meals and in **Table 1.6** for concentrates and isolates from rapeseed/canola, and reflect some of the previously commented effects. The ability of concentrates to bind water is enhanced when compared to meals. OAC was reported to be good when phytates and polyphenolic compounds are reduced during isolate

preparation.[17] Data corresponding to some chemically/enzymatically modified proteins are summarized in **Table 1.7**.

### 1.5.2. Solubility

The hydrophilicity/hydrophobicity balance, particularly at the protein surface, influences the protein solubility. Higher solubility is related to the presence of a low number of hydrophobic residues, the elevated charge, and the electrostatic repulsion and ionic hydration occurring at pH above and below the isoelectric pH. Denaturation, salting-in and salting out phenomena affects protein solubility due to alterations in the hydrophobicity/hydrophilicity ratio of the surface.[71] For food applications, protein (nitrogen) solubility is an important parameter that influences the extent of applications in different food matrices. In some cases, such as beverages, high protein solubility is a determinant for application as a fortification ingredient. Solubility of oilseed proteins is often influenced by the isolation method used. As many of these proteins are globulins (salt soluble), an evaluation of water solubility is not always beneficial.[17] Effect of operational conditions on protein solubility values for meals, and for concentrates and isolates are shown in **Tables 1.5–1.7**.

### 1.5.3. Emulsifying and foaming properties

Emulsions are two phase systems, with non-polar phase (oil droplets) dispersed in an aqueous continuous one. Foams are 2 phase systems composed of air bubbles surrounded by a continuous liquid lamellar phase.[80] Emulsions and foams are commonly found in food models and can be stabilized by either proteins or surfactants. In food applications, such as emulsion-type meat products, salad dressings and mayonnaise, emulsifying property of proteins is an important attribute and largely defines the protein's surface activity. Emulsifiers or foaming agents decrease the interfacial tension and facilitate formation of stable oil–water and air–water interfaces. Proteins as foaming agents have been studied mainly in terms of foaming capacity (FC) and foam stability (FS). FC is related to the readiness of proteins to bind to the air-water interface to form foam particles, whereas FS is related to the protein–protein interactions that form strong interfacial membranes that

**Table 1.5** Functional properties of oilseed meals.[71]

| Oilseed | Processing conditions | WAC (g/g) | OAC (g/g) | Solubility (%) | EAI (%) | ES (%) | FC (%ΔV) | FS | Reference |
|---|---|---|---|---|---|---|---|---|---|
| **Canola (*Brassica rapa*)** | Defatted by hexane in Soxhlet | - | - | - | 32.48% | 15.71 | 204.38 | 35.19% | Aluko and McIntosh[73] |
| **Canola (*Brassica napus*)** | a) Whole seed defatted by hexane for 18 h in Soxhlet | 3.08 | 2.81 | - | 57.3% | - | 128 | 146-37 ml ( foam vol. after 1 and 120 min) | Ghodsvali et al.[6] |
| | b) Dehulled seed defatted by hexane for 18 h in Soxhlet | 2.22 | 2.94 | - | 62.3% | – | 156 | 218-92 ml (foam vol. after 1 and 120 min) | |
| **Canola (*Brassica napus*)** | Commercial | 2.79 | 1.34 | 13.7 | 41.6% | 70 | 56 | 122-24 ml (foam vol. after 1 and 120 min) | Naczk et al.[14] |
| **Mustard white (*Sinapis alba*)** | Defatted by hexane in Soxhlet | - | - | 55.66 | 29.79 $m^2/g$ | 7.64 | 226.98 | 50.00% | Aluko et al.[74] |
| **Mustard greens (*Brassica juncea*)** | Defatted by hexane in Soxhlet | - | - | 67.70 | 58.14 $m^2/g$ | 58.59 | 196.72 | 51.92% | Aluko et al.[74] |
| **Rapeseed (*Brassica campestris*)** | Full-fat seed | 2.12 | 1.28 | 9.5 | 98% | 94.25 | 50 | 52.60-5.68 ml (foam vol. after 0.5 and 60 min) | Mahajan et al.[75] |
| | Defatted with petroleum ether for 1 h at room temperature | 2.50 | 2.31 | 13.5 | 25% | 90.75 | 90 | 64.25-11.11 ml (foam vol. after 0.5 and 60 min) | |
| | Full-fat seed soaked in water for 8 h at 26 °C and then fermented (pH 6.1) for 16 h at 30 °C | 2.42 | 1.04 | 6.5 | 91% | 64.75 | 60 | 64.75-4.40 ml (foam vol. after 0.5 and 60 min) | |
| | Defatted seed soaked in water for 8 h at 26 °C and then fermented (pH 6.1) for 16 h at 30 °C | 3.73 | 1.81 | 7.5 | 60% | 61 | 85 | 91.25-8.32 ml (foam vol. after 0.5 and 60 min) | |
| | Full-fat seed soaked in water for 8 h and then germinated for 72 h at 30 °C | 3.86 | 2.17 | 7.8 | 91% | 91.25 | 80 | 83.50-10.75 ml (foam vol. after 0.5 and 60 min) | |
| | Defatted seed soaked in water for 8 h and then germinated for 72 h at 30 °C | 4.33 | 3.12 | 8.2 | 97.25% | 86.50 | 100 | 106.2-17.25 ml (foam vol. after 0.5 and 60 min) | |

**Table1.5** Functional properties of oilseed meals.[71] *(continued)*

| Oilseed | Processing conditions | WAC (g/g) | OAC (g/g) | Solubility (%) | EAI (%) | ES (%) | FC (% ΔV) | FS | Reference |
|---|---|---|---|---|---|---|---|---|---|
| **Rapeseed (*Brassica campestris*)** | Full-fat seed roasted for 0.033 h at 240 °C | 3.31 | 1.67 | 4.0 | 99% | 49.25 | 0 | 0-0 ml (foam vol. after 0.5 and 60 min) | Mahajan et al.[75] |
| | Defatted seed roasted for 0.033 h at 240 °C | 4.25 | 2.06 | 5.1 | 48.75% | 56.25 | 0 | 0-0 ml (foam vol. after 0.5 and 60 min) | |
| | Full-fat seed soaked in water for 3 h at 26 °C and then puffed for 0.05 h at 250 °C | 1.97 | 1.37 | 4.9 | 49.25% | 51 | 45 | 44.75-0 ml (foam vol. after 0.5 and 60 min) | |
| | Defatted seed soaked in water for 3 h at 26 °C and then puffed for 0.05 h at 250 °C | 2.38 | 2.12 | 6.0 | 51.25% | 49.75 | 50 | 53.25-3.25 ml (foam vol. after 0.5 and 60 min) | |
| | Full-fat seed autoclaved for 0.12 h at 121 °C | 2.53 | 1.15 | 2.2 | 50.50% | 49.25 | 20 | 22.50-0 ml (foam vol. after 0.5 and 60 min) | |
| | Defatted seed autoclaved for 0.12 h at 121 °C | 2.99 | 3.12 | 1.1 | 52.75% | 55.50 | 25 | 25.75-0 ml (foam vol. after 0.5 and 60 min) | |
| | Full-fat seed soaked in water for 3 h and then microwave cooking was done for 0.003 h at 33 °C | 2.81 | 2.07 | 5.1 | 84.25% | 100 | 200 | 206.2-50.25 ml (foam vol. after 0.5 and 60 min) | |
| | Defatted seed soaked in water for 3 h and then microwave cooking was done for 0.003 h at 33 °C | 3.31 | 4.52 | 9.0 | 100% | 100 | 240 | 246.2-61.17 ml (foam vol. after 0.5 and 60 min) | |
| **Rapeseed (*Brassica carinata*)** | Defatted by hexane for 9 h in Soxhlet | 2.55 | 2.02 | - | 75.6% | 49.4 | 295 | 56.9 ml (foam vol. after 120 min) | Pedroche et al.[20] |
| **Canola (*Brassica napus*)** | Ground seed defatted by hexane for 18 h in Soxhlet | 3.9 | 2.09 | 66.42 | 48.58 ml/g | 11.17 ml water removed/g | 56.44 | ≈79% (after 120 min) | Khattab and Arntfield[72] |
| | Ground meal roasted at 180±2 °C for 15 min | 4.71 | 2.32 | 47.11 | 36.65 ml/g | 12.11 ml water removed/g | 8.92 | ≈0% (after 120 min) | |
| | Defatted meals boiled in water (1:5 w/v) for 15 min. | 5.11 | 2.51 | 37.86 | 32.5 ml/g | 13.08 ml water removed/g | 6.93 | ≈0% (after 120 min) | |

**Table 1.6** Functional properties of oilseed protein concentrates and isolates.[71]

| Oilseed | Processing conditions | WAC (g/g) | OAC (g/g) | Solubility (%) | EAI | ES (%) | FC (% ΔV) | FS (%) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| **Canola (*Brassica napus*)** | Extracted in 0.1M NaOH for 0.33 h at 23 °C. Recovered at pH 4.0 by isoelectric precipitation method and then freeze-dried. | - | - | - | 32.34 ml/g | 26.8 | 185.37 | 8.16 | Aluko and McIntosh[73] |
| | Extracted in 0.1M NaOH for 0.33 h at 23 °C. Recovery done at pH 6.0 in presence of $CaCl_2$ and then freeze-dried. | - | - | - | 28.27% | 71 | 189.15 | 24 | |
| **Canola (*Brassica napus*)** | Micellar extraction | - | - | - | 39.8% | 68 | 43.3 | 74.7 | Gruener and Ismond[76] |
| **Mustard white (*Sinapis alba*)** | Extracted in 0.1M NaOH for 0.33 h at room temperature. Recovered at pH 6.0 by isoelectric precipitation | - | - | - | 35.63 $m^2/g$ | 36.82 | 211.85 | 37.50 | Aluko et al.[74] |
| **Mustard greens (*Brassica juncea*)** | Extracted in 0.1M NaOH for 0.33 h at room temperature. Recovered at pH 6.0 by isoelectric precipitation | - | - | - | 42.54 $m^2/g$ | 35.79 | 189.15 | 37.82 | Aluko et al.[74] |
| **Rapeseed (*Brassica carinata*)** | Extracted in water at pH 10-12 for 2 h. Recovered at pH 5-3.5 by isoelectric precipitation and then freeze-dried. | 0.99 | 2.17 | 35 | 54% | 5 | 163 | 44.6 | Pedroche et al.[20] |
| **Canola meal (Crude commercial hexane defatted)** | Extracted in 0.4% w/v NaOH (glutelins) | - | - | 91.6 | - | - | - | - | Klockeman et al.[19] |
| | Extracted in 5.0% w/v NaCl (globulins) | - | - | 50.8 | - | - | - | - | |
| | Extracted in 60% v/v ethanol (prolamins) | - | - | 33.9 | - | - | - | - | |
| | Extracted in distilled deionized water (albumins) | - | - | 31.2 | - | - | - | - | |

**Table 1.7** Functional properties of some chemically modified oilseed proteins.[71]

| Oilseed | Processing conditions | Solubility (%) | WAC (g/g) | OAC (g/g) | EAI (%) | ES (%) | FC (% ΔV) | FS | Reference |
|---|---|---|---|---|---|---|---|---|---|
| **Canola (*Brassica napus*)** | Micellar extraction | 17.9 | - | 1.90 | - | - | - | - | Gruener and Ismond[76] |
| | Succinylation (61% acylated) | 70.6 | - | 4.05 | 100 | 96.7 | 157 | 7.5% | |
| | Acetylation (62% acylation) | 77.8 | - | 3.70 | 80 | 96 | 188 | 9% | |
| **Rapeseed (*Brassica campestris*)** | Succinylated with succinic anhydride in water at pH 8.0 for 2 h (room temperature) and then dialyzed | 90 | 1.79 | 2.0 | 44 | 55 | 230 | 57.3-1.6 ml (foam vol. after 0.5 and 60 min) | Dua et al.[77] |
| | Acetylated with acetic anhydride in water at pH 8.0 for 2 h (room temperature) and then dialyzed | 25 | 2.97 | 1.8 | 35 | 43 | 220 | 48.4-14.5 ml (foam vol. after 0.5 and 60 min) | |
| | Protein methylated with formaldehyde in water at pH 8.0 for 2 h (room temperature) and then dialyzed | 40 | 2.60 | 2.8 | 62 | 62 | 155 | 87.5-31.5 ml (foam vol. after 0.5 and 60 min) | |
| **Rapeseed (*Brassica napus*)** | Defatted with diethyl ether, protein extracted by isoelectric precipitation and freeze-dried. | 60 | - | - | - | - | 315 | 21.4% | Krause[78] |
| | Succinylation (91% N-blockage) | 100 | - | - | - | - | 342 | 16.6% | |
| | Acetylation (91% N-blockage) | - | - | - | - | - | 380 | 35.3% | |
| | Phosphorylation (10.1 mg phosphorus per g protein) | 95 | - | - | - | - | 367 | 29.4% | |
| **Canola (*Brassica napus*)** | Meal defatted with diethyl ether. Native 12S globulin extracted with 0.05M phosphate buffer (pH 8.0), precipitation till 90% saturation with $(NH_4)_2SO_4$, purification on Sephadex G-200 column | - | - | - | - | 0.55§ | 870* | 49 min | Gueguen et al.[79] |
| | Succinylated globulin (28% acylated) | - | - | - | - | 0.58§ | 860* | 68 min | |
| | Succinylation globulin (29% acylated) | - | - | - | - | 0.59§ | 910* | 65.6 min | |
| | Succinylation globulin (65% acylated) | - | - | - | - | 0.49§ | 1120* | 68 min | |
| | Succinylation globulin (83% acylated) | - | - | - | - | 0.46§ | 970* | 94.6 min | |
| | Succinylation globulin (93% acylated) | - | - | - | - | 0.47§ | 1160* | 64 min | |

§ volume fraction of oil in the creamed phase; * in terms of conductivity (μS/cm)

stabilize the foam particles.[5] Proteins present different surface activities, related to their conformation and ability to unfold at interfaces determined by molecular factors (flexibility, conformational stability, distribution of hydrophilic and hydrophobic residues in the primary structure) and external factors (pH, ionic strength, temperature, possible competitive adsorption of other proteins or lipids in the interface).[71] Emulsion and foam properties determined using several methodologies are summarized in **Tables 1.5 and 1.6** for meals, concentrates and isolates.

### 1.5.4. Gelation/gelling ability

A gel can be defined as an intermediate state between solid and liquid. In food systems the liquid is water and the molecular net is formed by proteins, polysaccharides or by a mixture of both. Gelation is favoured by the protein size, since large molecules form extensive networks by crosslinking in three dimensions, and by the flexibility and ability of the proteins to denature.[71] In order to form gels, partial denaturation is desirable, since unfolding of the tertiary structure gives long chains without breakage of covalent bonds. Other influencing factors are pH, ionic strength, reducing agents, urea, temperature and the presence of nonprotein components.[71] The gelling properties of canola proteins have been studied mostly in terms of least gelling concentration (LGC).[72] Khattab and Arntfield[72] studied LGC of raw and heat-treated canola meals and reported that neither roasting nor boiling caused a significant increase in the LGC value of different canola meals. Addition of phytates and phenolic compounds result in weaker and less elastic gels.[17] Gels from canola protein isolates were generally not as good as those from soybean protein.[17] To improve the function of canola proteins in gel-like food systems, different additives can be combined with these proteins to make convenient gels. Uruakpa and Arntfield[81] found that the gelling properties of CPI were greatly improved by the addition of κ-carrageenan or guar gum. Different ideal pHs were required for interaction between CPI and different types of polysaccharides with pH 6 being the optimum pH for CPI-κ-carrageenan emulsion and pH 10 being the optimum pH for CPI-guar gum emulsion.[81] Paulson and Tung[82] studied the thermally induced gelation of a rapeseed (canola) protein isolate heated to 72 °C. Gels were formed only at high pH (>9.5).

## 1.6. Modification of oilseed proteins for improving their functional properties

Most native proteins do not show functional properties desirable for food industries. Efforts have been made to improve the functionalities of oilseed proteins, and probably, the best documented of these efforts involve chemical and enzymatic modifications. Such modifications imply changes in both protein structure and conformation at different levels; and optimal characteristics of size, surface charge, hydrophobicity/hydrophilicity ratio and molecular flexibility of proteins can be achieved.[71]

The chemical modification that has been most used to improve functional properties of proteins from oil-producing plants is an acylation reaction (**Fig. 1.7**), where either acetic anhydride (neutral) or succinic anhydride (negatively charged) has been used (**Fig. 1.8**). These anhydrides interact with positively charged sites on the protein with the N-terminal amino group, ϵ-amino group of lysine being the most readily available. This results in a change in the net charge of the protein and at sufficiently high level of modification, denaturation of protein occurs.[17] Succinylation and acetylation have both been used to modify canola proteins with the degree of modification varying; levels up 62% acetylation or succinylation,[76] 84% succinylation[82] and 93% succinylation[79] have been used. For canola, low levels of modification led to improved foaming and emulsifying activities due to improved interfacial absorption kinetics,[79] but foam stability was adversely affected by acylation and emulsion stability decreased at higher levels of modification.[76] Heat-induced gel characteristics were also improved with the firmest gels being obtained at intermediate levels of succinylation.[82] Additional advantages of these modifications are related with delayed Maillard reactions, but the resulting derivative could be less susceptible to proteolysis.[71] In **Table 1.7**, the functional properties of canola/rapeseed proteins after chemical modification are shown.

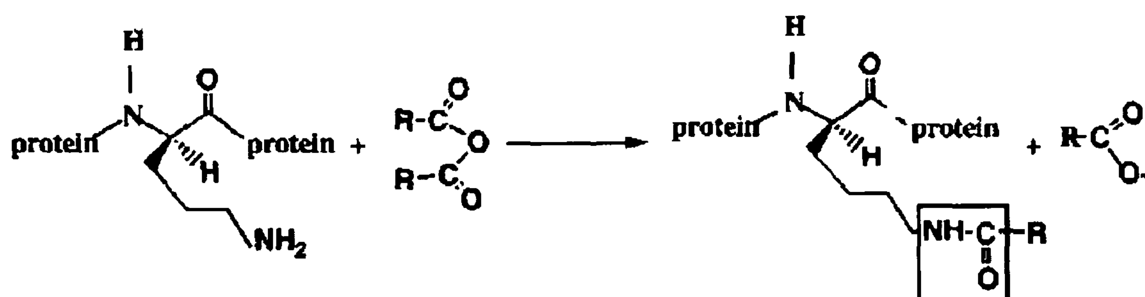



**Fig. 1.7** General scheme for protein acylation.

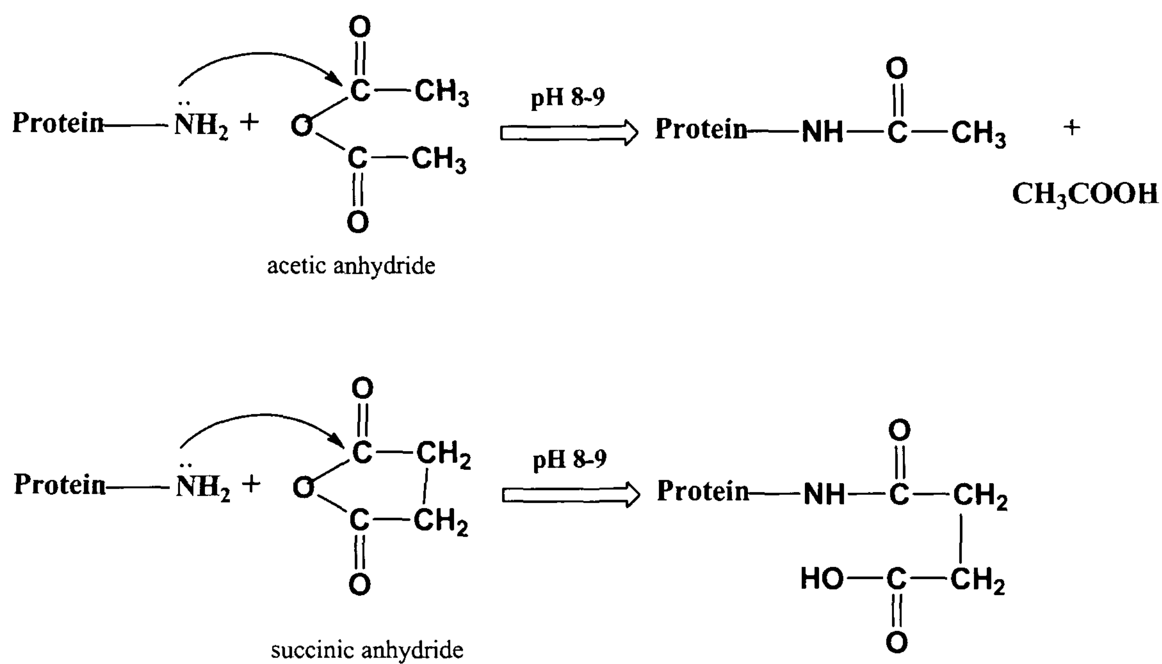



**Fig. 1.8** Schematic diagram of the acetylation and succinylation reaction of protein.

An alternative option is the enzyme modification through breakage of peptidic bonds in order to produce peptides of the desired size, charge and surface properties. A combination of endopeptidase (Alcalase) and exopeptidase (Flavourzyme), or these enzymes used individually, have been used to prepare protein hydrolysates. The main advantage of treating with these enzymes was an increase in solubility and the peptides were often found to be bioactive, having a role in disease prevention.[83] Limited hydrolysis (20%) improved foaming ability, but this advantage was reversed if the level of hydrolysis was too high.[17] While the potential for using enzymes to improve the functionality of oilseed proteins is demonstrated in many articles, application of enzymes on a large-scale may be quite expensive. Moreover, there are indications that all peptides do not contribute equally to these reported biological activities and thus the purification of active peptides is needed.[10]

### 1.7. Food applications of canola/rapeseed protein

There are a few available reports that mention about the food applications of canola/rapeseed protein concentrates or isolates.

Mayonnaise can be produced by partial replacement of the major stabilizer (egg yolk) with unmodified or enzymatically modified canola proteins.[21] This approach is mainly justified by economic considerations and possible whey/egg allergies in customers. Limited enzymatic modification permitted increased levels of canola incorporation into mayonnaise when compared to unhydrolyzed canola protein. The study suggests that limited hydrolysis can be used to produce proteins that will interact better with and form stable interfacial films around oil droplets, when compared to unhydrolyzed proteins.

Preparation of sausage with rapeseed protein concentrate has been reported by Yoshie-Stark et al.[84] An improvement in the taste, texture, and aroma was observed when steamed rapeseed protein concentrate replaced casein in the preparation of sausage.

An interesting study reported the generation of meat-like flavorings from enzymatic hydrolysates of proteins from canola/rapeseed (*Brassica* sp.).[85] The formation of meat aroma compounds was favored under lower pH conditions; at low temperatures, the aroma was

similar to that of cooked meat, and at higher temperatures, it was like roasted meat. From the various conditions studied, the best favored products were generated at 160 °C and pH 4.0.

**1.8. Other uses of rapeseed meal and its proteins**

The use of protein isolates to produce films and coatings is another area with great potential. Although rapeseed is one of the most common oilseed crops, little has been done to study its use in protein based bio-plastics or edible packaging material. **Table 1.8** lists the articles mentioning the use of rapeseed/canola meal or its protein for making bio-packaging materials. Studies relating to the improvement of performance of protein-based plastics from oilseed meal should be undertaken.

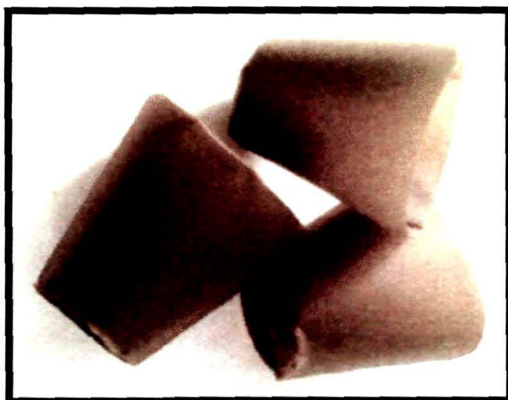


**Fig. 1.9** Plant pots prepared from a composite based on starch and rapeseed press-cake.[86]

**1.9. Future scope of rapeseed press-cake**

As the production of oilseed will continue to supply the demand for food grade oil and biodiesel production, there will be a great deal of meals available for valorization. The extraction, concentration and isolation of the protein and carbohydrates fractions from defatted meal are essential to the valorisation of the product.[2] Despite the nutritional value in proteins of oilseeds, their use in food systems may be restricted by antinutritional factors and 'less than ideal' functionality. Most of the studies highlighted the relative amount of

**Table 1.8** Use of canola/rapeseed meal or its proteins in the preparation of bio-plastics and bio-based packaging materials.

| Oilseed | Processing conditions | Features of packaging material | Reference |
|---|---|---|---|
| Rapeseed | Isolates were prepared from cold hexane defatted rapeseed meal by alkaline extraction followed by acid precipitation and ultrafiltration. Alkaline solutions (pH 12) of the isolates and PRF (32% oil) were prepared and cast into films. The PRF was further treated with supercritical CO2 to reduce the oil content and films of this material were cast in the same way. | • PRF (32% oil) material returned a water vapour permeability of 1370 $g/m^2 \cdot d$ and when deoiled with supercritical CO2 a water vapour permeability of 2230 $g/m^2 \cdot d$.<br>• Residual oil had a positive effect on water vapour permeability, although its effect on other properties was not noted.<br>• Films cast from the redissolved ultrafiltrated rapeseed isolate returned an $O_2$ permeability of 85 $cm^3/m^2 \cdot h \cdot bar$. | Wäsche et al.[87] |
| Rapeseed | 'Maehyang' strawberries were packed in rapeseed protein-gelatin (RG) film containing 1% grapefruit seed extract (GSE) as antimicrobial agent. | • Growth of pathogens like *Escherichia coli* O157:H7 and *Listeria monocytogenes* was inhibited.<br>• Populations of total aerobic bacteria and of yeast and moulds in the strawberries by 1.03 and 1.34 log CFU/g, respectively, after 14 days of storage, compared to that of the control.<br>• Sensory evaluation of the GSE-RG film-packaged strawberries produced better sensory scores than did the control. | Jang et al.[88] |
| Rapeseed | 'Seolhyang' strawberries were treated with fumaric acid (FA) and UV-C irradiation and packed with rapeseed protein-gelatin (RG) film containing 1% grapefruit seed extract (GSE). | • Initial population of total aerobic bacteria in strawberries was 2.9 log CFU/g, while that of samples treated with FA plus UV-C and packed with RG film was 1.3 log CFU/g.<br>• For yeast and mold populations, the control had 2.6 log CFU/g, while samples treated with the combined treatment had 1.0 log CFU/g. | Shin et al.[89] |
| Canola | • Meal proteins were solubilized at pH 12 (6N NaOH) and precipitated sequentially at pH values ranging from 11 to 3 in decrements of 1 pH unit.<br>• Meal flour was solubilized for 1 h (pH 12) and supernatant was precipitated at pH 5.0 (6N HCl) to get 'standard protein isolates'. To remove the hydrophilic protein fraction, protein supernatant solubilized at pH 12 was first precipitated at pH 11. After removing this fraction via centrifugation, the supernatant was again precipitated at pH 5.0 to get 'refined protein isolate'. | • Majority (76%) of the recovered proteins were precipitated at pH values at or below 7. Precipitates obtained at pH 11 showed the highest water holding capacity and lowest melting point.<br>• Bio-plastics prepared with refined protein isolates (with pH 11 fraction removed) showed higher water resistance, tensile, and flexural strength, toughness, and elongation values compared to those prepared with standard canola protein isolates. | Manamperi et al.[90] |

**Table 1.8** Use of canola/rapeseed meal or its proteins in the preparation of bio-plastics and bio-based packaging materials. (*continued*)

| Oilseed | Processing conditions | Features of packaging material | Reference |
|---|---|---|---|
| Rapeseed | • Protein (RP) was extracted from press-cake using distilled water and supernatant was freeze-dried<br>• To prepare RP film, 4% (w/v) RP was dissolved in distilled water and mixed with various amounts of emulsifier (polysorbate 20) and plasticizers (sorbitol and sucrose).<br>• RP-GC (*Gelidium corneum*, agarose-containing red algae) blend film was made by dispersing various amounts of RP and GC powder and was then mixed with 2% sorbitol, 0.5% sucrose, and 1.5% polysorbate 20 in 100-mL distilled water.<br>• RP-gelatin (RG) blend films were prepared by dispersing various amounts of RP and gelatin and were then mixed with 2% sorbitol, 0.5% sucrose, and 1.5% polysorbate 20 in 100-mL distilled water. | • RP blend films with the addition of GC or gelatin improved the physical properties of the film.<br>• Among the films, the RG blend film containing 3% RP/3% gelatin had the most desirable mechanical film property the highest tensile strength of 53.45 MPa | Jang et al.[91] |
| Rapeseed | • Starch, rapeseed cake, glycerol and urea were mixed in different ratios to form the composite.<br>• The mixtures were heated while stirring up to 85 °C during 15.20 min and stirred at this temperature for further 30 min. The films were cast from hot composite mixtures into polypropylene Petri dishes and dried in an oven at 25 °C for 48 h. | • The composite was used for the fabrication of disposable biodegradable nursery pots (**Fig. 1.9**).<br>• Their characteristics in the cultivation of tomato sprouts were studied.<br>• The evaporation of water rate from the plant pots prepared from the composites was found to be twice lower than that observed for the commercial peat plant pots.<br>• The fluctuations of the soil temperature were also lower than in the commercial peat pots.<br>• The average time of the biodegradation of the composites was about one month. | Treinyte et al.[86] |

antinutritional factors in the canola meal or its protein products; however there are not enough published data on efficient removal of such factors from the meal or its proteins. While the modified proteins have improved the possibilities for these proteins, there is a need to demonstrate how these proteins will behave in food products and what contributions they will make to these products.

Modified canola proteins have unquestionably been shown to possess improved features over the native proteins. Despite this, there are very few reports in the literature that contain detailed analyses of such modified proteins and their application for targeted food formulations. Given the essentially positive results that have arisen from a few food applications, it is important that more systematic studies are initiated to test for benefits in sensory and storage attributes of the food products supplemented with unmodified or modified canola proteins and the probable health effects upon consumption. Although, extensive literature is available on acetylation and succinylation of proteins and the characterisation of such products, however, reports on preparation and characterization of acylated protein derivatives using other dicarboxylic acid anhydrides apart from acetic and succinic anhydride are sparse.[92]

The production of films has been found to be effective for some of the oil-cake proteins. One would expect to see the outcomes of using canola/rapeseed proteins to examine the full range of properties that can be attained.

Minor components such as polyphenols, glucosinolates and phytates, that have been considered antinutritional and detrimental to protein functionality, may be recovered for use in pharmaceutical area, as these compounds have been shown to have properties that may have health benefits.[17]

Research in the nanotechnology field has skyrocketed over the last decade, and many researchers have synthesized bio-nanomaterials and their polymer nanocomposites from waste and biomass, mainly from greener sources of food industries. Lu et al.[93] reported such an endeavor from pomelo peel and the resulting carbon nanoparticle (CNP) was used for the detection of mercury ions in water. Wang et al.[94] prepared CNP from egg-shell membrane

and used it for designing a fluorescent probe for glutathione detection. The applications of nanotechnology in the food sector are only newly emerging, so it is well-known that uncertainties and lack of knowledge of potential effects, or lack of clear communication of risks and benefits, can raise concerns amongst the public. As rapeseed press-cake is rich in fibres and cellulose, its utility as a precursor of bio-nanoparticles can be envisioned, because in a closely related work, CNP was successfully prepared from used coffee grounds,[95] and its practicality in cell imaging and detection of angiotensin I and insulin was assessed. So, research should also be undertaken to explore this possibility in rapeseed press-cake, added with extensive *in vitro* and *in vivo* tests. This is a challenging but worthy concept as the use of waste materials is one of the most attractive options to reduce the raw material cost and also seems benign from ecological point of view.

The range of food products that can potentially include proteins from oil-producing plants is extensive. However, with respect to proteins for oilseeds, the term 'potential' is significant in that demonstrated uses are primarily at an experimental level and these proteins have yet to receive that commercial recognition seen by soy protein.[17] Nevertheless, for these proteins to become commercially viable, such experimental applications must be researched, mainly for improving its colour and flavour. Successful improvement of these attributes would be a crucial key to expand the spectrum of applications of canola proteins.

## 1.10. Objective of the present investigation

Keeping the current realities of the antinutritive factors and poor performance of rapeseed protein in view, along with possible benefits resulting from favorable processing of rapeseed meal, following objectives were chosen to offer a way for the best possible utilization of this industrial waste. The present thesis aimed to study the effect of different solvent systems in removing antinutrients from rapeseed meal prior to protein extraction, without affecting much of its physicochemical and functional properties. Recuperation of protein from partially detoxified meal was then optimized statistically, and the protein extracted under the suggested optimal condition was chemically modified with varying concentration of maleic anhydride (maleylation). The physicochemical, functional, *in-vitro* digestibility and cytotoxic attributes of modified rapeseed protein were compared with that of

an unmodified control. The oil-and-protein spent meal (residue left after oil and protein extraction) was further utilized as a precursor for synthesizing antioxidative, hemocompatible, fluorescent carbon nanoparticles, which can be effectively utilized as 'forgery-proof' ingredient in bio-packaging materials.

The objectives of the present thesis are as follows:

- To standardize the processing conditions for producing rapeseed meal with reduced levels of antinutritional compounds.
- To study the influence of antinutrients removal with acidified organic solvents from rapeseed meal on physicochemical and functional properties of rapeseed protein isolate.
- To optimize the process of preparing protein from treated rapeseed meal for improved yield, colour and emulsification properties.
- To analyze the physicochemical and functional properties of rapeseed protein recuperated under optimal condition and its chemically modified form (maleylation).
- To synthesize carbon nanoparticle from oil-and-protein spent meal and study its applicability in fabricating rapeseed protein-based nanocomposite film.

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# Removal of antinutrients from rapeseed press-cake with different solvent mixtures

## 2.1. Introduction

Currently there is a considerable impetus on the recovery, recycling and upgrading of wastes. Industries are more and more forced to find an alternative use for the residual wastes, because large volumes of generated bio-wastes pose potential disposal and pollution problems along with loss of valuable biomass and functional nutrients.[1] In many instances, food processing wastes might have a potential for recycling raw materials or for conversion into useful products of higher value as a by-product, or even as raw material for other industries, or for the use as food or feed/fodder. In this context, valorization of rapeseed meal is gaining special attention due to its high protein and polyphenols content. Among these, the content of phenolics in rapeseed flour is much higher than in other oleaginous seeds and amounts to about 30 times that in soybean flour.[2] Although plant polyphenols are usually regarded as valuable natural antioxidant, not all phenolics present in foods are beneficial and some may be of antinutritional concern. The presence of such high level of phenolics (6.4-18.4 mg/g rapeseed flour) or tannins (0.2-3% of defatted rapeseed meal)[3] or their oxidized products contributes to the dark colour, bitter taste and astringency of rapeseed products, lowering its palatability and being nutritionally detrimental in the diets of ruminants. Phenolic compounds have been identified as potent inhibitors of enzymes and iron ions in the gastrointestinal lumen, thus making the iron not available for absorption. In addition, adverse effects of sinapic acid (predominant phenolic acid of rapeseed flour) and tannins have been associated with the tainting of eggs, when incorporated into poultry rations.[4,5] Likewise, presence of phytates, glucosinolates, allyl isothiocyanates (AITC), etc., has thwarted the application of oilseed meal as functional food/feed additive. Phytates (5-7%) occupy the second largest position among the antinutrients in rapeseed/canola. They remain strongly associated with protein, starch and fiber matrices, and thereby reduce their digestibility. Also, they reduce the bioavailability of minerals in animal bodies mainly by chelation with divalent cations.[6]

At present there are few possibilities for the utilization of these wastes; the residues are thus disposed of in landfills or used as fertilizers. Incineration of oil-cakes

has been largely investigated but not strongly pursued due to the low calorific value and high water content.[1] In the past decade interest in the alternative use of agricultural waste streams beyond disposal or fertilization has increased drastically. Spurred by the above-mentioned factors along with high protein content, the oil-cakes were mostly used as animal feed in the past. However, in the past few years, owing to the increasing necessity to take into consideration the ill-effects of these antinutritional compounds on human/animal health and animal products, pre-treatment of the meal or waste treatment by detoxifying, neutralizing or destroying the undesirable compounds prior to feeding has been made stringent.[7] Accordingly, to produce rapeseed meal that can be incorporated into feed/food formulations, most of these compounds must be removed. Upgrading of vegetable residues to create a secondary use for the waste products has often being anchored with pre-treatment step(s), which has been designated as a key stage in the conversion of a biomass to useful products of higher value. Such pre-processing stages *must be simple and practically feasible. Certain methods (chemical treatments using* alkali or $Ca^{2+}$/ $NH_4^+$/$Fe^{2+}$ salts or heat treatment or fermentation)[6,8-11] proposed earlier for detoxifying rapeseed have neither been commercially exploited nor scaled up to practice so far, most probably due to incomplete removal, loss of useful protein, excessive cost, or tedious processing involving cumbersome and error-prone multi-steps.[12] Although there is a large body of literature focusing on the phenolic compounds in canola/rapeseed hull, knowledge of extraction of rapeseed phenolics and its other antinutritional compounds from the meal remains fragmentary and diverse. This makes the task of finding a satisfactory method very difficult and urgently needed. Moreover, no systematic attempt appears to have been made to study the simultaneous extractability of these major toxicants from rapeseed meal in a single extraction stage as a function of the composition, concentration and nature of acidic extraction solvents. The present chapter attempts such a methodical study.

## 2.2. Materials and methods

### 2.2.1. Chemicals and sample preparation

Rapeseed press-cake (from a mixture of local cultivars called "Lai behar" and "full behar") was obtained from Assam Khadi and Village Industries Board, Guwahati, India. Press-cake was ground to pass through 60 mesh size sieve, and then stored at -20 °C until use. All solvents and reagents were obtained from E. Merck® (India), of either high-performance liquid chromatography (HPLC) grade or analytical reagent grade, and were used without further purification.

### 2.2.2. Determination of the proximate composition of rapeseed press-cake

Moisture content, lipid content, crude fibre content, nitrogen free extract, ash and crude protein content (by Kjeldahl process) were determined by Association of Official Analytical Chemists (AOAC) methods.[13] Gross calorific value (digestible energy) was determined using an automatic adiabatic bomb calorimeter (Changsha Kaiyuan Instruments Co., 5E-1AC/ML).[14]

### 2.2.3. Preparation of the extracts

Meal (1 g) was treated with 10 ml of different solvents (mentioned in **Figs. 2.1a** and **2.2a**). The suspension was shaken at 213 x g for 2h (at 25±1 °C) in an orbital shaker (Sartorius Stedin Biotech, CERTOMAT® IS) and then centrifuged (SIGMA 3-18K Centrifuge) at 5367 x g for 20 min (at 4 °C). The supernatant (solvent extract) was filtered (Whatman paper no. 4) and stored at -20 °C for further analysis.

Each solvent extract was then dried under vacuum at 35±1 °C. The dried residue was dissolved in 25 ml of Milli-Q water and its pH was adjusted to neutrality (pH ≈6.8). This neutral solution was filtered through a 0.22 μm MCE syringe filter (BIOFIL), and then either used as-such for phytate and glucosinolate estimation, or extracted three times with a diethyl ether/ethyl acetate (1:1 v/v) mixture.[15] The pooled organic phase was finally reduced to dryness under vacuum using a rotary vacuum evaporator (EYELA, model CCA-1110, Japan) at 35±1 °C and was re-dispersed either in methanol (for quantification of polyphenols and DPPH-radical scavenging activity) or in dichloromethane (for AITC estimation).

The best selected solvent extract was dried under vacuum and neutralized according to the same method as described above. An aliquot of this neutralized solution was dispersed into phosphate buffer solution (PBS, pH 7.4) for hemolytic activity test. Likewise the residual pellet (meal remaining after solvent treatment) was also dried in a vacuum oven under reduced pressure (150 mmHg) at 35±1 °C for 42 h, and then extracted in PBS buffer for 2 h at 25±1 °C using an orbital shaker (213 x g). This aqueous meal extract was filtered through a 0.22μm MCE syringe filter (BIOFIL) and used for hemolytic test.

### 2.2.4. Quantification of antinutritional compounds

The amount of total phenolics (TP) in the extract was determined using the Folin–Ciocalteu reagent and sinapic acid as standard (sinapic acid equivalent, SAE) as described by Szydłowska-Czerniak et al.[16] Briefly, 0.05 ml of rapeseed extract was transferred into 10 ml calibration flask, 0.5 ml of Folin–Ciocalteu reagent added and shaken (3 min). Next, 1 ml of saturated sodium carbonate solution was added and made up to the mark with redistilled water. After 1 h, solution was centrifuged at 10,733 x g (5 min) and absorbance at 725 nm was measured against a reagent blank.

Total tannins (TT) and protein precipitable phenols (PPP) were estimated spectrophotometrically according to the protocols developed by Hagerman and Butler,[17] and Makkar et al.,[18] respectively, using tannic acid as standard (tannic acid equivalent, TAE). Two milliliters of BSA (1 mg/ml) was mixed with 1ml of the extract and allowed to incubate at 4 °C for 24 h. After centrifuging for 15 min at 3220 x g, the supernatant was discarded and the pellet was re-dissolved in 3 ml of SDS-triethanolamine solution (1% SDS (w/v) and 7% (v/v) triethanolamine in distilled water). One milliliter of ferric chloride solution (0.01M $FeCl_3$ in 0.01M HCl) was added and vortexed immediately. Absorbance was read at 510 nm after 15 min, against a reagent only blank (ferric chloride in SDS/TEA). The absorbance due to the tannin in the complex was calculated by ($A_s$ - $A_b$) X 1.5, where $A_s$ and $A_b$ are the absorbance of the sample and the blank, respectively.

Estimation of phytates was done using Wade reagent, according to the method described by Bhandari and Kawabata.[19] Results were expressed as sodium phytate equivalent (SPE) in mg per g dry meal. One milliliter of Wade reagent (0.03% solution of $FeCl_3$ containing 0.3% sulfosalicylic acid in water) was added to 3 ml of the sample solution and the mixture was centrifuged. The absorbance at 500 nm was measured using spectrophotometer, against the control (3 ml of water + 1 ml Wade reagent).

Glucosinolates content, in terms of sinigrin equivalent, was determined following the methodology developed by Tsao et al.[20] A Waters HPLC system was used, equipped with a Waters 2489 UV/visible detector and a Waters 1525 binary HPLC pump. A C18 column (5 μm, 4.6 × 250 mm, Waters, serial no. 024230005140-03, Part no. WAT054275, Ireland) was employed for analysis. The detector was set at 228 nm ($\lambda_{max}$ for sinigrin) and 20 μL of the sample was injected. The binary mobile phase was composed of 0.025M ammonium acetate (pH 6.75) (A) and acetonitrile (B). The flow rate was kept constant at 1.0 ml/min for a total run time of 12 min. The system was run with the following gradient program: 99% A/1% B (v/v) isocratic for 2 min, then linearly increased to 50% A/50% B in 0.5 min, held for 7.5 min, and then brought back to 99% A/1% B at 12 min. There was a 2-min post-run period between each sample injection.

For quantification of AITC, the protocol followed by Zhang et al.[21] was used with slight modifications. To the dried solvent extract residue (30-50 mg) were added 1 ml of water and 50 ml of dichloromethane, and then the mixture was sonicated in an ultrasonic water-bath (Labotec Inc., UK). AITC was extracted by dichloromethane from the residue for 10 min twice in the ultrasonic condition. The lower phase containing AITC was obtained by centrifugation at 6440 x g for 10 min. The content of AITC in dichloromethane was determined using ultraviolet spectrophotometry at 247.1 nm (its *maximum absorbance*).

### 2.2.5. DPPH-radical scavenging activity

One hundred microlitres of the sample was mixed with 3 ml of a methanolic solution of DPPH (0.1 mM). After 30 min of incubation in the dark (at room

temperature), absorbance was measured at 515 nm. The percentage scavenging was calculated according to the following equation:

$$\% \text{ scavenging activity} = [\{Abs_{control} - Abs_{extract}\}/Abs_{control}] \times 100 \quad (1)$$

where, $Abs_{control}$ is the absorbance of the reagent (without extract) after 30 min and $Abs_{extract}$ is the absorbance of extract after 30 min.

### 2.2.6. Hemolytic activity assay

The hemolytic test was performed following the protocol of Nair et al.[22] with slight modification. Briefly, fresh goat blood from a slaughter-house was collected in a centrifuge tube containing anti-coagulant, tri-sodium citrate (3.2%), and was centrifuged at 2500g for 10 min. The supernatant was discarded, and only the erythrocytes were collected. The erythrocytes were further washed three times with PBS (pH 7.4). A 10% (v/v) suspension of erythrocytes in PBS was prepared; 1.9 ml of this erythrocyte solution was placed in a 2 ml centrifuge tube and 0.1 ml of sample extract in PBS was added to it. The tubes were then incubated for 1 h at 37 °C. Triton X-100 (0.2%) and PBS were taken as the positive (+ve) and negative (-ve) controls, respectively, for comparison. After incubation the tubes were subjected to centrifugation at 2500g for 10 min. Then, 0.2 ml of the supernatant was added to 2.8 ml of PBS for dilution, and finally absorbance (Abs) was taken at 570 nm in a UV-visible spectrophotometer (CECIL 7400, 7000 Series, Aquarius).

$$\% \text{ Hemolysis activity} = \{(Abs_{sample} - Abs_{(-ve)\,control})/(Abs_{(+ve)\,control} - Abs_{(-ve)\,control})\} \times 100$$

### 2.2.7. Statistical analysis

All analyses were performed in triplicate, unless otherwise stated, and the mean value was calculated. Analysis of variance (ANOVA) and separation of means were carried out by Tukey's HSD test, using SPSS software (version 16.0, SPSS Inc., Chicago, USA) and considered significantly different at $p<0.05$. A multivariate analysis of the standardized data ((value-mean)/SD) was performed by Principal Components Analysis (PCA) with the STATISTICA (version 7, StatSoft, Oklahoma, USA) software package.

## 2.3. Results and discussion

### 2.3.1. Proximate analysis of rapeseed press-cake

**Table 2.1** shows the proximate composition of press-cake used in the current study. The meal contains high amount of crude protein (≈45 g/100g dry matter) and low residual oil content (11.4g/100g dry matter), indicating it to be a good source for extracting protein isolate/concentrate. This result is comparable with that of Thiyam et al.[23] Compared to the other studies,[24,25] slightly higher amount of ash content was measured (6.4 g/100g dry matter). Because of the high amount of proteins and minerals, nitrogen free extract and crude fibre were found to be only a minor part of the rapeseed cake.[24] The gross calorific value (digestible energy) of the press-cake, as determined by the bomb calorimeter was found to be 18,381.7 J/g dry matter, which is similar to those found by other investigators.[26,27]

**Table 2.1** Proximate analysis of rapeseed press-cake.

| Parameters | Mean±SD |
|---|---|
| Crude protein (g/100g dry meal) | 44.8±0.9 |
| Oil content (g/100g dry meal) | 11.4±1.2 |
| Crude fibre (g/100g dry meal) | 12.5±1.6 |
| Ash content (g/100g dry meal) | 6.4±0.5 |
| Moisture content (% dry wt. basis) | 21.9±0.6 |
| % Nitrogen free extract (by difference method) | 15.5 |
| Gross Calorific value (J/g dry meal) [a] | 18,381.7±31.7 |

[a] determined by Bomb Calorimeter. Values are mean of three replicates

Recently, Khattab et al.[28] and Thiyam et al.[29] analyzed sinapic acid derivatives of rapeseed and found numerous unidentified peaks in HPLC chromatogram due to lack of authentic standards. A method for simultaneous determinations of all rapeseed

polyphenolic compounds and their derivatives is still unknown, and therefore, it is difficult, based on available data, to select both appropriate standards and HPLC methodologies for separation and quantitation of each phenol involved.[30] So, the total polyphenol content of each solvent extract was quantified by the widely employed Folin-Ciocalteu assay, which is generally regarded as an index for overall yield.[16]

Moreover, the crude extract of the meal is expected to contain a series of oligomeric as-well-as polymeric phenolics (especially tannins), which differ in their specificity towards the reagents used for their determination.[5] This again makes the selection of appropriate methods for quantitation of tannins an intricate task. Lately attention has been focused on quantification of tannins based on their property of binding/precipitating proteins, as both the ecological and biological roles of tannins are attributed to its complexation with proteins, and it provides information which cannot be obtained with chemical assays.[17] Besides the condensed tannins, hydrolysable tannins can also precipitate proteins.[31] Therefore, in order to avoid complications associated with different classes of tannins and to distinguish polyphenols of nutritional concern from other low-molecular-weight phenolics that also occur naturally in these products, net content of tannins and protein precipitable phenols were determined by protein-precipitation assay, because this method is highly correlated with the biological value of tannin-rich food and feed.

Only one-stage extraction was performed here as Cai and Arntfield[32] and Wang et al.[33] found no statistical differences in the amount of extracted rapeseed phenolics with the number of extractions, or the extraction time.

### 2.3.2. Series I: Selection of suitable solvent(s) for simultaneous extraction of polyphenols, phytic acid and AITC

Selection of an extraction solvent is the preliminary parameter that should be critically considered before an extraction is started, as there is no single universal solvent applicable for all plant matrices. Absolute methanol or acetone or their aqueous mixture

is commonly used in rapeseed/canola polyphenol extraction.[2] Preliminary trials were performed using these solvents to determine the most efficient solvent(s) for the simultaneous extraction of TP, TT, PPP, phytates and AITC from the meal. Usually, the presence of a small amount of water along with acetone and/methanol has been reported to be essential to ensure high phenolics yield.[2] Conversely, large amount of water, e.g., >30% is undesirable, because the protein content is to some degree soluble in the water, and so a substantial amount of meal protein will be lost if too much water is used.[34] Therefore, in accordance with the common practice in the literature, aqueous organic solvent having water content >30% was not tested. **Fig. 2.1a** shows that the extraction of phenolics was dependent on the solvent used, with statistically significant differences ($p<0.05$) between the values for the sample. This variation is highly due to the relationship with variable degree of phenolic polymerization, solubility of phenolics, type of phenolic present, interaction between phenolics and other constituents in a particular extract, polarity and dielectric constant of the solvent(s) used, etc.[35,36] TP yield in absolute methanol extract (100% methanol) was the highest, which was closely followed by those in the water and 70% acetone extracts (**Fig. 2.1a**), whereas the highest yield of TT was obtained in the Me-Ac (1:1 v/v) mixture, which was followed by 100% methanol (**Fig. 2.1a**). Cai and Arntfield[32] also found refluxing with 100% methanol to be very efficient in removing phenolics from rapeseed.

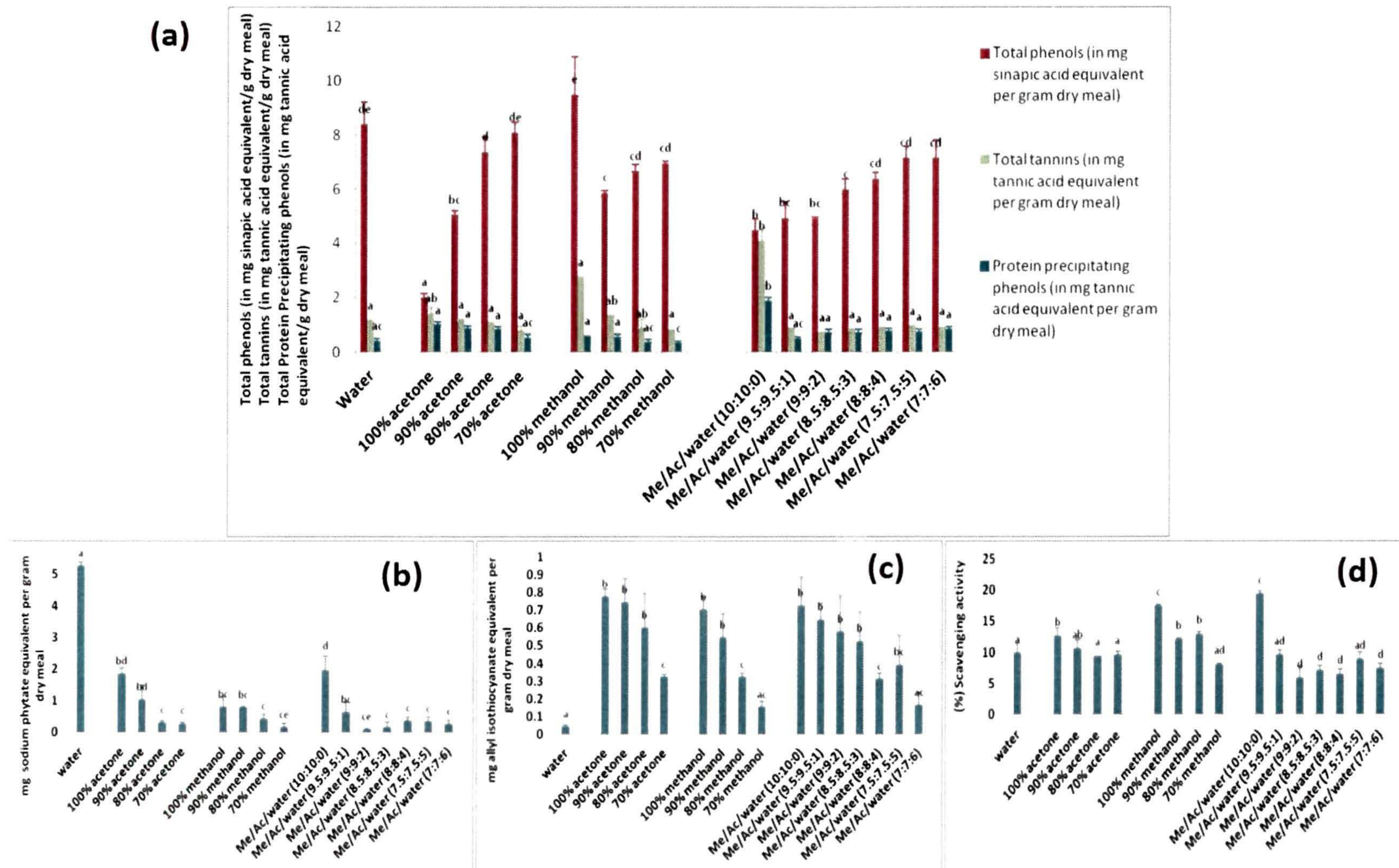


**Fig. 2.1.** Effect of absolute or aqueous solvent(s) on the yield of (a) polyphenols (b) phytic acid (c) allyl isothiocyanate (d) DPPH-radical scavenging activity. Bars with different letters indicate significant difference ($p<0.05$).

An increase in the percentage of water in methanol or acetone or Me-Ac mixture caused a progressive increase in TP yield. One of the possible reasons for this observed increasing trend may be due to the fact that aqueous methanol, due to its polarity, is more effective at extracting polyphenols linked to polar fibrous matrices. On the other hand, acetone-water mixtures are more useful for extracting polyphenols from protein matrices, because they appear to degrade the polyphenol–protein complexes.[37] As for TT extraction, irrespective of the presence of methanol or acetone, the yield decreased drastically as the water content in the extraction solvent increased. This can be explained according to the postulations of Dai and Mumper;[38] i.e., the extractability of higher molecular weight polymeric phenols decreases as the polarity of solvent increases. Wide discrepancies are apparent in the literature on rapeseed phenolics and tannin contents,[3] which may be due to the existing differences in the solvent extraction systems employed for their recovery and methods subsequently used for their quantitation;[2] hence comparison of the present values with the reported data is difficult.

The deleterious effect of tannins depends on the quantity and their protein-precipitating capacity. Naczk *et al.*[5] reported that tannins isolated from canola and rapeseed hulls exhibit a protein precipitation capacity or reduce protein digestibility by affecting enzyme activities. Therefore, PPP content in the solvent extracts also needs to be determined. The order of PPP extraction efficiency of the tested solvents was similar to that noted for TT, except that acetone-containing solvent extracts performed better than the ones obtained with methanol. Acetone is useful in dissolving the protein-bound phenolic complexes, especially less polar phenolics and highly methoxylated aglycone forms of polyphenols, and therefore considered as a good solvent to extract phenolics from protein-rich samples such as rapeseed meal.[36] Similar to our study, Naczk et al.[5] also found that the PPP correlated well with the TT content. Although the water extract showed high amount of TP, it exhibited very poor protein precipitating activity, signifying that water is not an appropriate solvent for releasing PPP from the meal. Me-Ac (1:1 v/v) extract showed the highest yield of ≈2mg TAE/g dry meal, which was found to be quite less compared to that obtained for TP and TT.

Liberation of phytates from the meal into these solvent media was also analyzed. Water extract showed the highest yield of phytic acid (**Fig. 2.1b**). This is due to the fact that water leaches out a large quantity of meal protein and sugars, onto which the oilseed phytates are mostly attached.[39] Although the use of water for extracting phytic acid seems lucrative, it causes heavy loss of meal protein, which is highly valued in food and cosmetic industries. Moreover, if any extended application or assay of this water extract is desired, removal of unwanted proteinaceous matter from the extract would require additional processing, thereby making this solvent less attractive for further use. Following the water extract, the Me-Ac (1:1 v/v) extract presented the second highest phytate yield, which in-turn is closely followed by the 100% acetone extract (**Fig. 2.1b**). A general decreasing trend in phytate yield was observed with increasing water content in the organic solvents or their mixtures. This may be due to the release of more phytate-related phospholipids into the organic solvents than in the aqueous ones. Phytates have been reported to show a high correlation with organic phosphorus content of many fruits, vegetables, crops, etc.[40] Varying the percentage of water in aqueous Me-Ac mixture gave comparable phytic acid yield ($p>0.05$), so the presence of water gave no significant improvement.

*Brassica* and other cruciferae have long been known to contain high levels of undesirable thioglucosides commonly known as glucosinolates. In the current study, glucosinolate content in the meal and the solvent extracts was found to be below the detection level (<0.15 μg sinigrin equivalent/g dry meal or per ml of extract), which perhaps may be due to its autolysis by the endogenous myrosinase enzyme liberated alongside during cold-pressing of seeds for oil extraction. A similar observation was noted by Xu et al.[41] in yellow mustard meal. Tsao et al.[42] showed that sinigrin dissolved into aqueous media from ground mustard seed, was very quickly degraded by co-dissolved myrosinase into AITC. This seems to be the plausible cause for the much reduced glucosinolate content in all the solvent extracts and the meal. Varying manifestations of toxicity, from depressed weight gain to enlarged thyroids and kidneys to carcinogenesis to death, are observed in rats and other species ingesting glucosinolate

hydrolytic products.[43] Consequently, the major hydrolytic product of glucosinolates i.e. AITC was estimated. AITC yields of different extracts are presented in **Fig. 2.1c**. It is clear that owing to the non-polar nature of AITC, its solubility and hence the yield was more facilitated in organic solvents, devoid of water, than in the aqueous ones. AITC content perpetually declined in aqueous solvents. This remains true, no matter what the solvent used.

Rapeseed polyphenols, AITC and phytates have been well-recognized as strong antioxidants. With a premonition to find the antioxidative power of these solvent extracts, DPPH-radical scavenging activity was performed. The model using scavenging of the stable DPPH-radical is a widely used assay to evaluate antioxidant activity in a relatively short time compared with other methods. The decrease in absorbance occurs when the DPPH-radical accepts an electron or hydrogen from an antioxidant. The radical becomes a stable molecule that is visually noticeable as a colour change from purple to yellow. The percent scavenging activities of the crude extracts toward DPPH-radical are depicted in **Fig. 2.1d**. Comparable scavenging activity has been achieved for acetonic and methanolic extracts (except 100% methanol). As can be seen from the results, the highest variations were found in the aqueous Me-Ac (1:1 v/v) extracts. Extract from Me-Ac (1:1 v/v) mixture showed the highest scavenging activity, which may be ascribed to its high TT and PPP contents.

### 2.3.3. Series II: Augmenting the solvent efficacy using hydrochloric acid (HCl), acetic acid ($CH_3COOH$), perchloric acid ($HClO_4$), trichloroacetic acid ($CCl_3COOH$) and phosphoric acid ($H_3PO_4$)

Many authors have stated that the most efficient extraction of polyphenols and phytates can be achieved by using acidic solvent. Since the prime focus of this work was to increase the effectiveness of commonly used extraction solvent(s) for removing antinutritional antioxidants from rapeseed meal, the second step of the preliminary study (Series II) was the selection of the most appropriate solvent system from Series I, and then the addition of various concentrations of different acids into it, for further boosting its efficiency to extract TP, TT, PPP, phytates and AITC from the meal. Finally,

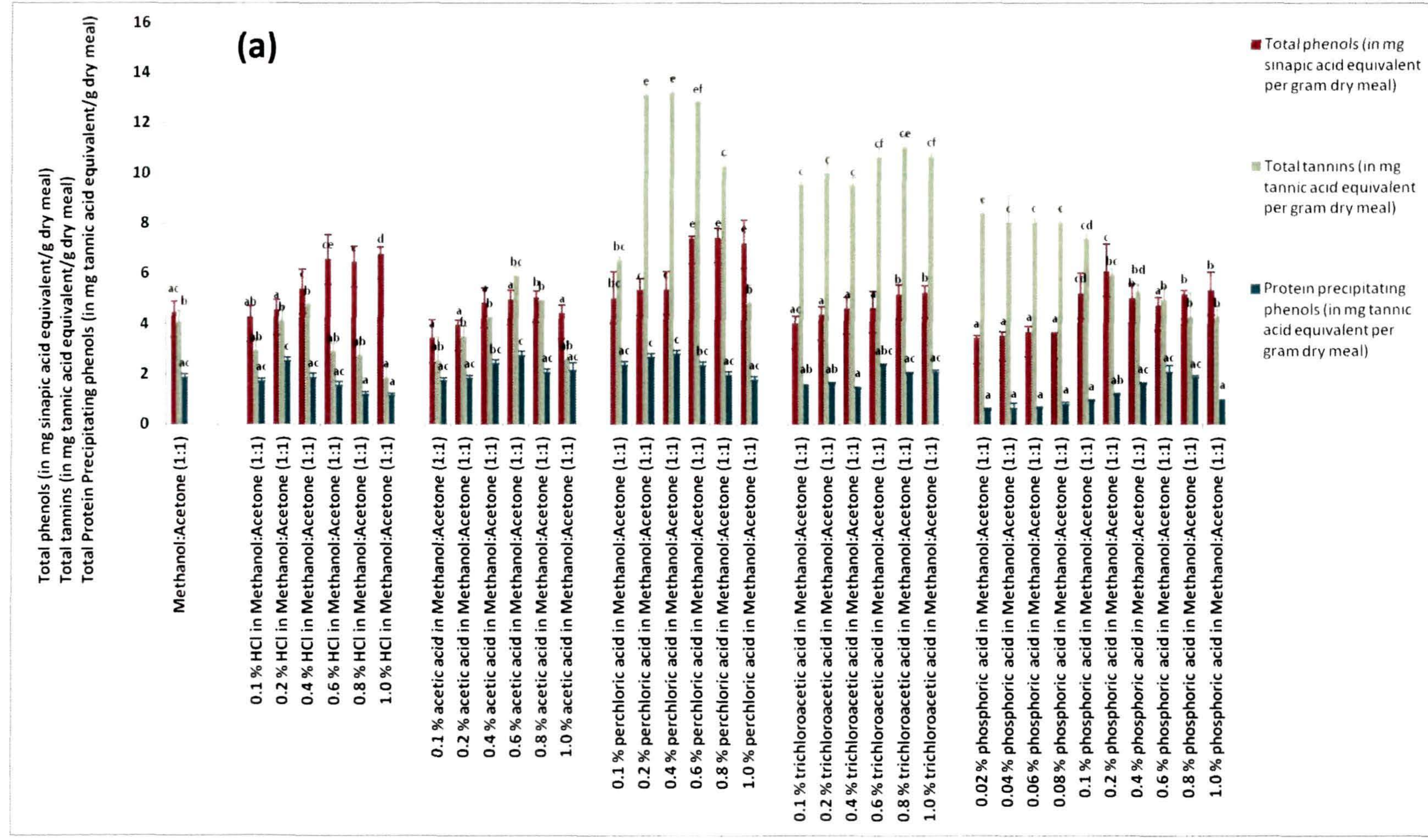


**Fig. 2.2a** Effect of acidified solvent mixture on the yield of polyphenols from rapeseed meal. Bars with different letters indicate significant difference ($p<0.05$).

enhancement or reduction in DPPH-radical scavenging potency of these acidified solvent extracts was also assessed. Me-Ac (1:1 v/v) mixture was selected for Series II, as it showed the highest yield of TT, PPP and scavenging activity, and fairly good yield of TP, AITC and phytates, among all the tested solvents of Series I. Our choice partly corroborated with that of earlier workers; Naczk et al.[44] used a mixture of acetone/methanol/water (7:7:6 by volume) to obtain a high amount of phenolics from rapeseed/canola, whereas, Krygier et al.[15] employed 70% methanol-70% acetone (1:1) to do the same. Based on the implication of Dai and Mumper,[38] concentration of acids above 1% in Me-Ac (1:1 v/v) mixture, was not tested in the current study.

On average, solvent extract containing higher concentration of acids (≥0.6%) gave higher TP yield, especially HCl and $HClO_4$ (**Fig. 2.2a**). The most efficient solvents were 0.6%—1.0% $HClO_4$ in Me-Ac (1:1 v/v), presenting ≈7.42—7.21 mg SAE/g dry meal ($p>0.05$). Likewise, the TT extractability remarkably increased in $HClO_4$, $CCl_3COOH$ and $H_3PO_4$ containing solvent systems (**Fig. 2.2a**). In case of TT yield, the 0.4% $HClO_4$ in Me-Ac (1:1 v/v) proved the most efficient solvent (≈13.18 mg TAE/g dry meal), followed by 0.2% $HClO_4$ in Me-Ac (1:1 v/v) and 0.6% $HClO_4$ in Me-Ac (1:1 v/v) (≈13.07 and ≈12.75 mg TAE/g dry meal, respectively). An increasing trend in TT yield was observed with increasing concentration of $CCl_3COOH$ in solvent mixture (**Fig. 2.2a**). A low concentration of $H_3PO_4$, ranging from 0.02% to 0.1%, was found to be suitable for tannin extraction. It can be inferred that acid breaks the molecules liberating the bound phenolic compounds from the plant matrices. The presence of an acid in the solvent increases the extraction capacity as-well-as the rate of extraction of these desired compounds.[45] However, the acidity of the extraction solvent may cause hydrolysis of some labile phenolic compounds and/or may affect the chemical modification of these compounds, thereby changing their sensitivity to the reagents used in the assays.[38] The latter may be one of the probable reasons for explaining the decreasing trend of TT yield vis-a-vis the perpetually increasing trend of PPP yield in the extracts having ascending concentration of $H_3PO_4$ (**Fig. 2.2a**), although TT and PPP were expected to show correlation as predicted by many authors.

As can be seen from **Fig. 2.2a**, the highest variations in the PPP yield were found in the $CH_3COOH$ and $CCl_3COOH$ containing solvent mixtures. The variations may be attributed to polarities of different compounds present in the meal. Lower concentration of $H_3PO_4$ (<0.2%) was found as not very suitable for extracting PPP. Likewise very high concentration of acids also gave poor result. This can be attributed to the possible degradation of some labile phenolics under such harsh conditions or there is a possibility of the formation of insoluble-coloured decomposition products in the extracts having high acid level,[46] which may not be able to form complexes with soluble protein during the assay. The best result was obtained with 0.2%-0.4% $HClO_4$. These results were similar to those obtained with 0.2% HCl and 0.6% $CH_3COOH$, and slightly higher than that obtained for an extraction with Me-Ac (1:1 v/v).

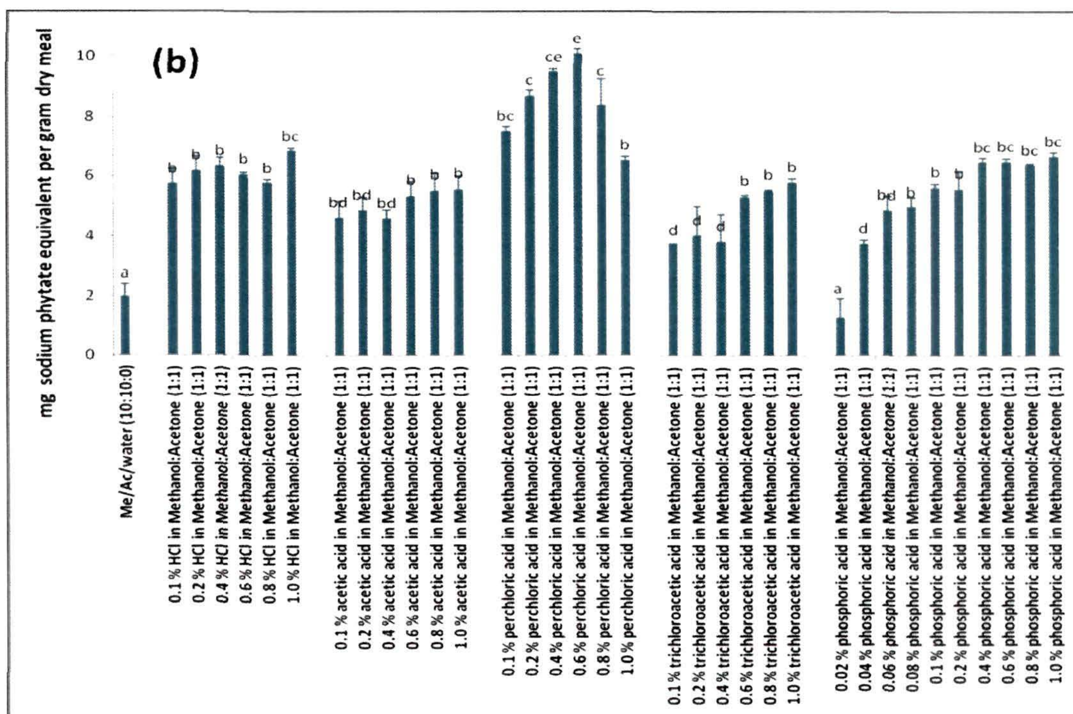


**Fig. 2.2b** Effect of acidified solvent mixture on the yield of phytic acid from rapeseed meal. Bars with different letters indicate significant difference ($p<0.05$).

The content of phytic acid in the acidified solvent mixtures increased greatly compared to those in non-acidified solvent systems (**Figs. 2.1b** and **2.2b**). Usually, phytates are extracted with the aid of an acid. Solvents having $HClO_4$, HCl and $H_3PO_4$ were more efficient in phytate extraction than $CH_3COOH$ and $CCl_3COOH$ containing ones (**Fig. 2.2b**). Levels of 0.6%, 0.4% and 0.2% $HClO_4$ in Me-Ac (1:1 v/v) were the most efficient solvents resulting in ≈10.06, ≈9.45 and ≈8.66 mg SPE/g dry meal, respectively. Acid concentration dependent phytic acid yield was observed in the $CCl_3COOH$ and $H_3PO_4$ acidified solvents; application of higher concentration of acid gave comparatively higher yield. This may be due to the acidic hydrolysis of linkages existing between phytate molecules with other plant components.[47] Phytate contents of solvent extracts containing HCl were comparable between themselves and the $CH_3COOH$ containing ones.

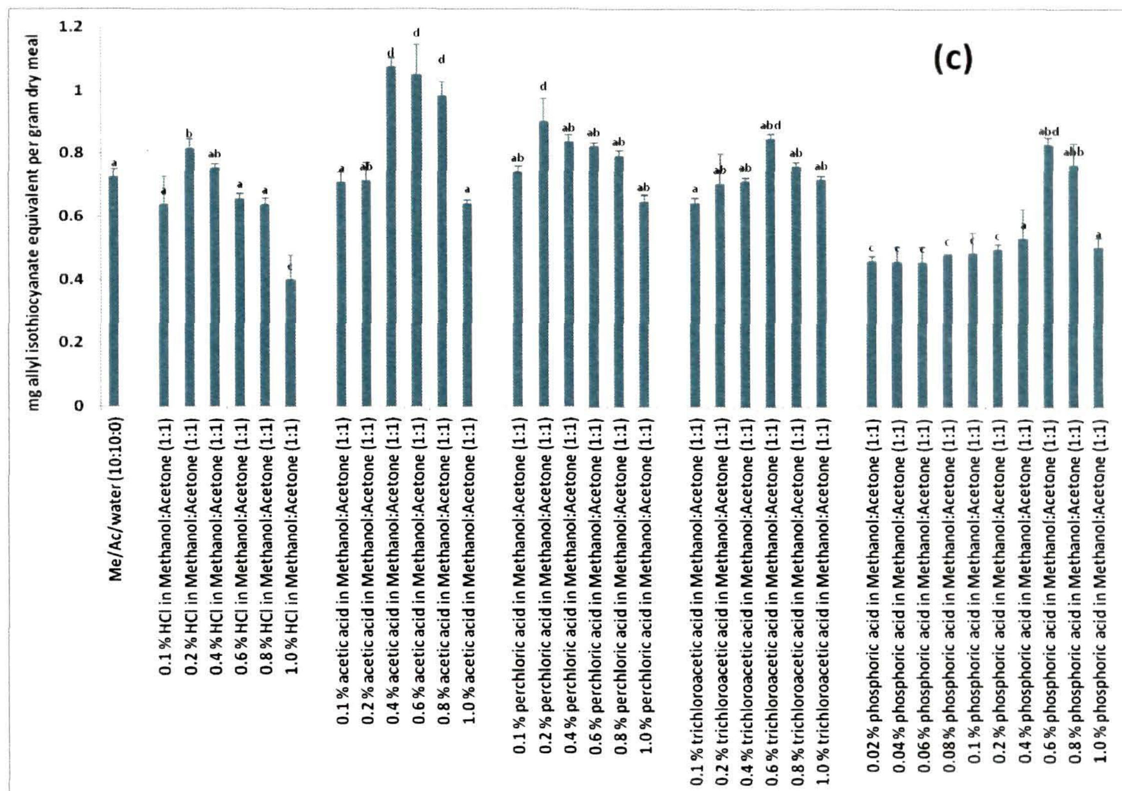


**Fig. 2.2c** Effect of acidified solvent mixture on the yield of allyl isothiocyanate (AITC) from rapeseed meal. Bars with different letters indicate significant difference ($p<0.05$).

Regarding the AITC content, the extracts prepared from 0.4%-0.8% $CH_3COOH$ contained substantially higher amounts compared to other solvents (**Fig. 2.2c**). Sinigrin and AITC are potentially prone to degradation if exposed to ambient conditions and pH<5.[42] This may be one of the possible explanations for the decreasing trend in AITC yield noted in the case of acidified solvents, particularly when HCl, $CH_3COOH$ and $HClO_4$ were used at a concentration of ≥0.2%. Some exceptions were observed in case of $CCl_3COOH$ and $H_3PO_4$ containing solvents; even so their concentration >0.6% pointed towards pretty much alike decreasing tendency.

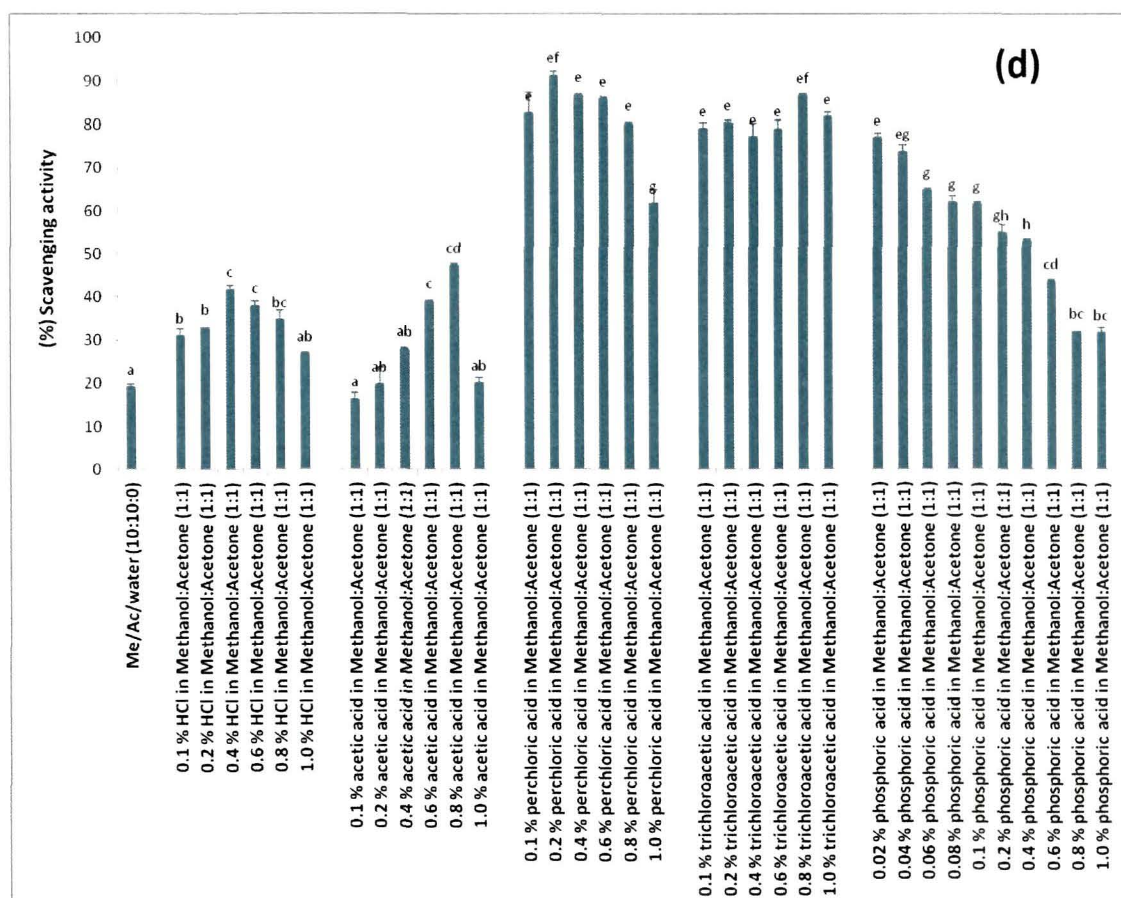


**Fig. 2.2d** The DPPH-radical scavenging activity of the different solvent extracts from rapeseed meal. Bars with different letters indicate significant difference (p<0.05).

The different acidified solvents clearly influenced the antioxidant activity of the extracts (**Fig. 2.2d**), with statistically significant differences ($p<0.05$) between them. $HClO_4$ and $CCl_3COOH$ containing mixtures gave much higher scavenging activity than $H_3PO_4$ containing ones, which can mainly be attributed to the higher quantities of tannins and phytic acid in these extracts. Moreover, in the extracts obtained with $H_3PO_4$, the scavenging effect decreased with the increasing acid concentration, indicating the hydrolysis of the antioxidative compounds; whereas the opposite was observed in the extracts having $CH_3COOH$. For HCl acidified solvents, a comparatively higher activity was observed at intermediate acid levels. In HCl and $CH_3COOH$ containing mixtures, the scavenging activities were significantly lower compared to the other acidified mixtures. Nevertheless, the DPPH-radical scavenging activity of all the tested acidified solvent systems was found to be either much higher or comparable with that of Me-Ac (1:1 v/v). There may be 2 probable reasons behind the increased antioxidant activity of the acidified Me-Ac (1:1 v/v) extracts than the non-acidified one: the higher dissolubility of phytates, AITC and polymeric phenols in acidic media imparted more DPPH-radical scavenging, or the use of acids in extraction media hydrolyzed the sinapine and sinapic acid derivatives from the meal into free sinapic acid, which is reported to have very high antioxidant potential.[29]

### 2.3.4. Principal Component Analysis (PCA)

In order to find the most appropriate solvent system for obtaining higher yield of the selected antioxidative phytochemicals (polyphenols, AITC and phytates) from rapeseed meal concomitantly in a single run, PCA was carried out using data listed in **Fig. 2.2**. By the cross-validation technique, it was established that three PCs were significant (Eigen value >1.0) for explanation of total cumulative variability of 92.24%; PC1, PC2 and PC3 accounted for 42.93%, 28.70% and 20.61% of total variability, respectively (**Table 2.2**).

The PCA plots (PC1xPC2) provided an overview of the similarities and differences between samples and their selected variables. All the studied parameters (variables) fell in the right-hand area of PC1 (located on 1$^{st}$ and 2$^{nd}$ quadrants) (**Fig.**

**2.3a**). Being clustered tightly together, the loading plot (**Fig. 2.3a**) clearly showed that the DPPH-radical scavenging activity of the extracts was positively correlated to tannin content (Pearson correlation coefficient, r=0.92). Other parameters also exhibited similar close associations among themselves; however Pearson correlation coefficients were found to be <0.9. To understand more about the relationship between the variables and PCs, Eigen vectors were studied.[48] Among the Eigen vector values (**Table 2.3**), those showing absolute values >0.1 were adopted to explain the projection of the samples on the factor-plane.[48] PC1 was positively correlated with all the studied variables. PC2 showed positive correlation with TP, PPP, phytates and AITC content, and negative correlation with TT and DPPH scavenging activity. PC3 showed high negative correlation with TP and marginal association with the others.

The score values for the first two PCs (PC1 and PC2) are often used to represent the characteristics of the samples. The score plots (**Fig. 2.3b**) enabled differentiations among quality characteristics of the extracts tested. It was noticeable that the 0.2%, 0.4% and 0.6% $HClO_4$ containing solvent extracts were closely clustered separately from all of the other samples in the 1<sup>st</sup> quadrant of the plot, indicating their similarity in overall extraction efficiency. It is also clear that processing with these solvent systems generally results in a shift closer to the studied variables (towards the right-hand side of the plot), i.e., these solvent extracts reflects higher amount of polyphenols, phytates, AITC and DPPH activity compared to the others. The separated locations of these extracts in the multivariate space clearly revealed differentiations between the large numbers of samples and also helped in drawing a compromise between the partially incommensurable quality traits of these solvent extracts. Out of these three solvent systems, extract from 0.2% $HClO_4$ in Me-Ac (1:1 v/v) can be selected for further use because of practical reasons (acids should be used at the lowest possible concentrations) and for its highest DPPH activity. Therefore, the use of multivariate tool (PCA) enabled us to comprehensively and systematically assess the potentiality of the solvent(s) that need to be used in the processing industries.

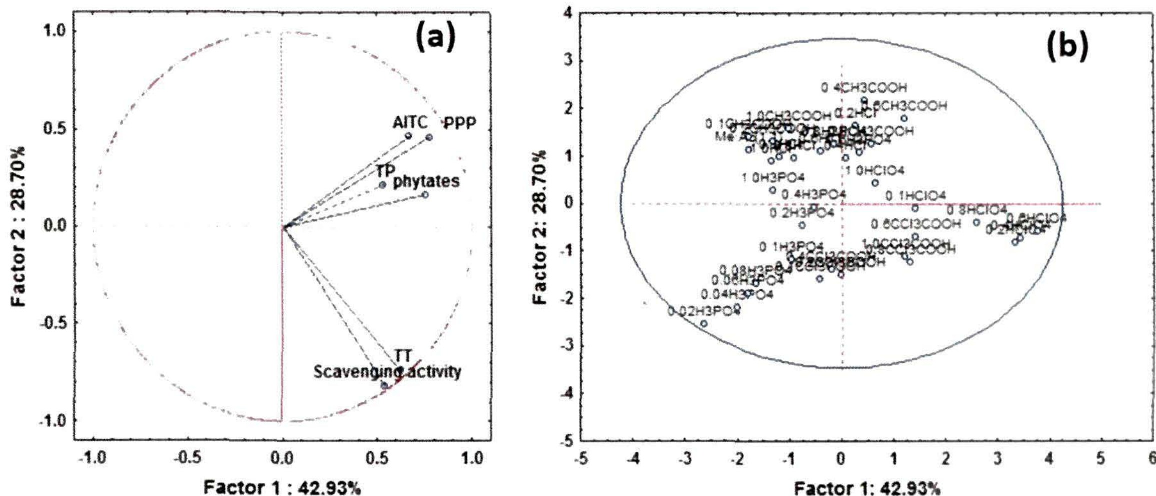


**Fig. 2.3** Distribution of (a) variables (loading plot) and (b) solvent extracts (score plot) on the multivariate domain (PC1xPC2).

**Table 2.2** Eigen-values of correlation matrix and related statistics.

| Principal Component | Eigen-value | % Total variance | Cumulative Eigen-value | Cumulative (%) |
|---|---|---|---|---|
| 1 | 2.576 | 42.928 | 2.576 | 42.928 |
| 2 | 1.722 | 28.698 | 4.298 | 71.626 |
| 3 | 1.237 | 20.614 | 5.534 | 92.240 |
| 4 | 0.317 | 5.282 | 5.851 | 97.522 |
| 5 | 0.090 | 1.506 | 5.942 | 99.028 |
| 6 | 0.058 | 0.973 | 6.000 | 100.00 |

**Table 2.3** Eigen-vectors of correlation matrix.

| Variables | PC1 | PC2 | PC3 | PC4 | PC5 | PC6 |
|---|---|---|---|---|---|---|
| Total phenolics (TP) | 0.332 | 0.161 | -0.664 | 0.629 | 0.103 | 0.132 |
| Total tannins (TT) | 0.388 | -0.564 | 0.168 | 0.001 | -0.175 | 0.688 |
| Protein precipitating phenols (PPP) | 0.484 | 0.351 | 0.328 | -0.139 | 0.709 | 0.116 |
| Phytic acid | 0.470 | 0.122 | -0.451 | -0.682 | -0.284 | -0.125 |
| DPPH radical scavenging activity | 0.336 | -0.626 | 0.040 | 0.132 | 0.196 | -0.662 |
| Allyl isothiocyanate (AITC) | 0.416 | 0.356 | 0.468 | 0.321 | -0.580 | -0.205 |

### 2.3.5. Hemolytic activity assay

With the anticipation of utilizing such high antioxidative plant extract for food or pharmaceutical or cosmetic application, the hemocompatibility of such an extract was first tested, as it would eventually come in direct/indirect contact with blood as soon it is exposed to the host's body. The 0.2% $HClO_4$ containing Me-Ac (1:1 v/v) solvent extract was selected for this assay, as it showed the highest reductive potential (DPPH-radical scavenging activity). Since this extract also contains high concentrations of phytates, AITC and polyphenols, and could therefore be considered as potentially more hemo-toxic; its impact was checked by erythrocyte hemolytic assay taking PBS and TritonX-100 as negative and positive controls, respectively (**Fig. 2.4**). Hemolytic activity of the solvent extract was found to be ≈65.89%, which is much higher than that observed for negative control (**Fig. 2.4**). Thus the compounds present in the extract were found to be toxic and non-compatible with blood as they caused lysis of the erythrocytes. This hemolytic assay is crucial and provides an obvious reason for removing such harmful phytochemicals from rapeseed meal before it can be used as a food/feed item.

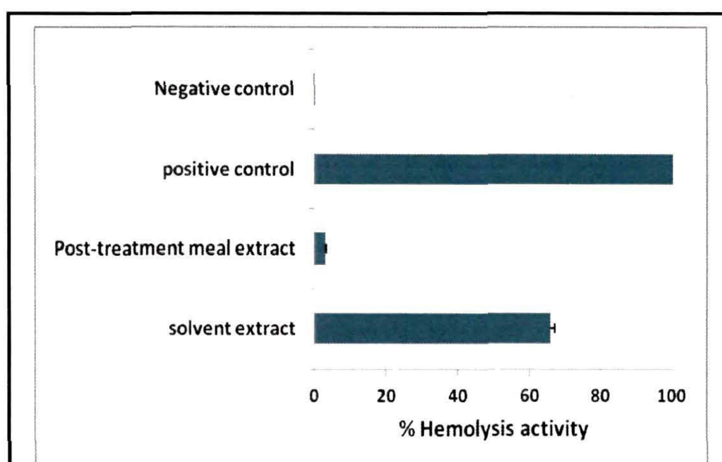


**Fig 2.4** Percent Red Blood Corpuscles hemolytic activity of the 0.2% $HClO_4$ containing Me-Ac (1:1 v/v) solvent extract and its treated meal.

Furthermore, the meal remaining after the treatment with the 0.2% $HClO_4$ containing Me-Ac (1:1 v/v) solvent was extracted with PBS buffer, and the buffer-extract of treated meal (post-treatment meal extract) was also tested for hemolytic activity, in order to assess the suitability of this solvent-treated meal as food/feed-stuff. The activity of the post-treatment meal extract (≈3.15%) was much

reduced (a 20.9 fold) compared to that of solvent extract, which again vouched the fact that the treated meal contained very little or negligible amount of residual antinutrients, and hence can be recommended for feed/food purposes.

Moser[49] used toxic gossypol from cottonseed for increasing the resistance of biodiesels against oxidation. So, considering the high reductive potential and toxicity of the solvent extracts, it is feasible to believe that the antinutrients from rapeseed press-cake may hold a lot of promise for as antioxidant for non-food items.

## 2.4. Conclusion

Arising from this work, we can conclude that the antinutrients of rapeseed meal can be effectively reduced by using organic solvent mixtures, containing suitable level of acid. The results can contribute to the selection of the most efficient extraction solvents to be used when removal of the above-mentioned antinutritive compounds are to be made. The characteristic differences in the efficacy of these wide varieties of solvents were studied by principal components analysis, based on which the mixture 0.2% perchloric acid in methanol-acetone (1:1 v/v) was deemed as 'the best' for detoxification of rapeseed meal. The high reductive potential (excellent DPPH-radical scavenging activity) of such solvent extract is attributable to the myriad of phytochemicals present in it; however, it cannot be recommended for food or drug application due to its high toxicity.

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# Influence of antinutrients removal with acidified organic solvents from rapeseed meal on physicochemical and functional properties of meal protein

## 3.1. Introduction

The usefulness of rapeseed protein is mainly restricted by the presence of undesirable components such as polyphenols, phytates, allyl isothiocyanates (AITC) and glucosinolates. Despite the introduction of rapeseed varieties with very low glucosinolate content (i.e., canola), the use of rapeseed meal as a source of food protein is still thwarted by the presence of high amounts of polyphenols and phytates. These antinutritional compounds must be removed from the meal and its protein products as-much-as possible prior to human consumption. To date, several works in the literature, addressing the removal of antinutrients from rapeseed meal or its protein preparations have been available, but all reported problems with incomplete removal, loss of protein, or excessive cost.[1] Moreover, knowledge of the influence of varied extraction techniques and purification methods on structural and functional properties of rapeseed/canola proteins has either not been fully understood or is inconsistent on the basis of different works.

The choice of extraction solvent(s) usually comes from a systematic study of several mixtures because the solvent employed must allow extraction of the principal compound(s) of interest, concurrently avoiding chemical changes in the resulting protein and/or solubilizing proteins to reduce the losses. However, appropriate processing treatments that lead to structural modification can often improve functional properties and enhance utilization of proteins as ingredients in food industries.[2] Rapeseed press-cake valorization has been the focus of our ongoing research. To complete our previous work regarding antinutrients extraction from rapeseed press-cake using various solvent systems,[3] the work reported in this chapter focuses on the investigation of the influence of the removal of antinutrients from rapeseed flour on the physicochemical and functional properties of rapeseed protein.

## 3.2. Materials and methods

### 3.2.1. Chemicals and sample preparation

Rapeseed press-cake was obtained from Assam Khadi & Village Industries Board, Guwahati, India. Ground press-cake was passed through 60 mesh size sieve, and then stored at -20°C until use. All solvents and reagents were obtained from E. Merck® (India), of either high-performance liquid chromatography (HPLC) grade or analytical reagent grade, and were used without further purification.

Based on the results obtained in the earlier chapter (Chapter-2), solvents for detoxification of rapeseed meal were deemed 'acceptable' when the simultaneous extractability of all the known antinutrients (i.e., total phenols, total tannins, protein-precipitating phenols, phytates, glucosinolates and AITC) from the meal by the solvent is either equal to or higher than that of the solvent system having only methanol and acetone mixture (Me-Ac, 1:1 v/v). This criterion was fulfilled by 15 different solvent systems containing Me-Ac (1:1 v/v) in combination with different acids: 0.1, 0.2, 0.4, 0.6 or 0.8% perchloric acid ($HClO_4$); 0.6, 0.8 or 1.0% trichloroacetic acid ($CCl_3COOH$); 0.4, 0.6 or 0.8% acetic acid ($CH_3COOH$); 0.6 or 0.8% phosphoric acid ($H_3PO_4$); and 0.2 or 0.4% hydrochloric acid (HCl). As such, samples used for the preparation of protein isolates included (i) meal without any solvent treatment as control (untreated), (ii) meal treated with diethyl ether for removing lipids (defatted), (iii) defatted meal treated with Me-Ac (1:1 v/v) without any added acid, and (iv) defatted meal treated with Me-Ac (1:1 v/v) added with various concentrations of different acids as stated above.

### 3.2.2. Preparation of solvent-treated meals

Meal (1 g) was treated with 10 ml of different solvents (mentioned above) according to the protocol mentioned in Chapter-2. Treated meals were dried in a vacuum oven at 40 °C for 72 h.

### 3.2.3. Preparation of protein isolate from untreated or solvent-treated meals

Meal (20 g) was extracted with water (meal:solvent ratio=1:20 (w/v)) at room temperature for 1 h under constant agitation (213 x g) in an orbital shaker (Sartorius Stedin Biotech, CERTOMAT® IS) and then centrifuged at 5367 x g for 20 min at 4 °C

(SIGMA 3-18K Centrifuge) to get aqueous extract. The pellet was re-extracted as before with NaOH solution at pH 11. Both the extracts were combined, to which ammonium sulfate was added up to 85% saturation.[3] The resulting precipitate was redispered in water, adjusted to pH 7, dialyzed against Milli-Q water and finally freeze-dried to obtain rapeseed protein isolate (RPI).

The respective meal and its corresponding isolate were denoted on the basis of the solvent used for their processing (mentioned in **Figs. 3.2a** and **3.2b**). Protein purity of all the isolates was above 80% (dry basis), based on Kjeldahl nitrogen (Nx6.25). The yield was calculated as follows:

$$\%\ \text{protein yield} = \frac{\text{weight of protein isolate} \times \%\ \text{protein in isolate}}{\text{weight of starting meal} \times \%\ \text{protein in starting meal}} \times 100 \qquad (1)$$

### 3.2.4. Quantification of antinutritional compounds

Protocols for estimating phytates, glucosinolates and AITC are same as those reported in earlier chapter (Chapter-2). Sinapine content was determined according to the method developed by Wanasundara et al.[4] Freeze-dried protein was extracted with 99.8% methanol (1:20, w/v) in a round-bottom flask for 48 h with stirring. The extract was filtered through cotton wool, concentrated under vacuum, to which internal standard (N,N-Dimethyl formamide) was added and then re-suspended in deuterium oxide (1 ml). Proton NMR signals were recorded at 400 MHz using a JEOL $^{1}$H-NMR system. Singlet peak at $\delta$=3.25 ppm was identified as phenylpropanoid ester (sinapic acid ester or sinapine).

### 3.2.5. Analytical methods

*(a) Hunter Colour parameters*

Colour was determined with Hunter Lab colorimeter (Ultrascan, VIS-Hunter Associates Lab., USA). The instrument (including 65°/0° geometry, D25 optical sensor, 10° observer, specular light) was calibrated using white and black reference tiles

provided by the manufacturer. Majority of the isolates displayed a similar off-white colour (**Fig. 3.1**). However upon dissolution in water, their solution showed a light brown colour of different intensities; an observation that was consistent with a previous report.[5] So, following the protocol of Xu and Diosady,[5] colorimetric evaluation was performed by scanning their aqueous solution (7 mg/ml). Measured values were expressed as L (lightness), a (redness/greenness), b (yellowness/blueness) colour units. Whiteness was calculated as follows:[6]

$$\text{Degree of Whiteness} = 100 - \left[(100 - L)^2 + a^2 + b^2\right]^{1/2} \quad (2)$$

*(b) Ultraviolet (UV)-Visible (vis) spectroscopy*

UV-vis spectroscopy was performed on a UV-vis CECIL 7400 spectrophotometer (Aquarius, 7000 series) by scanning aqueous protein solution (2 mg/ml).

*(c) Free sulfhydryl group (SH) and disulfide bond (SS)*

Free sulfhydryl group (SH) and disulfide bond (SS) were assayed by the method of Tang et al.[7] and Deng et al.[8] Protein samples (75 mg) were dissolved in 10 ml of Tris-Gly buffer (0.086M Tris, 0.09M glycine, and 0.04M EDTA, pH 8.0) containing 8M urea. The solution was gently stirred overnight until a homogeneous dispersion was achieved. For SH content determination, 4 ml of the Tris-Gly buffer was added to 1 ml of protein solution. Then, 0.05 ml of Ellman's reagent (5,5'-dithio-bis-2-nitrobenzoic acid in Tris-Gly buffer, 4 mg/ml) was added, and absorbance was measured at 412 nm after 5 min. For total SH content [SH + reduced SS] analysis, 0.05 ml of β-mercaptoethanol (β-ME) and 4 ml of Tris-Gly buffer were added to 1ml of the protein solution. The mixture was incubated for 1 h at room temperature. After an additional hour of incubation with 10 ml of 12% trichloroacetic acid (TCA), the mixtures were centrifuged at 5000g for 10 min. The precipitate was twice resuspended in 5 ml of 12% TCA and centrifuged to remove β-ME. The precipitate was dissolved in 10 ml of Tris-Gly buffer. Then 0.04 ml of Ellman's reagent was added to 4 ml of this protein solution, and the absorbance was measured at 412 nm after 5 min. The calculation was as follows:

$$\mu M\ SH/g\ protein = (73.53 \times A_{412} \times D)/C \quad (3)$$

$$\mu M\ SS/g\ protein = (SH_{total} - SH_{free})/2 \quad (4)$$

where $A_{412}$ is the absorbance at 412 nm, C is the sample concentration (mg/ml), D is the dilution factor, 5 and 10 are used for SH and total SH (SH + reduced SS) content analysis, respectively, and 73.53 is derived from $10^6/(1.36 \times 10^4)$; $1.36 \times 10^4$ is the molar absorptivity, and $10^6$ is for the conversion from molar basis to μM/ml basis and from mg solids to g solids.

*(d) Surface hydrophobicity* ($S_o$)

Surface hydrophobicity ($S_o$) was measured by using the SDS binding method,[9,10] with slight modification. Sodium dihydrogen phosphate dihydrate buffer (0.02 mol/L, pH 6.0) was prepared in demineralized water. Proteins were soluted in sodium dihydrogene phosphate buffer (w/v = 1.0 g/litre). SDS reagent was prepared in sodium dihydrogen phosphate buffer (w/v = 40.37 mg/litre). Methylene blue was dissolved in sodium dihydrogen phosphate buffer (w/v = 24.0 mg/litre). Protein solution and SDS reagent were mixed (v/v = 1/2) and incubated for 30 min at 20°C. Subsequently, the SDS-protein solution was dialyzed against sodium dihydrogen phosphate buffer (v/v = 1/25) for 24 h at 20 °C. Mixtures of 0.5 ml of dialysate, 2.5 ml of methylene blue, and 10.0 ml of chloroform were centrifuged at 2500g for 5 min. The transmittance (%T) of the chloroform (lower) phase was assessed spectrophotometrically at a wavelength of $\lambda = 655$ nm. Chloroform served as solvent blank.

$$\text{Surface hydrophobicity } (\mu g\ SDS/500\ \mu g\ protein) = 62.305 \times \log [1/(\%T/100\%)] - 0.8349 \quad (5)$$

*(e) Intrinsic fluorescence emission spectra*

Intrinsic fluorescence spectra were recorded in a PerkinElmer LS55 fluorescence spectrophotometer (Perkin Elmer Corp., Boston, MA, USA) using 2 mg/ml protein solution and background calibrated with Milli-Q water. To minimize the contribution of tyrosine residues, the solution was excited at 290 nm.[3]

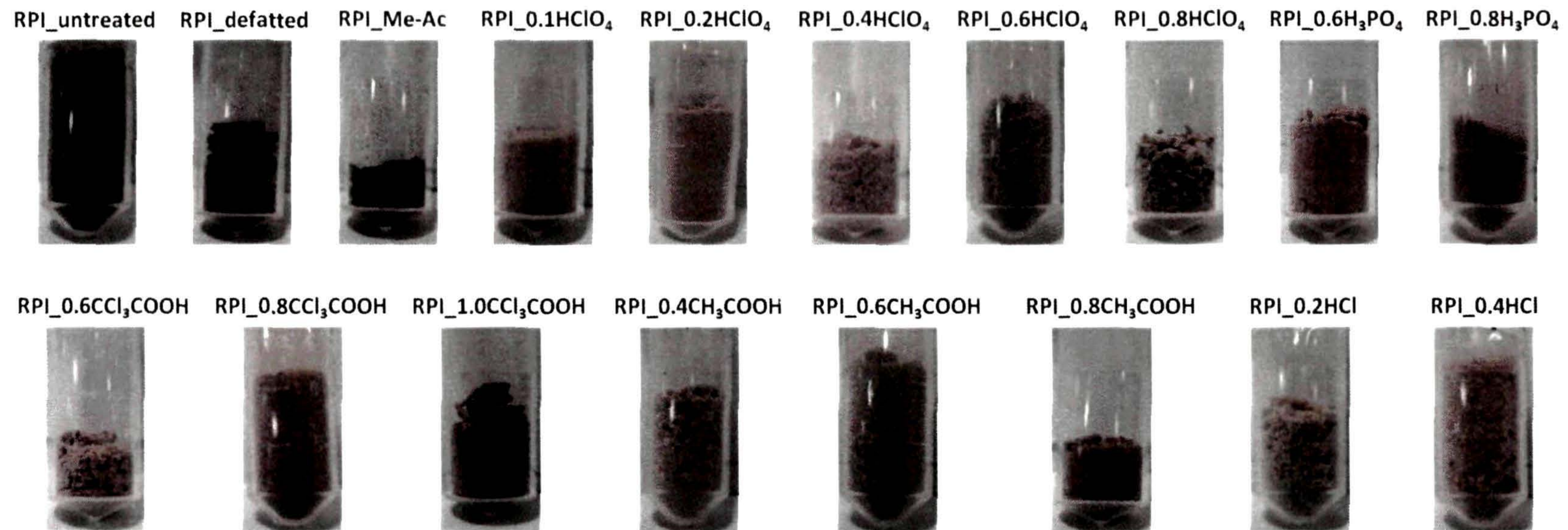



**Fig. 3.1** Photograph showing freeze-dried protein isolates extracted from different solvent-treated rapeseed meals.

*(f) Fourier transform infrared (FTIR) spectroscopy*

FTIR was carried out on solid samples by KBr pelleting method using PerkinElmer Spectrum-100 model.

*(g) Differential Scanning Calorimetry* (DSC)

DSC analysis was performed in a DSC-60 (Schimadzu, Japan), using 5 mg freeze-dried sample according to the methods of Manamperi et al.[11] and Li et al.[12] Denaturation temperature ($T_d$) and enthalpy change ($\Delta H$) were acquired from the thermogram using TA-60WS software (Schimadzu, Japan).

*(h) Non-denaturing Polyacrylamide Gel Electrophoresis (Native-PAGE) and Sodium Dodecyl sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

SDS-PAGE was performed in a Bio-Rad vertical electrophoresis system (Bio-Rad Laboratories, California, USA) with 12% separating and 5% stacking gels (12.5cmX14cm and 0.75mm thickness), following the procedure provided by the manufacturer. Freeze-dried samples (4g/l) were solubilized in either non-reducing or reducing (2% β-mercaptoethanol (β-ME)) buffer solution, heated for 5 min in boiling water and then centrifuged at 10,733 x g for 3 min prior to loading. Fifteen microlitres of each sample was loaded onto a gel lane. Constant current of 45 mA per gel was applied for 1 h (approx.), stained with Coomassie Brilliant Blue (0.125%) in 25% methanol and 10% acetic acid, and then destained with 40% methanol and 10% acetic acid. GelDoc system equipped with Vilber Lourmat® image analysis software was used for scanning the gel. Premixed protein markers (high range, Catalog no. 623110375001730; and medium range, Catalog no. 623112375001730, Genei™, Bangalore, India) [myosin (205 kDa), phosphorylase b (98 kDa), bovine serum albumin (68 kDa), ovalbumin (44 kDa), glutathione-S-transferase (29 kDa), and lysozyme (16 kDa)] were used for molecular weight (MW) comparison.

Native-PAGE was performed on 7% separating gel and 3% stacking gel. Main differences from the SDS-PAGE assay were the absence of SDS and β-ME in the procedure, and no thermal treatment before electrophoresis.

*(i) Protein solubility*

Protein solubility (at neutral pH) was measured by the Lowry method using bovine serum albumin as standard.[13,14] Results were expressed as percentage of the nitrogen protein in the starting isolates (determined by Kjeldahl).

*(j) Foaming capacity (FC) and foam stability (FS)*

FC and FS were evaluated according to the protocol of Hassan et al.[15] Forty millilitres ($V_{initial}$) of protein solution (3% w/v) was agitated for 3 min in a vortex mixer, poured into graduated cylinder and the foam volume was recorded immediately ($V_{final}$). Foam was left undisturbed at 25 °C for 30 min, and the volume of liquid ($V_0$) generated beneath the foam was measured.

$$FC = V_{final} / V_{initial} \quad (6)$$

$$FS = (V_{initial} - V_0) / V_{initial} \quad (7)$$

*(k) Emulsion capacity (EC) and emulsion stability (ES)*

EC and ES were also studied according to the methodology of Hassan et al.[15] after incorporation of a slight modification from Kalashnikova et al.[16] Oil-in-water (o/w) emulsions were prepared by blending soybean oil with aqueous protein solution (7 mg/ml) at a ratio of 3:7 (v/v). The mixture was sonicated to form an emulsion using an ultrasonic water bath (Labotec Inc., Nottingham, UK) for 10 min. Forty millilitres of emulsion ($V_T$) was centrifuged at 3220 x g for 5 min, and the volume of emulsified fraction ($V_{F1}$) was recorded. The emulsion was heated in a water bath at 80°C for 30 min and cooled to room temperature, and again centrifuged at 3220 x g for 5 min. Volume of the remaining emulsified fraction ($V_{F2}$) was recorded.

$$EC\ (\%) = (V_{F1}/V_T) \times 100 \tag{8}$$

$$ES\ (\%) = (V_{F2}/V_T) \times 100 \tag{9}$$

A 15 μl of the resulting emulsion was added to 1 ml of distilled water, stirred gently, and then a single drop was poured onto a glass-slide and observed via light microscope (BX51 Olympus) at 40x magnification. Droplet diameter (μm) was measured by ImageJ® software and average droplet diameter ($d_{43}$) is expressed as[17]

$$d_{43} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \tag{10}$$

where $n_i$ is the number of droplets of diameter $d_i$

### 3.2.6. Statistical analyses

All analyses were performed in triplicate, unless otherwise stated, and the mean value was calculated. Analysis of variance and separation of means were carried out by Tukey's HSD test, using SPSS software (version 16.0, SPSS Inc., Chicago) and considered significantly different at $p<0.05$.

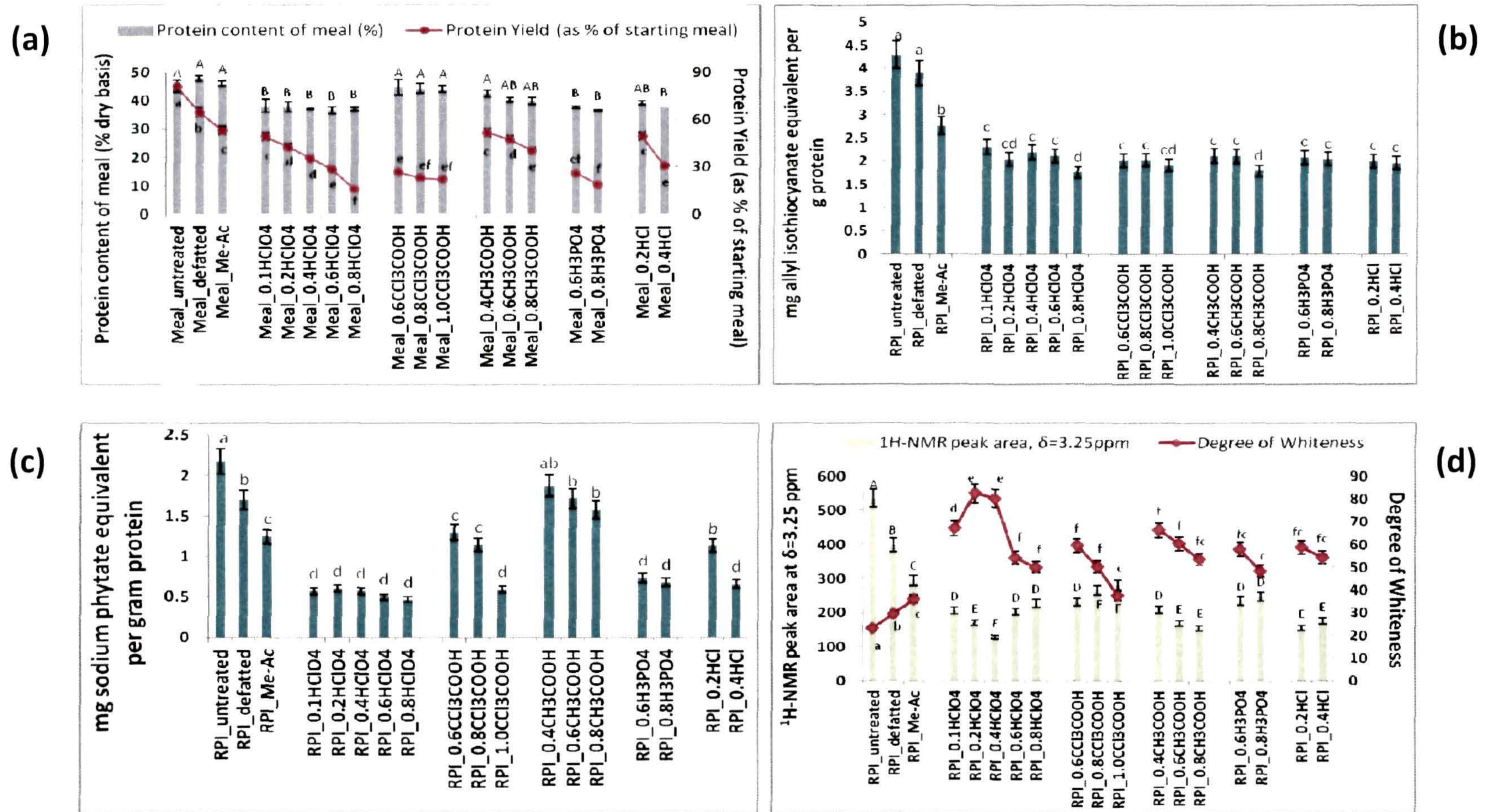



**Fig. 3.2** (a) Protein content of rapeseed meal treated with different solvents and their corresponding protein yield; Impact of solvent treatment on (b) AITC; (c) phytates; (d) sinapine content and Degree of Whiteness of the produced isolates. Bars or points with different letters in each type of analysis indicate significant difference ($p<0.05$).

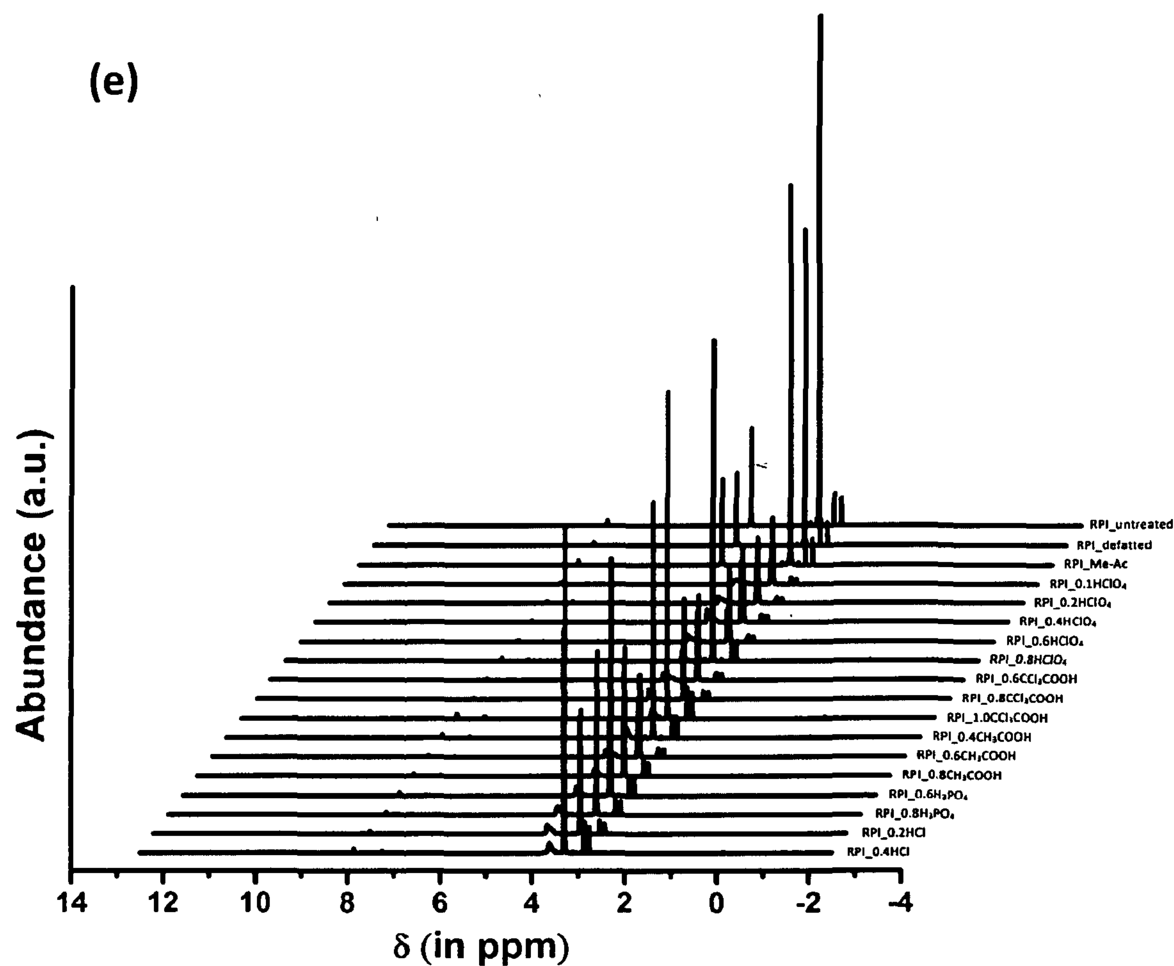



**Fig. 3.2e** Estimation of Sinapine, Betaine and Choline content, using $^1$H-NMR spectra, in the isolates obtained from untreated or solvent-treated meals.

### 3.3. Results and discussion

Untreated or treated meals were first analyzed by Kjeldahl process for checking their protein content and to evaluate whether the treatments led to any protein loss. The protein content of the treated meals was within a range (36.7-48.1%) similar to that of the control (44.8%) (**Fig. 3.2a**). Marginal increase in protein level of defatted and Me-Ac treated meals were expected, due to leaching of lipids and nitrogen-free compounds. Slight reduction of protein in $HClO_4$ and $H_3PO_4$-treated meals may be due to the removal of non-protein nitrogen in the form of glucosinolates, triamine compounds like betaine, choline, sinapine and free amino acids. As the use of nitrogen content to estimate protein levels of oilseeds and meal has been the norm for the oilseed industry,[4] so greater elimination of these nitrogen-containing components by the acidified solvents reduced the crude protein level in such meals. This may also be the reason for the decreasing trend of protein content in $CCl_3COOH$ and $CH_3COOH$-treated meals with increasing acid concentration. Overall, the actual protein loss from the meal during solvent treatment was minimal due to the presence of high concentration of non-polar solvents (methanol and acetone), instead of any aqueous medium, which ensures negligible protein dissolubility. Unfortunately, the treatments diminished protein yield, which dropped almost linearly from >81% to 15.6% with the rise in acid concentration, indicating dramatic decrease in protein solubility and denaturation. It seems highly likely that the acidified organic solvents (acetone in particular) produced a dehydrating effect and underwent different interactions with the proteins, leading to non-extractable complexes/aggregates. Pretreatment of rapeseed/canola meal with a two-phase solvent system ($CH_3OH/NH_3/H_2O$-hexane) also resulted in a similar reduction in protein yield.[18]

### 3.3.1. Effect on antinutritional factors

Glucosinolate level was undetectable in all of the isolates (<0.15 μg sinigrin equivalent/g protein), indicative of its complete elimination or rapid degradation into AITC during processing. Defatting and successive treatment with Me-Ac mixture, led to a progressive decrease in AITC content of the respective isolate (**Fig. 3.2b**). Isolates from

acid-treated meals were characterized by a lower content of AITC as compared to RPI_untreated ($p<0.05$), the extent of decrease being almost similar for any such isolate. With regard to phytates, a distinct decreasing trend with increasing acid concentration was found, confirming that each treatment indeed removed some phytates from the meal, and thus from the isolates (**Fig. 3.2c**). The phytate level of all the isolates were well below the value reported for soy protein isolate containing flour-type infant foods (>230 mg/100 g), estimated using Wade reagent.[19] Samples from $HClO_4$-treated meals presented the lowest value (0.47-0.57 mg/g), which supported our earlier findings (Chapter-2) that $HClO_4$ helps in maximum removal of phytates among the tested acids.

Phenolic compounds (chiefly sinapine, choline ester of sinapic acid) are important factors when considering rapeseed protein in food formulations,[1] because they are found to cause unacceptable darkening. As anticipated, successive treatment with higher levels of acids resulted in remarkable reduction in sinapine content (**Fig. 3.2d**), manifested from their NMR peak area (**Fig. 3.2e**). Sinapine level in RPI_untreated (as 100%) was considerably higher than that of RPI_defatted (74.8%) and RPI_Me-Ac (55.3%). For isolates from $HClO_4$ (RPI_0.6$HClO_4$ and RPI_0.8$HClO_4$), $CCl_3COOH$ and $H_3PO_4$-treated meals, the increase in sinapine was mainly contributed by preferential association of polyphenols with some specific proteins to form protein-polyphenol complexes, which can be augmented to a great extent by the presence of denatured proteins. The higher acid concentration used in this study denatures protein (discussed later). Denatured protein provides additional binding sites for phenolic compounds.[20] Charlton et al.[21] proved that intermolecular binding between polyphenols and protein/peptides is dominated by stacking of polyphenolic rings onto planar hydrophobic surfaces and is strengthened by multiple cooperative bindings of phenolic rings. When the coating is sufficiently extensive to provide polyphenol bridges, precipitation occurs. Such polyphenols may readily oxidize into colour-forming quinones during protein extraction in an alkaline medium which cannot be eliminated later.[14] This is an indirect indication that such isolates with high phenolic content or their oxidized forms would possess darker appearance (as witnessed in **Fig. 3.1**). The above-stated reasons probably accounted for

the observed general increase in degree of whiteness of the isolates with decrease in their sinapine content (**Fig. 3.2d**), except for the samples obtained from $CH_3COOH$-treated meals, in which the decrease in sinapine paralleled the decrease in whiteness value. Exact reason for this discrepancy could not be deduced. RPI_0.2$HClO_4$ and RPI_0.4$HClO_4$ had the highest values, which can be ascribed to their least sinapine content. Compared to the extent of decrease in sinapine (1.3-4.1 fold), the extent of increase in whiteness (1.3-3.6 fold) was slightly less. Data indicated that the influence of the treatment with acidic organic solvents on the whiteness of the isolates was mainly due to the reduction in protein-bound polyphenols or their oxidized forms; however, high concentration of acid in the treatments to remove phenolics may not give a satisfactory lighter product.

### 3.3.2. Effect on physicochemical properties

UV spectra of the protein solutions showed similar profile irrespective of the acid used for treating the meal (**Fig. 3.3**). Strong UV absorbance in tryptophan (>290 nm) and phenylalanine (<275 nm) regions were not detected. The spectra mainly revealed changes in the position of peak maxima in the tyrosine region (275-290 nm), which give evidence of re-arrangement in the protein structure. Tyrosine peak maxima of RPI_defatted and RPI_Me-Ac showed a red shift with reference to RPI_untreated. Proteins obtained from the meals treated with $HClO_4$ (0.1-0.6%), $CH_3COOH$ and HCl also presented a red shift with respect to the control. This bathochromic effect could be attributed to a more polar environment of tyrosine residues.[22] Among others, RPI_0.8$HClO_4$ and the proteins recuperated from $CCl_3COOH$ and $H_3PO_4$-treated meals produced a shift of the maxima to lower wavelengths (blue shift) in comparison to the control. Appearance of blue shift could be due to the exposure of chromophores present at the interface of the subunits or from the interior of the molecule due to unfolding or dissociation, followed by rapid re-aggregation or non-native refolding of denatured molecules.[23] A second weaker peak within 322-332 nm was seen in all spectra. Absorbance in this region may correspond to the residual phenolic compounds (mainly sinapic acid or sinapine) complexed with rapeseed proteins.[24] Phenolics in rapeseed bind to the protein through a variety of

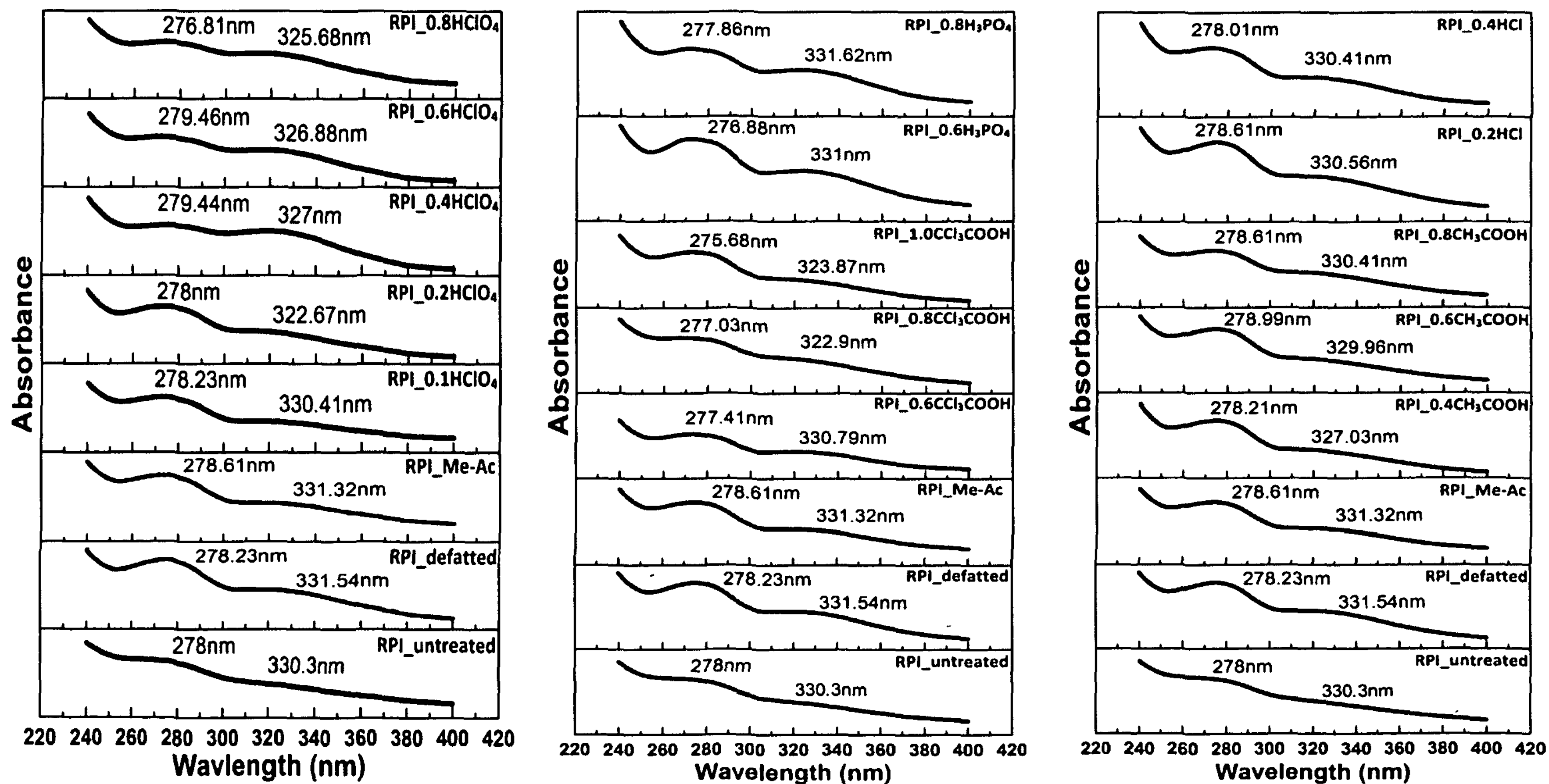



**Fig. 3.3** UV absorption spectra of soluble proteins in Milli-Q water from different meals.

mechanisms in aqueous media, including hydrogen bonding, covalent binding, hydrophobic interactions, and ionic binding.[1] Impact of the various acids on structural changes of phenolics and their cross-linking with protein might be different in each case, in terms of degree and site/domain of interaction,[6] which could be one of the possible reasons for the slight differences in the position of this second absorption band. Thus the extent of structural changes due to denaturation and complexation differs for each protein sample.

**Fig. 3.4a** shows free SH and SS contents of the isolates. Treatment with acidified solvents affected their number as opposed to that of untreated meal protein ($p<0.05$). SH group of $CH_3COOH$, $H_3PO_4$ and HCl-treated samples were comparable to those of RPI_defatted and RPI_Me-Ac; whereas the level markedly increased in $HClO_4$-treated ones. The increase can be ascribed to the higher removal of polyphenols from such samples and subsequent exposure of SH groups. Covalent attachment of the phenolic compounds to protein via its hydroxyl and carbonyl groups are known to cause the blocking of amino and thiol groups.[7,25] A drastic reduction of SH in $CCl_3COOH$-treated samples was possibly because most or all of the available free SH groups had reacted. It is worth noting that the nature of aggregates i.e., spatial distribution and accessibility of SH groups might also play an important role in causing such differences.[26,27] In case of SS content, all of the acid-treated samples showed much higher values compared to the control, with special emphasis to $CCl_3COOH$ (51.5-30.4 fold increase) and $H_3PO_4$ (54.9-30.6 fold increase) treated ones. Presence of such high amount of S-S in these samples clearly explains their poor solubility/dispersibility and high aggregate formation, as observed in SDS-PAGE and FTIR results. It is feasible to believe that the formation of SS bond was in agreement with the decrease in thiol content.[26] Organic solvent treatment followed by alkaline extraction induced unfolding of protein and subsequent exposure of SH groups from the interior of protein molecules, which possibly underwent oxidation to form S-S linkages. Also, the conformation of protein is severely compromised in the presence of higher acid concentration, which then competes with the formation of non-native intermolecular interactions giving rise to the S-S bond.[28] Although this supposition

seems to be suitable for explaining the rise in S-S content in the majority of the samples; in case of $HClO_4$-treated isolates, the presence of high SH group was not accompanied by an increase in S-S level. This may possibly be due to the presence of very low phenolic compounds in these samples: factually, it has been suggested that plant polyphenols are oxidized to quinones. Quinones may then form peroxides under extreme conditions, which are highly reactive oxidizing agents, and could facilitate oxidation of thiol and amino acid residues, and polymerization of proteins i.e., favouring S-S formation.[29]

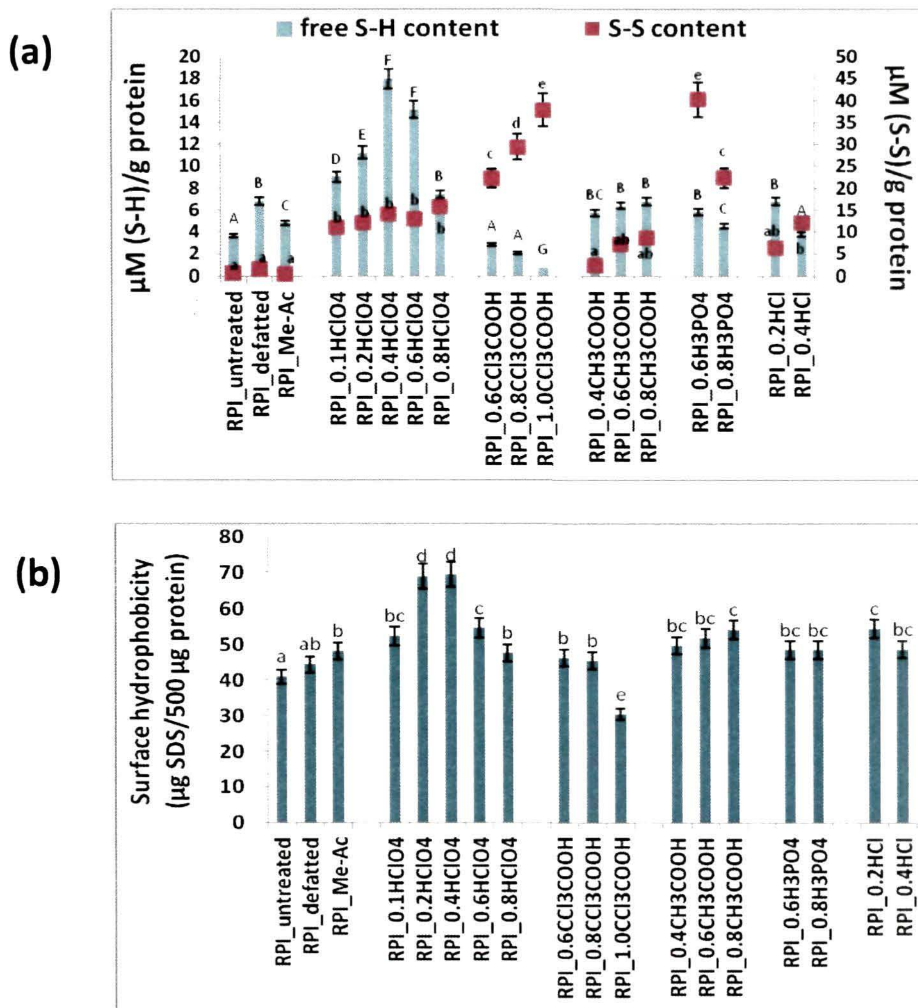



**Fig. 3.4** (a) Free sulfhydryl and disulfide content; (b) Surface hydrophobicity of isolates prepared from different meals. Bars or points with different letters in each type of analysis indicate significant difference ($p<0.05$).

$S_o$ is an indicator of the number of hydrophobic groups on the protein surface that are in contact with the polar aqueous environment. In other words, the level of protein unfolding can be measured by its $S_o$ value. Overall, $S_o$ of oilseed proteins is known to be significantly higher than those of other food proteins.[30] $S_o$ slightly increased at low acid treatment (0.1-0.4% $HClO_4$ and 0.4-0.8% $CH_3COOH$), presumably due to partial unfolding and subsequent uncovering of more buried hydrophobic moieties such as thiol group (**Fig. 3.4b**). Further application of higher concentration of acids might have caused greater extent of conformational changes, promoting hydrophobic interactions between the unfolded and/or dissociated molecules to form rapid aggregation, thus causing restricted access to buried hydrophobic moieties.[26,27] Markedly lower $S_o$ values of such samples corroborates this supposition.[31] Again, many researchers proposed that complexation of protein with polyphenols reduces hydrophobicity.[25] Acidified solvent treatment initiates the unfolding of the native protein structure, and exposes the hydrophobic groups to interact with the residual polyphenols, thus blocking off the reactive residues. This might account for the low $S_o$ values in samples having high sinapine content.

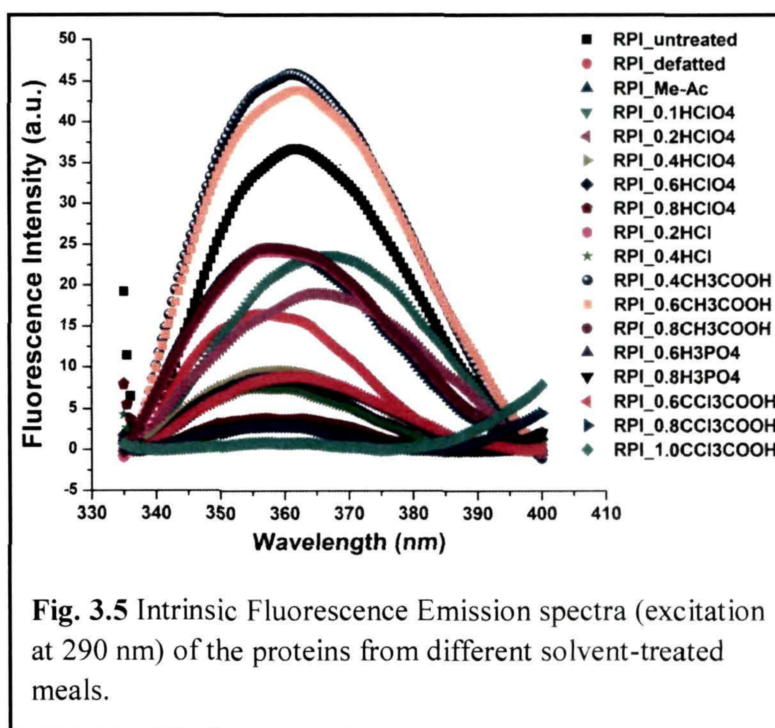
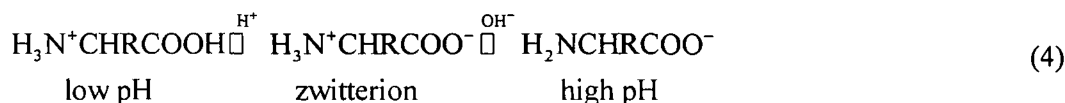



**Fig. 3.5** Intrinsic Fluorescence Emission spectra (excitation at 290 nm) of the proteins from different solvent-treated meals.

Intrinsic fluorescence emission spectrum of protein is usually dominated by tryptophan (Trp) and provides sensitive detection of tertiary conformation of the protein involved. Emission spectra of all the isolates are collectively shown in **Fig. 3.5** and the spectra of protein samples from each type of acid used are segregated individually

(**Fig. 3.6**) along with RPI_untreated, RPI_defatted and RPI_Me-Ac for ease of comparison. Spectra of the isolates are typical of those generally obtained for Trp-containing proteins. Emission maximum ($\lambda_{max}$) of Trp residues buried within the protein is expected below 330 nm in a non-polar environment and around 350 nm in a polar environment,[32] whereas $\lambda_{max}$ of pure napin is reported at 333±1 nm.[22] RPI_untreated showed $\lambda_{max}$ at around 362 nm. Exposure of Trp residues on protein surface, possibly fostered by heat generated during mechanical oil pressing in concert with alkaline protein extraction technique, might have led to such deviation. Solvent-exposed Trp is mostly observed above 335 nm,[32] which was apparent in all of the tested samples. Changes in $\lambda_{max}$ are likely to occur in response to polyphenol binding.[33] Except for RPI_0.4$CH_3COOH$ and RPI_0.6$CH_3COOH$, the isolates presented dramatic reductions in fluorescence intensity to a varying extent compared to RPI_untreated, together with a blue shift in $\lambda_{max}$ (movement toward lower wavelengths) in case of majority of the samples. Indeed, the emission intensity in RPI_defatted and RPI_Me-Ac reduced by 2.2 and 1.5 folds, respectively. This implies less polar surroundings of Trp, i.e., Trp residues are shielded from bulk water medium or moved closer to more hydrophobic core.[34] A plausible explanation for the lower fluorescence intensity in the isolates from acid-treated meals could be the more compact tertiary conformation of protein. It is well-known that exposure of protein to organic solvent and/or acidic condition leads to perturbations in its native structure (partial or complete unfolding). Upon rehydration by solvents such as ethanol, methanol, water and dimethyl sulfoxide, the protein probably refolds back[35] to compete with the formation of non-native intra-/intermolecular interactions or aggregation, as discussed earlier. This phenomenon possibly resulted in burial/confinement of the fluorophores once again into the non-polar environment.[23] This statement is partly in agreement with the FTIR result. In **Fig. 3.6b**, emission intensity decreased up to ≈9.6 fold with the successive increase in $HClO_4$ concentration, indicating a quenching effect of the acid. This was also seen with other tested acids (HCl, $H_3PO_4$ and $CCl_3COOH$). Apart from the acids, the presence of varying degree of polyphenols in the isolates can contribute to the observed quenching effect. A red-shift in $\lambda_{max}$ was

observed in RPI_0.1$HClO_4$ and RPI_0.2$HClO_4$, which suggests that the Trp surrounding became more polar i.e., the chromophores are more exposed to the hydrophilic environment. Further increase in $HClO_4$ concentration caused a blue shift in $\lambda_{max}$. Use of $CH_3COOH$ in the treatment solvent did not shift $\lambda_{max}$ significantly in case of RPI_0.4$CH_3COOH$ and RPI_0.6$CH_3COOH$, although an increase in their intensities was noted with respect to RPI_untreated. A lack of spectral shift pinpoints that Trp is not exposed to any change in polarity. However, their enhancement in intensity could be explained by the positive overall net charge of protein molecules under acidic condition (Eq. 4) because of which the sensitive areas in the backbone of protein molecule acquire more like charges, causing internal repulsion or perhaps lose charges that were previously involved in attractive forces holding the protein structure together.[34] This in-turn leads to a less compact structure, increasing the possibility of exposure, or accessibility of more buried fluorophores, thereby enhancing emission intensity.




(4)

FTIR spectroscopic patterns of the proteins were nearly identical, with no significant differences in the major bands (**Fig. 3.7**). Amide B band (≈3100 $cm^{-1}$) originates from N-H stretching of protein vibrations, with contribution from O-H stretching of intermolecular H-bonding. Amide I band (within 1600-1700 $cm^{-1}$) arises predominantly from C=O stretching vibrations (70-85%), whereas Amide II region (within 1500-1600 $cm^{-1}$) represents N-H bending vibrations (40-60%) coupled to C-N stretching vibration (18-40%). Spectral changes in Amide I region have been widely used for the conformational analysis of the secondary structure of proteins. So, secondary structure quantitations were performed by Gaussian curve-fitting of the spectra in the Amide I region (**Fig. 3.8**) using OriginPro software (version 8.0, OriginLab Corp., USA).

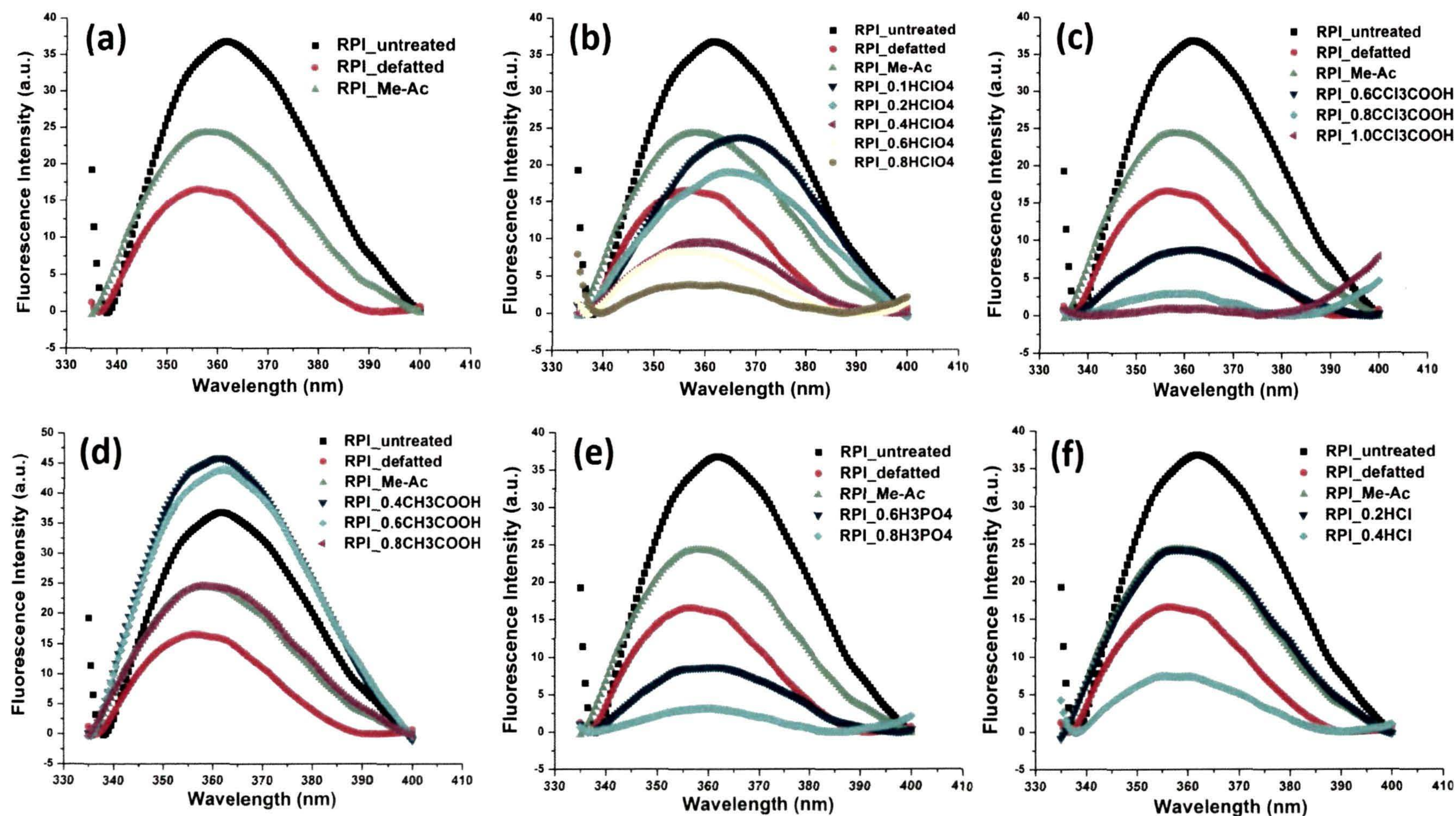



**Fig. 3.6** Comparative representation of emission fluorescence spectra of isolates from different solvent-treated meals (Excitation at 290 nm).

Peak positions were assigned at those positions where the second-order derivative had local minima. Bands were assigned to various elements of secondary structure (α-helix, β-sheet and others) and the data are compiled in **Table 3.1**. Correlation coefficient (adj. $R^2$) between the original and fitted spectra was ≥0.998, and the error of estimate (Chi-square value) was $\leq 1.08x(10)^{-5}$. The percentage of each secondary structure was calculated from the integrated areas of the component bands. Schmidt et al.[22] studied rapeseed napin and found that the protein is primarily composed of α-helices (45-57.9%) and β-sheets (7-15.3%) as a function of pH 3-12. He et al.[2] also showed that native rapeseed protein comprises of 43.6% α-helix and 25.8% β-sheet (β-strand + β-turn). These data are consistent with the result presented in **Table 3.1**.

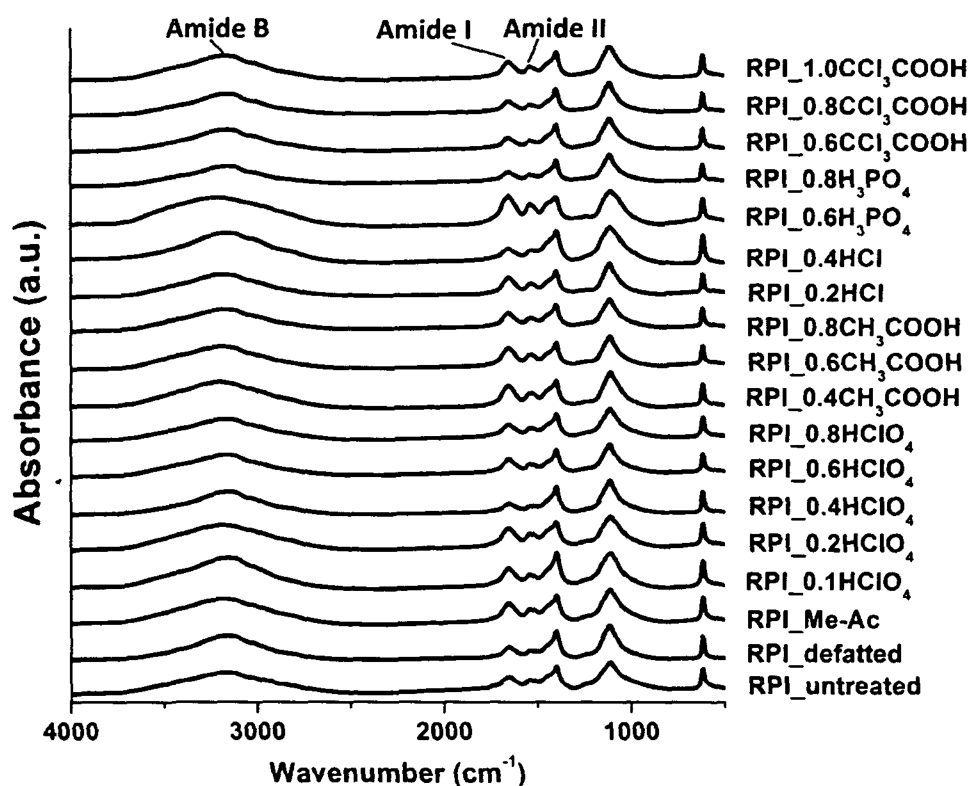



**Fig. 3.7** FTIR spectra of isolates from different meals.

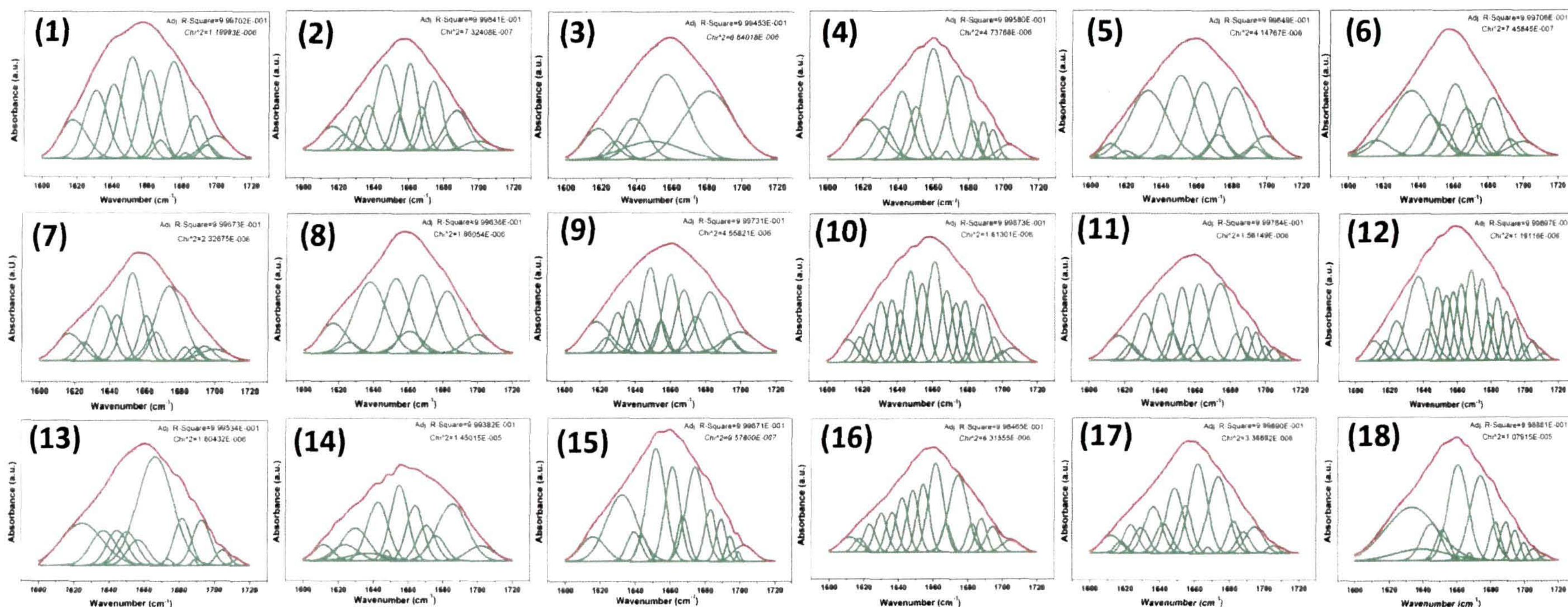



**Fig. 3.8** Curve-fitted Amide I bands of (1) RPI_untreated, (2) RPI_defatted, (3) RPI_Me-Ac, (4) RPI_0.1$HClO_4$, (5) RPI_0.2$HClO_4$, (6) RPI_0.4$HClO_4$, (7) RPI_0.6$HClO_4$, (8) RPI_0.8$HClO_4$, (9) RPI_0.4$CH_3COOH$, (10) RPI_0.6$CH_3COOH$, (11) RPI_0.8$CH_3COOH$, (12) RPI_0.2HCl, (13) RPI_0.4HCl, (14) RPI_0.6$H_3PO_4$, (15) RPI_0.8$H_3PO_4$, (16) RPI_0.6$CCl_3COOH$, (17) RPI_0.8$CCl_3COOH$, and (18) RPI_1.0$CCl_3COOH$. The red line represents the protein IR spectra, which coincide with the results of the Gaussian curve-fitting. Green lines are the individual Gaussian bands fitted to the spectra. Spectral correlation coefficients between the original IR spectra and the Gaussian curve-fitting were calculated (adjusted $R^2$) and error of the estimate is represented as Chi-square value ($\chi^2$).

**Table 3.1** Percent of secondary protein structures obtained in the Infrared (Amide I) spectral region of various proteins from untreated or solvent-treated meals.

| Proteins | Area (%) | | |
|---|---|---|---|
| | α-helix | β-sheet | Others |
| **RPI_untreated** | 44.68±0.8$^{a}$ | 29.81±0.63$^{a}$ | 25.51±0.17$^{a}$ |
| **RPI_defatted** | 41.69±1.0$^{b}$ | 34.80±0.91$^{b}$ | 23.51±0.28$^{a}$ |
| **RPI_Me-Ac** | 44.85±0.5$^{a}$ | 33.58±0.48$^{b}$ | 21.57±0.66$^{a}$ |
| **RPI_0.1HClO$_4$** | 46.29±0.26$^{a}$ | 35.54±0.32$^{b}$ | 18.18±0.67$^{b}$ |
| **RPI_0.2HClO$_4$** | 47.43±0.51$^{a}$ | 38.27±0.55$^{c}$ | 14.30±0.39$^{c}$ |
| **RPI_0.4HClO$_4$** | 35.30±0.43$^{c}$ | 42.37±0.64$^{d}$ | 22.33±0.51$^{a}$ |
| **RPI_0.6HClO$_4$** | 32.98±0.71$^{c}$ | 47.77±0.89$^{e}$ | 19.25±0.1.3$^{b}$ |
| **RPI_0.8HClO$_4$** | 33.52±0.11$^{c}$ | 41.56±1.02$^{d}$ | 24.93±0.99$^{a}$ |
| **RPI_0.6CCl$_3$COOH** | 31.85±0.9$^{c}$ | 50.77±1.1$^{f}$ | 17.38±0.11$^{b}$ |
| **RPI_0.8CCl$_3$COOH** | 24.53±0.49$^{d}$ | 54.62±0.12$^{g}$ | 20.86±0.83$^{b}$ |
| **RPI_1.0CCl$_3$COOH** | 18.70±0.56$^{e}$ | 55.16±0.88$^{g}$ | 26.15±1.7$^{e}$ |
| **RPI_0.4CH$_3$COOH** | 43.77±0.31$^{a}$ | 31.64±0.75$^{b}$ | 24.59±2.0$^{a}$ |
| **RPI_0.6CH$_3$COOH** | 43.02±0.26$^{a}$ | 37.13±0.44$^{c}$ | 19.85±0.26$^{b}$ |
| **RPI_0.8CH$_3$COOH** | 38.47±0.44$^{c}$ | 40.73±0.79$^{d}$ | 20.80±0.59$^{b}$ |
| **RPI_0.6H$_3$PO$_4$** | 35.33±0.13$^{c}$ | 47.64±0.22$^{e}$ | 17.03±0.44$^{b}$ |
| **RPI_0.8H$_3$PO$_4$** | 30.32±0.73$^{e}$ | 53.08±0.61$^{g}$ | 16.61±1.6$^{b}$ |
| **RPI_0.2HCl** | 35.29±0.82$^{c}$ | 39.36±0.19$^{c}$ | 25.35±0.21$^{a}$ |
| **RPI_0.4HCl** | 24.00±1.9$^{d}$ | 40.88±0.43$^{d}$ | 35.12±0.31$^{f}$ |

Values are mean±SD (n=2). Values followed by same superscript letter within a column are statistically not significant ($p>0.05$)

The α- and β-contents of RPI_untreated, RPI_deftatted and RPI_Me-Ac were found to vary minimally, which strongly suggests structural similarity between these samples. Estimated α:β ratio decreased with increasing acid concentration ($p<0.05$) (**Fig. 3.9**). This clearly reflects that acidic solvents induced an increase in β-sheet formation (non-native protein aggregates) accompanied by a decrease in α-helical conformation (indicative of organized native structure), the effect being much stronger on proteins recuperated from $CCl_3COOH$-treated meals. These results complement the findings of Ayutsede et al.[36] where formic acid treatment was reported to increase β-sheet with a concomitant decrease in α-helix of silk protein. The β-sheet probably arises at the expense of α-helix (partial unfolding of α-helices and subsequent aggregation).[26]

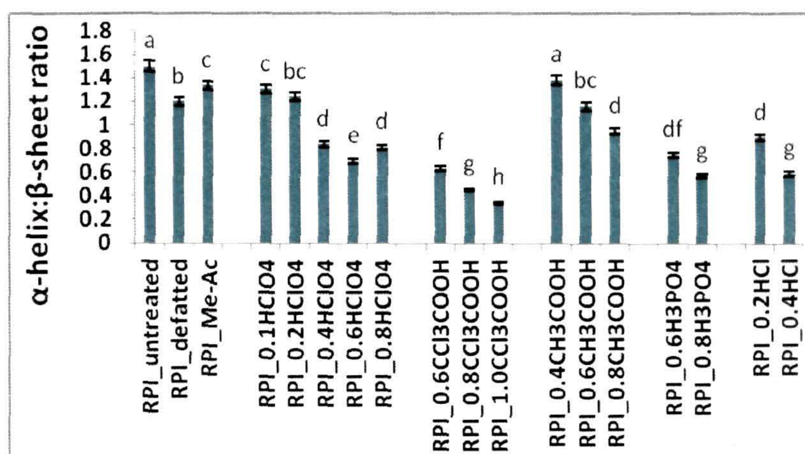



**Fig. 3.9** Estimated ratio of α-helical to β-sheet conformation of isolates from different meals.

ΔH reflects the extent of ordered structure of protein as the transition from native to denatured state takes place and $T_d$ provides information on the temperature needed to unfold the structure.[37] Thermograms of the majority of the samples displayed one broad endothermic peak (not shown), possibly corresponding to the overlapping denaturation of

12S (cruciferin) and 2S (napin) rapeseed proteins.[12] Progressive decrease in $\Delta H$ from 9.82 to 6.7 J/g with defatting, followed by Me-Ac treatment revealed solvent-induced protein unfolding (**Fig. 3.10**). Regardless of the acid type, proteins from meals treated with higher acid concentration exhibited very low $\Delta H$, confirming denaturation and more disordered conformation; the effect being higher in RPI_0.8$HClO_4$, RPI_0.8$CCl_3COOH$, RPI_1.0$CCl_3COOH$ and RPI_0.8$H_3PO_4$ which were denatured almost completely (having a flat trace with negligible sign of transition). It could be speculated that the native structure of RPI_untreated had not been completely lost with acidified solvent treatment; proteins from meals treated with lower acid level partially conserved their native structure (manifested by their higher $\Delta H$), especially in RPI_0.1$HClO_4$, RPI_0.2$HClO_4$, RPI_0.4$CH_3COOH$ and RPI_0.6$CH_3COOH$. It is feasible to believe that treating the meal with low acidic solvent exposes protein to conditions of minimum harshness, and therefore, yields a protein with least structural aberrations. The current enthalpy data are somewhat comparable to those reported by He et al.[2] Regarding $T_d$, solvent-treated species presented lower values ($p<0.05$) in comparison with RPI_untreated, in agreement with the generally described unfolding effect of organic solvents on protein. Unlike $\Delta H$, $T_d$ of the samples varied within a narrow range of 64-79.5 °C. This observation is partly similar to that reported for buckwheat protein isolates obtained from organic solvent-treated flours,[7] for which the $T_d$ value of 13S globulin was found to be nearly unaffected by the removal of polyphenols with cold organic solvents; however $\Delta H$ was found to increase significantly. In **Fig. 3.10**, all the isolates presented lower $T_d$ than that of RPI_untreated. Although, there was no significant effect ($p>0.05$) due to concentration for any of the acids tested, the isolates from $CCl_3COOH$ and $H_3PO_4$-treated meals underwent a slight rise in $T_d$ with increasing acid level. Taking into account the data from FTIR, higher $T_d$ can be expected due to the tendency of proteins to aggregate into a more compact moiety after unfolding, especially in a highly denaturing condition. As a comparison, $\Delta H$ and $T_d$ values of rapeseed protein obtained in this study were inferior to those reported earlier.[12,38,39] Partial protein denaturation due to commercial oil expression and alkaline extraction procedure could explain this difference. In addition, most authors

perform DSC analysis of protein solution in different ionic strength buffers instead of dry protein powder, which in-turn causes remarkable differences in thermal properties,[40] due to the stabilizing effect of salt (non-specific charge-shielding effect)[37] and water.[41]

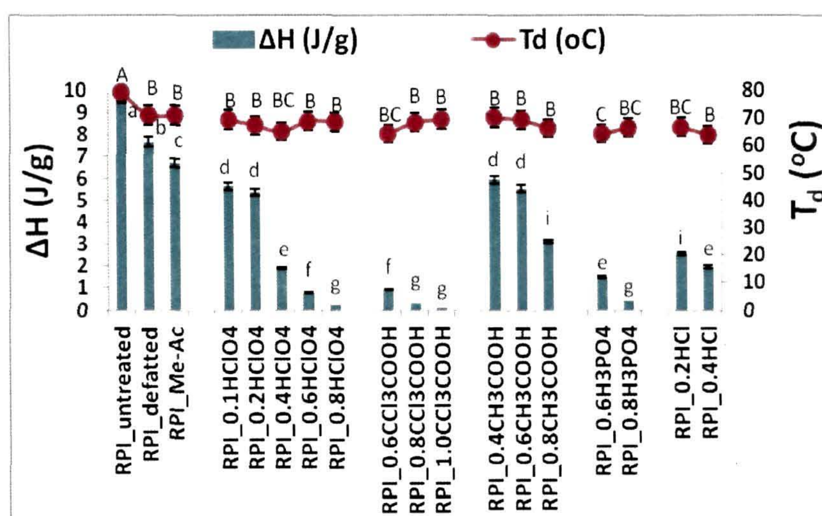



**Fig. 3.10** Thermal transition characterization of isolates from different meals. Bars or points with different letters in each type of analysis indicate significant difference ($p<0.05$).

Of 18 tested samples, Native-PAGE of only 10 samples could be traced (**Fig. 3.11a**). High MW proteins (>205 kDa) were caught at the top of the lanes as smears. Electrophoretograms of these isolates did not present any detectable difference from the control, precluding that these samples are relatively native-like. Remaining samples did not enter the separating gel and are presumed to be complex(es) of very high MW, perhaps due to protein aggregation. MW of cruciferin, one of the major canola/rapeseed proteins, was reported to be in the range of 230-240 kDa;[38] whereas other studies suggest its MW above 300 kDa.[42] Since MWs of the proteins could not be calculated according to their mobility by Native-PAGE, the samples were further analyzed by SDS-PAGE.

Examining the gel in **Fig. 3.11b** shows that the very same samples, which presented diffused bands in Native-PAGE, also yielded similar electrophoretic patterns under non-reducing condition (without β-ME), except RPI_0.4HCl and RPI_0.4$HClO_4$, and contained mainly globulin (cruciferin) subunits which were composed of bands with MW above 20 kDa (56-49.6, 35-28 and 20.2-19.2 kDa), and albumin (napin) subunits (ranging from 15.9 to 14.8 kDa). This result is consistent with earlier reports.[12,38] Aluko and McIntosh[43] also reported 4 major polypeptide bands in canola with MWs of 16, 18, 30 and 53 kDa in non-reducing SDS-PAGE. RPI_0.4HCl gave feeble bands of high MW aggregates, seen as smears at the top and RPI_0.4$HClO_4$ failed to enter the resolving gel. Upon addition of β-ME to cleave SS bonds (reducing condition), the bands within 56-49.6 and 15.9-14.8 kDa disappeared and concomitantly two additional bands with estimated MWs of 13.9-12.5 and 10 kDa appeared (**Fig. 3.11c**). The latter (10 kDa) probably corresponds to dissociated napin components (reported MW is 10-9.5 kDa).[38,39] The disappearance of canola protein bands having MWs of 59, 53 and 47.5 kDa in the presence of β-ME has been shown earlier.[38,43] Under reducing condition, cruciferin dissociated into acidic (37.8-28.5 kDa) and basic (21.4-20 kDa) polypeptides, in agreement with a previous report.[39] Protein profile of RPI_0.4HCl exhibited changes compared to the pattern described under non-reducing condition, displaying fewer bands having lower MW ($\approx$11.7 kDa). So the high MW aggregate of this isolate detected in **Fig. 3.11b**, was stabilized by S-S linkages. Dissociation followed by reaggregation after acidic treatment (pH $\leq$3) has been reported in a number of oilseed proteins.[23] Surprisingly in case of the remaining samples, even after the addition of β-ME, diminished concentration of soluble aggregates were able to enter the gel, which suffered pronounced decrease in band intensity (**Fig. 3.11d**). The coincidental occurrence of polymerized proteins at the bottom of the loading wells and at the junction between stacking and resolving gels was clearly noticeable (**Fig. 3.11d**). Protein denaturation in such samples was reflected by the diffusive and unresolved bands of very low electrophoretic mobility. It is apparent that the results follow the trend observed in FTIR (predominance of β-sheet formation), and the formed aggregates are not soluble in buffer used for electrophoresis. These moieties

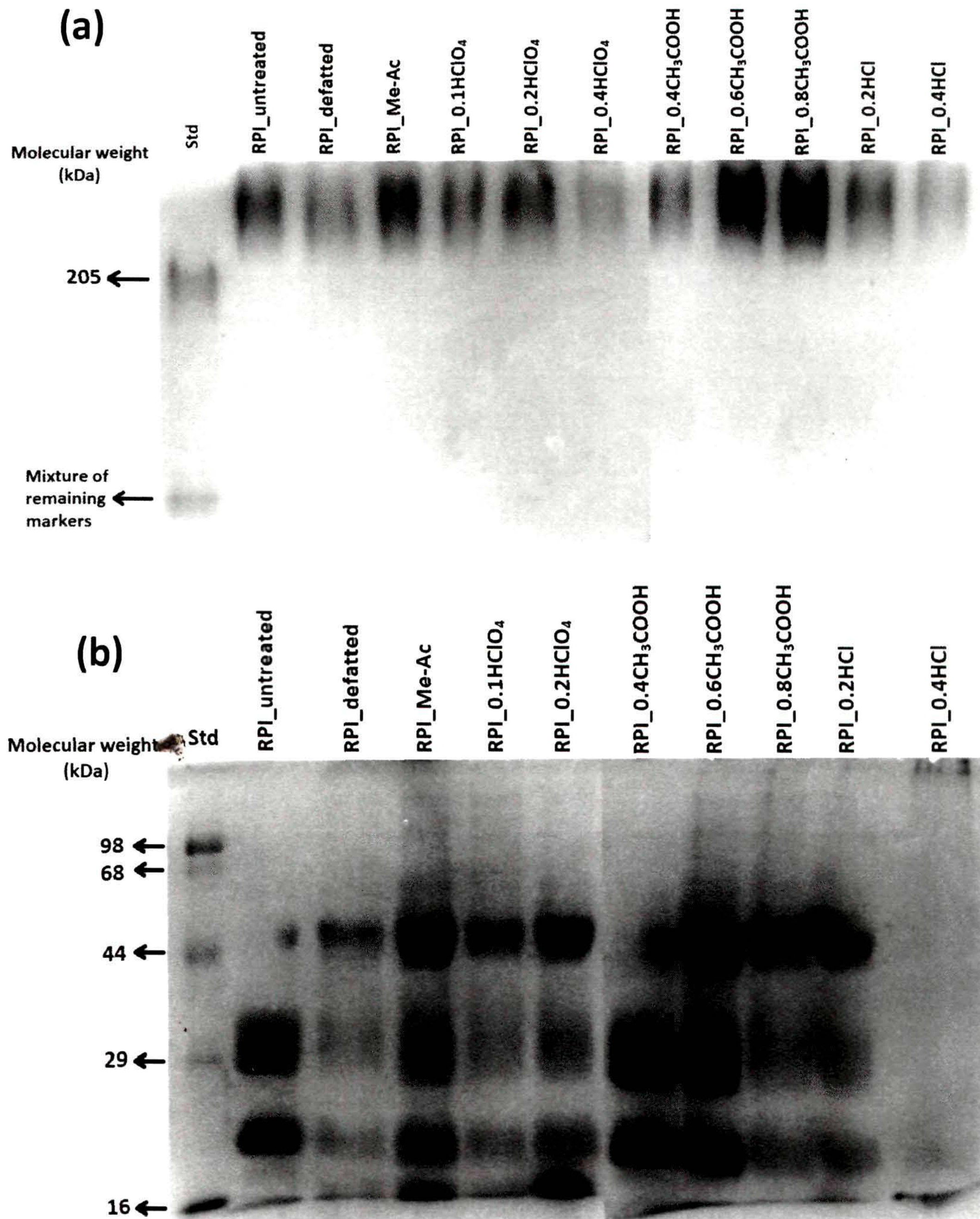



**Fig. 3.11** (a) Native-PAGE; and (b) Non-reducing SDS-PAGE profiles of isolates from different meals.

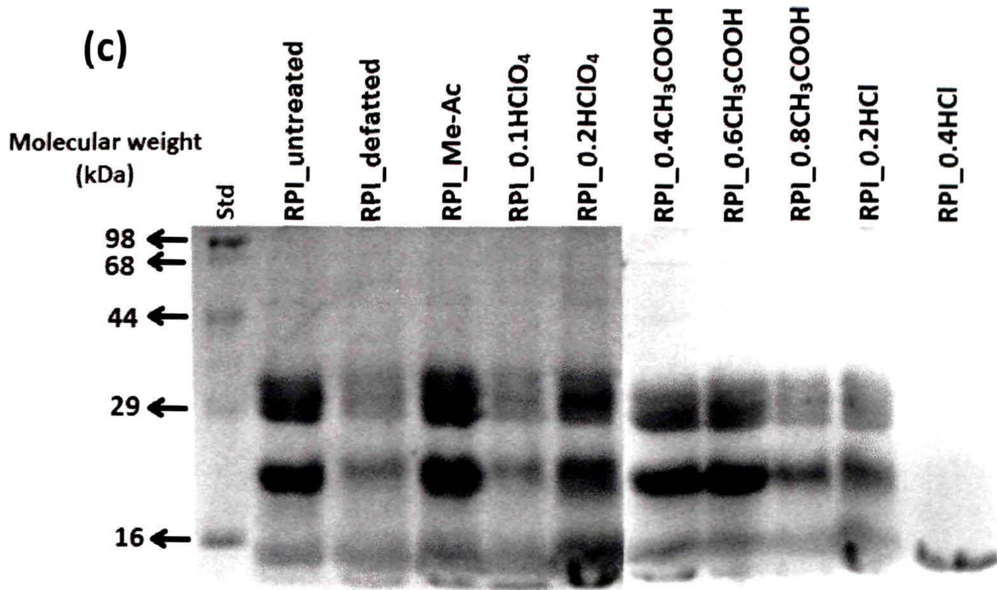



**Fig. 3.11** (c) Reducing SDS-PAGE profiles of isolates from different solvent-treated meals.

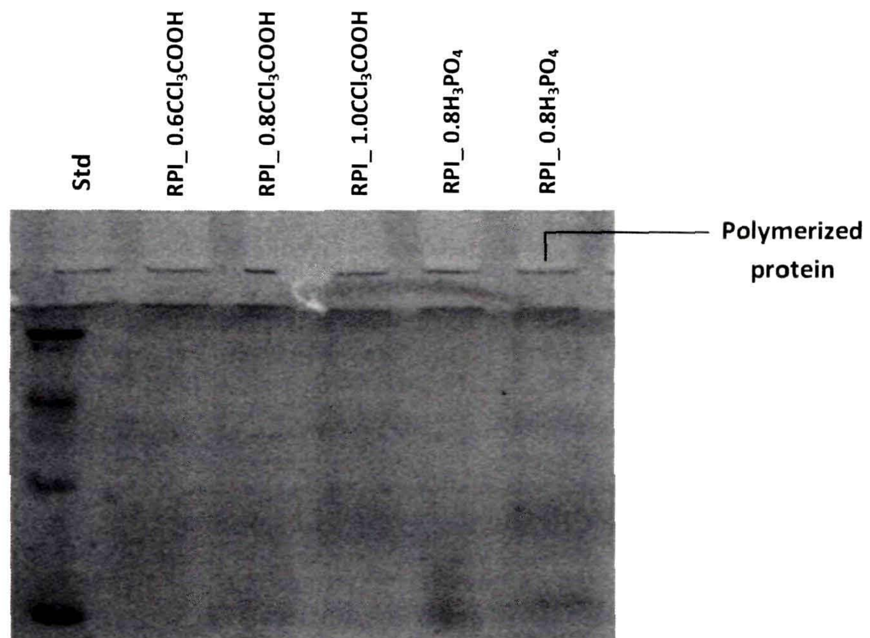



**Fig. 3.11** (d) Reducing SDS-PAGE patterns of proteins from $CCl_3COOH$ and $H_3PO_4$-treated meals. First lane represents the protein molecular weight markers.

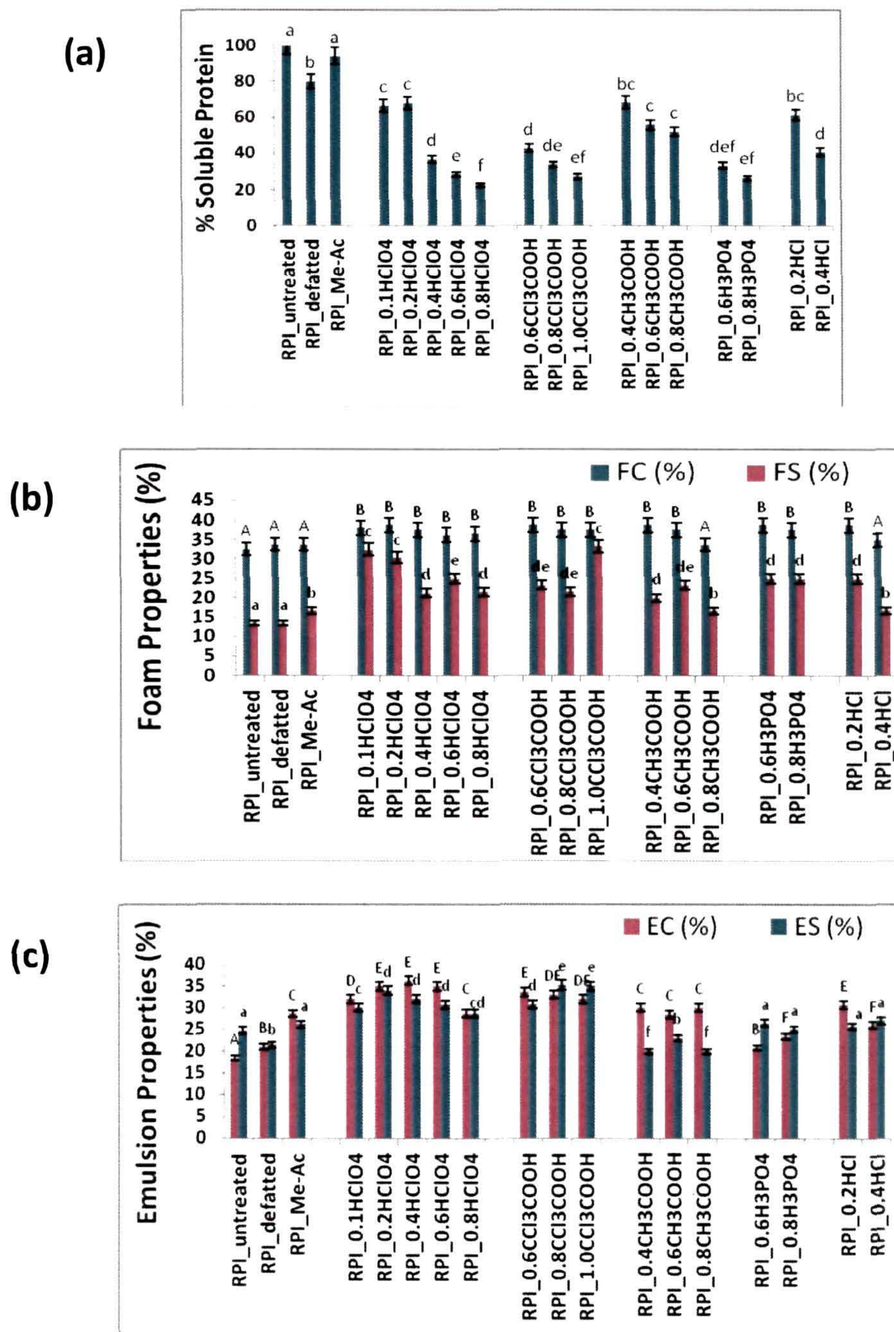



**Fig. 3.12** (a) Protein solubility (at pH 7.0); (b) Foaming capacity (FC) and stability (FS); and (c) Emulsion capacity (EC) and stability (ES) of isolates from different meals. Bars with different letters in each type of analysis indicate significant difference ($p<0.05$).

are more likely stabilized by covalent bonds in addition to S-S linkages. Covalent bonding occurring locally in the deep of protein structure especially in highly denatured state cannot be excluded.[37] The assumption that most of the S-S cross-links are located deep or hidden in the hydrophobic core due to conformational change or protein folding in a manner that limits the accessibility of the reducing agent (β-ME), may also corroborate such a result.[26,37]

### 3.3.3. Effect on functional properties

Protein solubility (at neutral pH) of RPI_untreated (as 100%) was reduced to 80% and 94% in RPI_defatted and RPI_Me-Ac, respectively (**Fig. 3.12a**). Slightly higher value of the latter could be due to hydration of protein caused by methanol (in comparison with diethyl ether), preventing severe dehydrating effect on the protein.[44] Decrease in solubility with increasing acid concentration can be ascribed to a number of factors: first, it may be a consequence of solvent-induced protein denaturation or due to the varying level of residual antinutrients remaining associated with protein. It is well known that increasing the level of phytates and/or polyphenols in protein products decreases nitrogen-solubility.[45,46] However, despite the highest level of phytates and polyphenols in RPI_untreated, it shows maximum solubility because of the partial native state of proteins from pristine rapeseed meal as-well-as differences in the nature of phytate-protein complexes or phenolic-protein complexes in the protein from pristine meal compared with the isolates from solvent-treated meals.[46] Second, varying degree of physico-chemical changes caused by the solvents may be responsible for these deviations, such as non-availability or blocking of the residual thiol groups leading to sustained insolubility. Floris et al.[27] proved that the insoluble aggregate is the result of SH group oxidation and not the result of SH/SS exchange reactions. It was demonstrated that SH/SS rearrangement takes place in the aqueous environment and reshuffling of S-S groups, which can occur when free SH groups are present, is very important in the process of protein re-solubilization.[27] Thus, it is difficult to ascertain which influence

would play a predominant role, or their concerted effects may dictate the solubility property of different isolates.

Proteins, being amphiphilic, show a strong tendency at fluid interfaces (reducing interfacial tension), which allow them to play a major role in the formation and stabilization of emulsion and foam. Literature data on rapeseed protein-stabilized foams and emulsions are conflicting. As reviewed by Moure et al.[47] EA and ES of rapeseed/canola protein (concentrates and isolates) widely range from 28.3% to 54% and from 5% to 71%, respectively. In case of FC and FS, the respective value varies from 43.3% to 211.9% and from 8.2% to 74.7%. Dispersion interactions between protein and the oil-water interface are always attractive, whereas that between protein and the air-water interface are generally repulsive. Because of this difference proteins are adsorbed much more readily at the oil-water interface than at the air-water interface.[48] Thus the emulsification behavior of a protein may not be analogous to its foaming ability. Apparently, proteins from meals treated with lower concentration of $CH_3COOH$ and HCl showed slightly higher ability to form foam (**Fig. 3.12b**), which can be partly ascribed to their higher $S_o$ and solubility.[47] Highly ordered proteins possess poorer FC than the ones that have disordered structure,[48] due to their inability to migrate into the air-water interface of gas bubbles to facilitate a more cohesive film. This might be the cause for inferior FC and FS in untreated sample. Thus structural and conformational features of protein as a whole rather than solubility alone appear to be the primary determinants for foaming properties.[46] For RPI_0.1$HClO_4$ and RPI_0.2$HClO_4$, a pronounced increase in FS was found (**Fig. 3.12b**), which seems to be related with their high SH content. Introduction of thiol groups in protein has been shown to increase FS significantly, promoted by SH/SS interchange reaction at the interface,[48] which provides a highly viscoelastic film resisting coalescence. In contrast, $CCl_3COOH$-treated samples, although with very low SH group content, showed improved stability; the influence being very pronounced in RPI_1.0$CCl_3COOH$. This was tentatively attributed to the presence of high protein aggregates, which could behave as surface-active solid particles begetting a

Pickering foam.[49] Such particles could attract each other, increasing jamming in the fluid interstices between bubbles and the formation of a firmer shell.[49]

EC was found to vary within 18.32%-36.28%, with all treated samples showing higher values than the untreated one (**Fig. 3.12c**). This is because solvent-induced partial denaturation of protein generally improves surface activity.[48] Reduction in $S_o$ and/or high level of phenolics in RPI_untreated and RPI_defatted impeded their emulsion properties.[25] Excessive denaturation may impair EC by rendering the protein insoluble, as a highly rigid protein cannot unfold/reorient at the interface rapidly, and thus may not form the film around the oil droplets effectively. This may be the probable reason for decreased EC in samples obtained from high acid-treated meals. EC of such samples may also be affected by their $S_o$ and solubility,[47] although a high solubility is not a pre-requisite for good interfacial properties.[48] Usually EC shows positive correlation with $S_o$; the greater the number of hydrophobic patches on a protein's surface, the higher is the probability of its adsorption and retention at the interface, albeit highly hydrophobic proteins may precipitate and cause instability.[48] As reflected in **Figs. 3.4b** and **3.12c**, EC and ES of proteins from treated meals partly paralleled their $S_o$ values. An exception to this observation was the TCA-treated samples, which showed highest ES. The increase in acid level in the processing treatment did not present any close relationship with ES. $CH_3COOH$-treated samples showed the least stability, which may be due to their high phytate content. Our observation is in line with those of Dev and Mukherjee.[46]

In light of the differences among the wide number of systems available, the results of a single method can give only a reductive suggestion of the emulsion properties of the sample. For that reason, morphological changes occurring in the droplets of emulsions stabilized by different isolates were visualized. At t=0 h (**Fig. 3.13a**), the emulsion droplet morphology stabilized by RPI_untreated compares well with those of RPI_defatted, RPI_Me-Ac, RPI_0.1$HClO_4$ and RPI_0.2$HClO_4$. Although a smaller droplet size was found in the emulsions stabilized by RPI_0.4$HClO_4$ and RPI_0.6$HClO_4$, insoluble protein aggregates were visible in the matrix. Such aggregates were also

detected in emulsions formed by $CCl_3COOH$-treated samples. Poor emulsifying properties of $H_3PO_4$ and HCl-treated samples can be anticipated from their flocculated droplets with large diameters. This is congruent with their EC values. At t=24 h, the droplets displayed a significant level of flocculation (**Fig. 3.13b**). The spherical shape of oil droplets was severely distorted; the burst of large droplets dominated, and some emulsion collapsing was observed, such as those stabilized by defatted, $CH_3COOH$ and HCl-treated samples. These phenomena seem to denote their inferior ES. Interestingly, the droplet diameters were initially larger in emulsions formed by $CCl_3COOH$-treated samples, but their stabilities were found to be outstanding, although these samples possessed very low $S_o$ and protein solubility. This vast improvement can be attributed to insoluble protein aggregates, which were mainly located at the junction between oil droplets, favouring network stabilization by retarding droplet coalescence. However, the intense colour of these isolates should be taken into account when considering their potential applications.

Additionally, changes in droplet size of these emulsions (denoted by $d_{43}$) are shown in **Fig. 3.13c**. Emulsifying ability of a surfactant can be assessed by $d_{43}$; the smaller the particle size, the better the ability. At t=0 h, emulsion from all solvent-treated samples presented comparatively smaller particle sizes, indicating higher EA, which is due to their $S_o$. Except RPI_0.8$HClO_4$, perchloric acid-treated samples had lower $d_{43}$ values compared to others. The droplet size of emulsions decreased markedly when $HClO_4$ concentration increased from 0.1% to 0.4%; however, further increase in acid level produced larger sizes. This can be related to their $S_o$ and soluble protein content.[48] The initial increase in hydrophobicity facilitated enhanced interaction between the oil and aqueous phases.[13] As the acid level increased, a point was reached where the unfolded polypeptide chains reconstructed (SS linkages with polymerization), which made it prone to aggregation rather than to adsorb onto the interface.[13] When the soluble protein amount is limited, there is no longer sufficient protein to fully stabilize the droplet interface, and therefore larger particles may be formed as a result of coalescence or bridging flocculation. In case of emulsions stabilized by other protein samples, larger particle sizes

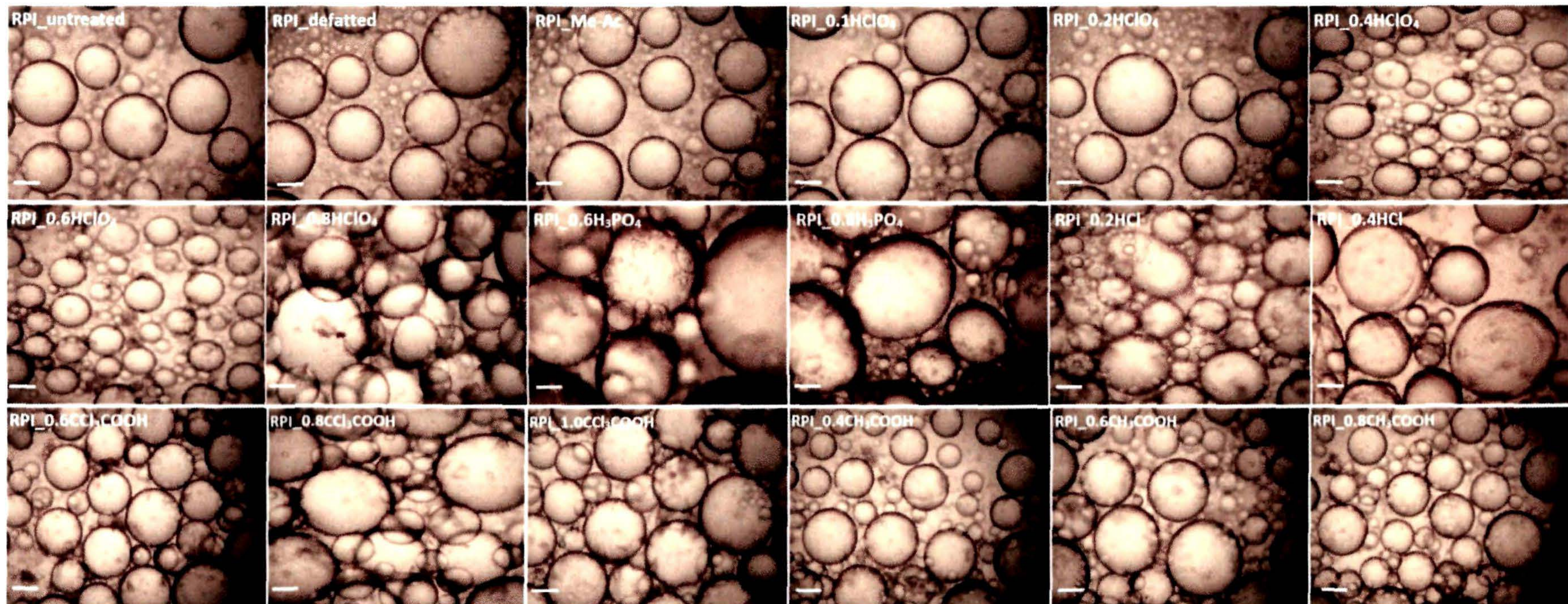



**Fig. 3.13a** Optical micrographs of the o/w emulsions stabilized by protein isolates from different meals at t=0 h (Scale bar represents 55 μm; images procured at 40x magnification).

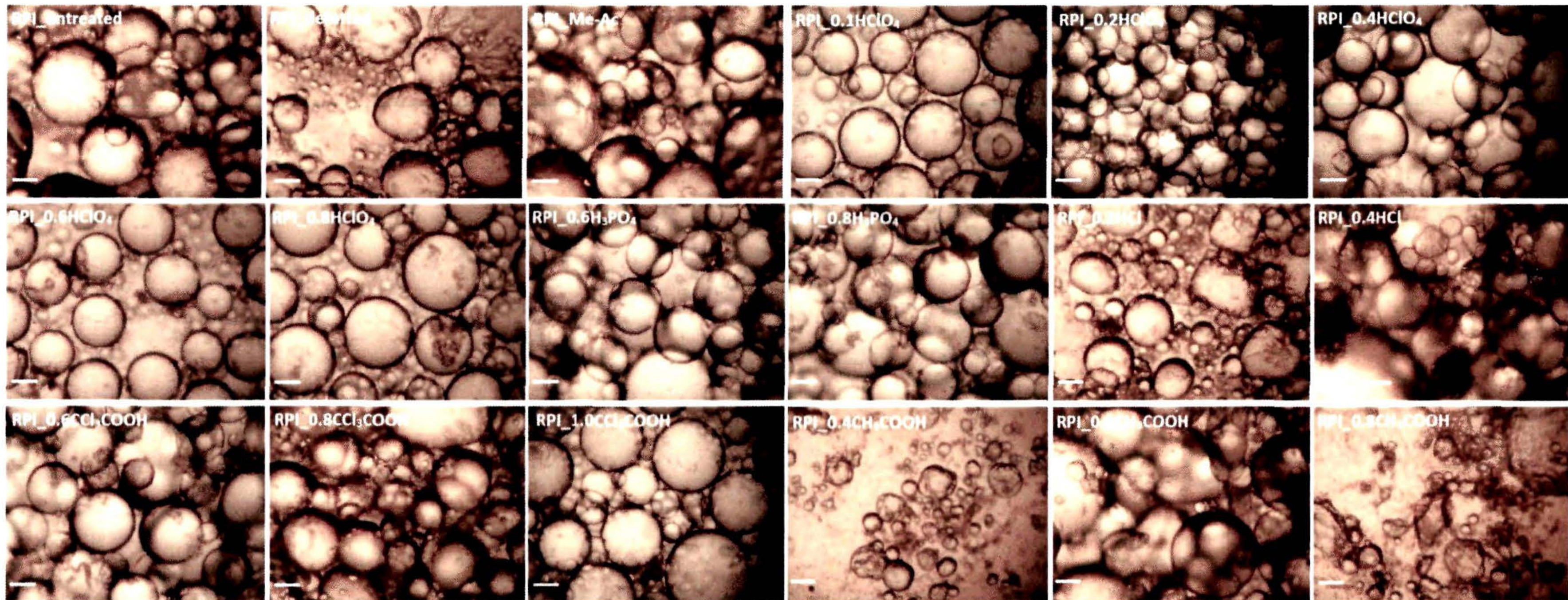



**Fig. 3.13b** Optical micrographs of the o/w emulsions stabilized by protein isolates from different meals at t=24 h (Scale bar represents 55 μm; images procured at 40x magnification).

were observed. The decreased $S_o$ might have precluded the migration of such proteins to the interface. A similar trend in $d_{43}$ values was observed for these systems at t=12 h, so the factors accounting for emulsion droplet sizes during storage may be similar to those stated previously. Overall, the particle size of emulsions gradually increased with increasing storage time, due to individual droplet growth (Ostwald ripening) or droplet aggregation (flocculation or coalescence).[25] At t=24 h, the studied emulsions presented a significant amount of ruptured/collapsed droplets, because of which their $d_{43}$ values could not be calculated. Taken together, these data suggest that an efficient method to stabilize the oil-water or air-water interface consists in using protein from a meal treated with low acidic solvents, having an intermediate degree of hydrophobicity.

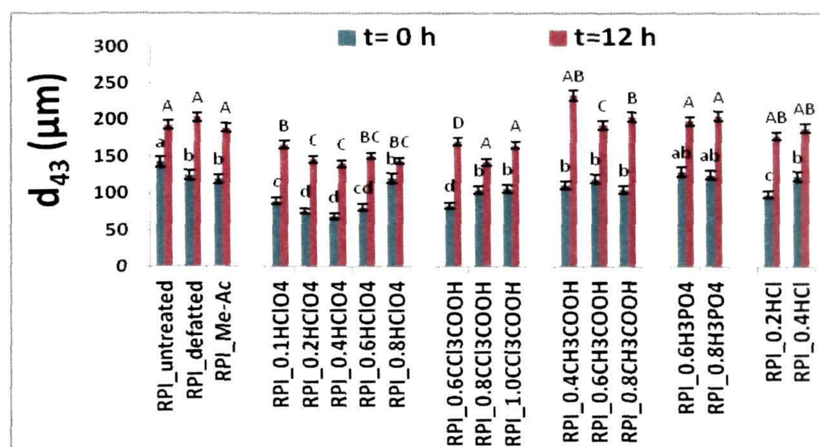



**Fig. 3.13c** Impact of different isolates on the average droplet-size ($d_{43}$) of o/w emulsions stored at 25°C for t=0 h and t=12 h. Bars with different letters in each type of analysis indicate significant difference ($p<0.05$).

### 3.3.4. Hemocompatibility of the isolates recuperated from untreated and solvent-treated meals

The presence of high amount of antinutrients in rapeseed meal has been shown to have detrimental effect on mammalian RBCs in our earlier chapter (Chapter-2). So

concerning the use of these protein isolates, derived from acidified solvent-treated meals, as a food ingredient, their hemocompatibility was tested. According to ASTM E2524-08 standard, hemolysis >5% indicates that the test material causes damage to RBC and releases hemoglobin from lyzed cells; this criterion was exceeded in RPI_untreated , RPI_defatted and isolates from $CH_3COOH$-treated meals (**Fig. 3.14**). Thus it is confirmed that the level of residual antinutrients (such as phytates, polyphenols, AITC, tannins, etc.) present in these isolates are high enough to cause lysis of mammalian RBCs. Health implications of phytates, AITC and polymeric phenols have been reported in numerous publications and scientific papers. High hemolytic activity of the isolates from untreated and defatted meal provides an obvious reason for removing such harmful phytochemicals from rapeseed meal before it can be used as food/feed item. Hemolytic test of the remaining samples were within the defined maximum limit of 5% and did not display any consistent trend. The result vouched the fact that treating the meal with $HClO_4$, $CCl_3COOH$, $H_3PO_4$ or HCl-containing Methanol-Acetone mixture (1:1 v/v) was indeed efficient in eliminating the antinutrients to a substantially low level to ensure product safety.

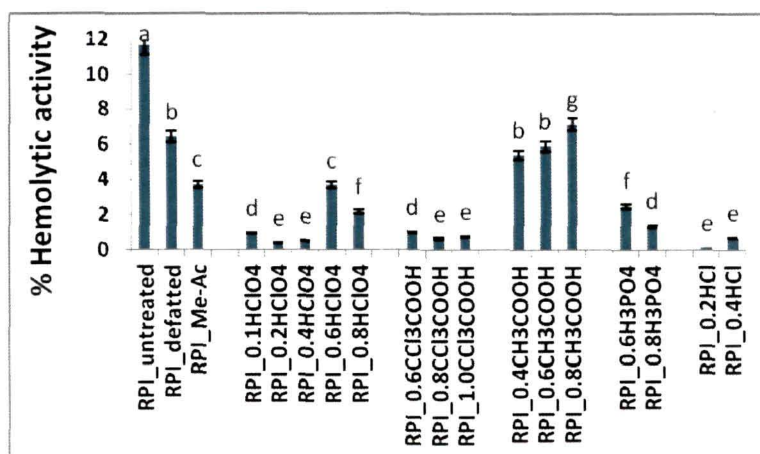



**Fig. 3.14** Percent Red Blood Corpuscles hemolytic activity of the isolates obtained from untreated or solvent-treated meals. Bars with different letters indicate significant difference ($p<0.05$).

### 3.4. Conclusion

Efficiency of the removal of polyphenols and phytates from protein treated with $HClO_4$ acidified solvent was much better than that with other acids, but the extent of AITC removal was similar for all the acids tested. Isolates were essentially free of glucosinolates. The solvent treatment lowered protein yield and its solubility; conversely their colour and interfacial properties were enhanced, but the results did not necessarily match with those obtained from highly acidified solvent-treated meals because the mechanism of denaturation and structural changes caused by different solvent systems were not the same. The treatment also resulted in distinct changes in proteins' secondary and tertiary conformations, especially higher levels of acid led to detrimental effects of protein unfolding and aggregation, which seemed to be more likely consecutive events. Such aggregation-prone conformation tended to enhance thermal stability (higher $T_d$). From a calorimetric point of view, meals treated with lower level of acids seemed to consist of considerably structure-preserved proteins relative to the control. Further evidence that these samples have not undergone appreciable denaturation comes from their electrophoretic patterns. It is evident that 0.2% $HClO_4$ in Me-Ac (1:1 v/v) constitutes a valid extraction mixture giving results that are either higher than or comparable to the other solvents tested, and also does not affect the protein much, as is shown by its physicochemical and functional features. Further investigation should be undertaken to improve the techno-functional properties of such isolates.

### 3.5. References

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# Chapter-4

# Optimization of protein extraction from treated rapeseed meal

## 4.1. Introduction

Isolation of valuable materials from rapeseed oil-cake, such as protein, is crucial for its optimal use. In the course of harnessing this industrial waste, its protein production volume (overall yield), consumer relevance, possible technical application and associated harmful compounds derived along with protein (if any), are pertinent for food or pharmaceutical or any other related industries. The recuperation of proteins present in rapeseed meal, making it feasible for use in human food, is important due to its balanced amino acids composition,[1] and excellent techno-functional properties which are comparable with those of soy, sunflower and other leguminous proteins.[2] However compared to soy protein industry, the rapeseed/canola protein has not had as much opportunity or volume to develop, mainly due to its poor dark brown-black appearance, caused by association of oxidized or polymerized polyphenolic compounds with protein, especially during conventional alkaline extraction. In this connection, several attempts have been made for the production of oilseed protein with reduced colour, using membrane-based extraction, ultrafiltration, diafiltration, ion-exchange and protein micellar mass methods.[3,4] However, all these processes failed to make any noticeable improvement in either the colour of the isolate or the protein content.[5] An effective search for efficient and cheap means for obtaining light-coloured rapeseed protein still remains a challenge.

For rapeseed protein to occupy a good position in the commercial chain like other vegetable proteins, extraction steps need to be followed up and improved. As alkaline protein extraction is the most widely used technique among the authors, so simple feasible changes need to be incorporated in specific parts of the process. In particular, attention must be paid to those parts of the extraction protocol which may conceal sources of pitfalls, thereby deteriorating the product quality. In practice, attention is now being focused on the production and use of protein concentrates/isolates from partially dephenolized meal,[6] because recovery of vegetable proteins, devoid of co-extracted polyphenols, from the rapeseed/canola meal is not possible due to the strong covalent bond of polyphenol-protein complex.[7] Also, very low level of polyphenol-protein

complex seems to have beneficial effects in imparting superior techno-functional properties,[8,9] without imparting much negative effect on the colour of the resulting protein concentrate/isolate. Perusal of literature reveals that variation of physicochemical parameters such as pH, ionic strength, temperature, extraction time and solid-to-liquid ratio influence protein extraction efficiency. There is a multitude of references related to the solubilisation of vegetable proteins in water or NaCl solution with widely varying pH levels (controlled by the addition of NaOH or HCl), with or without the presence of reducing agent such as sodium sulfite or ascorbic acid.[10,11] Although the effects of all those process parameters on protein extraction from different plant sources have been assessed separately, a systematic study of their concerted application is still lacking. Subsequent retrieval of protein by acidic isoelectric precipitation is very common. Recently, this traditional isoelectric precipitation method has been found to impart adverse effects on extraction yield (due to multiple isoelectric points of oilseed proteins)[1,2] and on functional properties of the protein isolate.[12] Therefore, protein recovery should be maximized by use of alternative precipitation methods in order to optimize the overall protein yield and its functional properties. In this perspective, protein precipitation by ammonium sulphate is preferable since it allows precipitation of maximum amount of dissolved proteins.[13,14] It is also of particular interest in the field of proteomics, because this salt does not cause any adverse denaturation to the resulting isolate, thereby granting an extra asset to the product.[12]

Only scant attention has been paid to the optimization of protein extraction from oilseed meal. When exploring the suitability of aforementioned factors for their potential use in rapeseed protein extraction, it would be rational to select an extraction system, which would efficiently extract high yield of whiter protein, having higher emulsification activity and lower phytate level. Protein emulsions are encountered in many areas including cosmetics, food, pharmaceutics, etc. Optimization of emulsification ability of a vegetable protein is advantageous because protein emulsions, due to their satisfactory stability over a certain storage time and high bioavailability, have attained particular interest as delivery systems for bioactive substances.[15] Proteins are preferred over low molecular weight surfactants for emulsification purposes in foods.[16] Liu et al.[12] showed

that the peanut protein obtained during isoelectric precipitation gave poorer functional properties than those obtained by ammonium sulphate precipitation, particularly those related with emulsifying properties. Manamperi et al.[17] optimized the effect of solubilisation and precipitation pH values on the yield and functional properties of canola protein isolate, wherein the authors found that emulsification property of canola protein is sensitive to solubilisation pH, giving better results at high alkaline pH. Nonetheless, no work has been reported regarding the emulsification properties of rapeseed protein, extracted using alkaline dissolution and ammonium sulphate precipitation. In the same way, optimization of extraction conditions for phytate reduction from rapeseed protein has also been overlooked. Presence of phytate in rapeseed and its products has greatly maligned its application as functional food additive. Phytate is present in canola meal at levels as high as 5-7%.[18] Even if the work of Harland and Morris[19] suggests that phytic acid may have some positive anticarcinogenic and antioxidant effects, it is also well established that phytic acid, being an anti-nutritional factor, acts as a strong chelator, forming protein and mineral-phytic acid complexes; the net result being reduced protein and mineral bioavailability. In addition, it inhibits the action of gastrointestinal tyrosinase, trypsin, pepsin, lipase and amylase.[20] For reducing phytates from soy or rapeseed protein, few authors have suggested the use of ultrafiltration,[21] bipolar membranes electrodialysis[22] or phytase enzyme;[23] however application of enzymes or sophisticated membrane technologies at an industrial scale is challenging due to their high cost.[24] As such, it is of interest to develop alternative extraction process for the production of rapeseed protein with low phytic acid.

While numerous articles have mentioned the usefulness of rapeseed press-cake as a potent source of high-quality edible protein; but none of the study till date has proposed an optimization strategy for simultaneous improvement in yield, whiteness, phytate reduction as-well-as emulsification capacity of the recuperated protein. A methodical optimization study was conducted and the results are reported in this chapter.

## 4.2. Materials and methods

### 4.2.1. Materials

Rapeseed press-cake was procured from Assam Khadi and Village Industries Board, Guwahati, Assam, India. The press-cake was ground to pass through 60 mesh size sieve, and then stored at -18°C for further analysis. All solvents and reagents were obtained from Merck® (India), of analytical grade. Bovine serum albumin (BSA) was procured from Sigma Chemicals Co. (St. Louis, MO, US).

### 4.2.2. Preparation of defatted, partially dephenolized meal (treated meal)

Ground meal was defatted using diethyl ether for reducing the lipid content to <0.1% (by Soxhlet method). Since the dark colour of oilseed protein is mainly caused by its phenolic compounds, as a result of the removal of them the colour of the product is expected to become lighter. So, defatted meal was extracted with solvent containing 0.2% perchloric acid in methanol:acetone (1:1 v/v) mixture at a meal-to-solvent ratio of 1:20 (w/v) by mixing the suspension at 200 rpm for 2 h (at 25 °C) in an orbital shaker (Sartorius Stedin Biotech, CERTOMAT® IS). Suspension was then centrifuged (SIGMA 3-18K Centrifuge) at 5000 rpm for 20 min (at 4°C), and the residue (defatted, partially dephenolized meal or simply 'treated' meal) was dried in a vacuum oven under reduced pressure (150 mmHg) at 35±2°C for 42 h and was ground again to pass through a 60 mesh sieve to obtain fine powder and then stored at -18°C until use.

### 4.2.3. Screening of extraction pH (Series 1)

In a preliminary trial, the effect of different pH level on rapeseed protein yield and colour was determined. Aqueous suspension of defatted meal was prepared with water (10:1 v/w); the pH of the suspensions was adjusted to values in the range of 2-13 with HCl or NaOH solution; the suspensions were mixed (200 rpm) for 2 h at 25 °C in the orbital shaker, followed by centrifugation at 8000 rpm for 20 min at 4 °C. The supernatant was filtered through Whatman filter no. 41 and the volume of clarified extract was noted. Ammonium sulfate was added into the supernatant to 85% saturation[12] and the mixture was kept in ice bath for 3 h with gentle stirring and then centrifuged. The obtained protein precipitate was re-dispersed in Milli-Q water (Millipore Water Purification System, Model-Elix, USA). A known aliquot of this protein solution was

analyzed for protein content determination by Lowry method, using BSA as standard.[5] From these results, optimal pH value was chosen for further optimization process.

### 4.2.4. Optimization of extraction parameters (Series 2)

Rapeseed protein was extracted from defatted, partially dephenolized meal, with selected 31 combinations of independent variables such as extraction time (1–5 h), solvent:meal ratio (10:1–30:1 v/w), NaCl concentration (0–0.2 M) and sodium sulfite concentration (0–0.4%) as per central composite design (CCD) (**Table 4.1**). Meal extracts were prepared by constant mixing of treated meal-solvent mixture in an orbital shaker set at 200 rpm (25 °C). The solvent consisted of an alkaline solution of pH 11, to which sodium chloride and sodium sulfite were added at each of the indicated concentrations in the design.[25,26] Subsequently, the slurry was centrifuged, filtered and the protein was recuperated and then re-dispersed in Milli-Q water by the same method as described in Series 1. A known aliquot of this protein solution was analyzed for protein content by the Lowry method, using BSA as standard. Relating the protein amount of the extract to that of the rapeseed meal used (44.8% of dry matter, determined by Kjeldahl method), protein extractability was calculated as relative protein yield (%), as it is of importance in overall protein turn-over of the production process as a whole.[27]

### 4.2.5. Analyses

#### 4.2.5a Colour measurement

All the precipitated protein isolates displayed a similar off-white colour. However, upon dissolution in water, their solutions showed light brown colour of different intensities; an observation that was consistent with previous report on canola protein isolates.[7] Therefore, following the protocol of Xu and Diosady,[7] colorimetric evaluations were performed by scanning their aqueous solution, using Hunter Lab Colorimeter (Ultrascan, VIS-Hunter Associates Lab., USA). Measurements of Hunter-L (absolute lightness=100; absolute darkness=0), Hunter-a (+a=redness; -a=greenness) and Hunter-b (+b=yellowness; -b=blueness) values were taken and denoted as Whiteness Index, as described in our earlier chapter (Chapter-3).

#### 4.2.5b Estimation of phytates

Definite aliquots of known protein concentration (2 mg/ml) were used for phytate determination using Wade reagent according to the method of Bhandari and Kawabata,[28] as described in our earlier chapter (Chapter-3). Results were expressed as mg sodium phytate equivalent per 100 g protein.

#### 4.2.5c Emulsion capacity and emulsion stability

Emulsion properties were studied according to the method described by Hassan et al,[29] the details of which is given in Chapter-3.

### 4.2.6. Statistical analyses

Data from central composite design (CCD) were approximated to a second-order polynomial equation and analysis of variance (ANOVA) was generated. Statistical analysis was performed using STATISTICA (version 7, StatSoft, Oklahoma), MINITAB (version 15, Minitab Inc., US) and Design-Expert (version 6, Stat-Ease Inc., MI, USA) softwares. Effects of variables on responses were discussed by evaluation of Standardized Pareto charts. Relative standard error of the estimate (RSEE), observed between the experimental and predicted results was determined from Eq. (1).

$$RSEE(\%) = \frac{100}{n}\sum_{i=1}^{n}\left|\frac{Y_{exp} - Y_{mod}}{Y_{exp}}\right| \quad (1)$$

where, $Y_{exp}$ and $Y_{mod}$ are the values obtained from experiments and from the model, respectively. n is the number of points at which measurements were carried out.[30]

## 4.3. Result and discussion

### 4.3.1. Influence of pH on protein yield and colour

In order to develop a viable protein recovery process, the influence of pH on protein yield and colour was explored in preliminary trials and the most suitable range of pH for protein extraction was used for subsequent process optimization. Protein extractability increased markedly as the pH was increased, especially above 9 and reached a maximum at pH 12, confirming the trend reported by earlier authors.[4,5,7] Generally, oilseed proteins have higher solubility in strong alkaline solutions and high solubility is a basic requirement for high overall yield.[17] Alkali present in the aqueous solvent system loosens the texture of protein matrices and enhances its solubility.[2] Aside from major effects of pH on protein yield, the expected strong dependence of pH on the protein solution colour became evident. A shift of the colour from faint yellow to dark brownish-green with rising pH levels was observed (**Fig. 4.1**). This observation is in agreement with Xu & Diosady[7] and Pickardt et al.[27] Alkaline oilseed protein extraction typically yields unpleasant dark-colour, mainly due to oxidation of phenolic compounds present in meal. So, dephenolization prior to alkaline protein extraction is indispensible for production of light-coloured protein, and therefore further optimization tests were performed using defatted, partially dephenolized meal. High alkaline pH of 12 has been mostly used in rapeseed/canola protein production; however, operating above pH 11 is impractical because exposing the protein to high pH ($\geq$12) increases the risk of lysinoalanine formation, which would render the protein toxic. Furthermore, at very high pH values ($\geq$13) (especially with elevated temperatures), alkaline hydrolysis of peptide bonds can take place causing uncontrollable cleavage of the protein molecules.[17] Since, raising the pH above 11 resulted in only a slight increase in protein yield ($p>0.05$); we therefore selected pH 11 for all further extraction experiments.

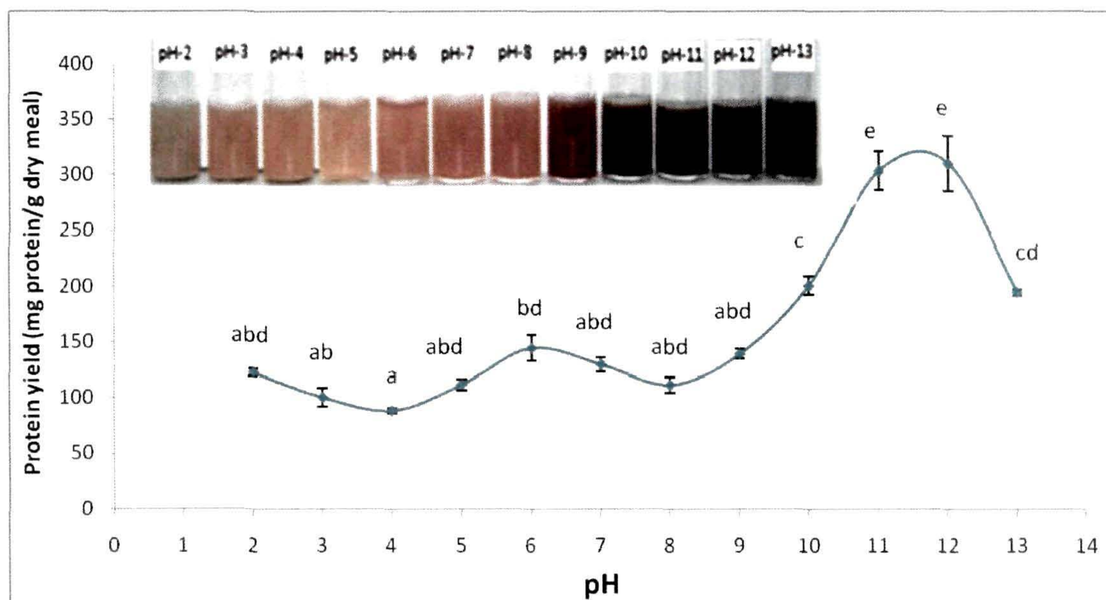



**Fig. 4.1** Effect of different pH levels on protein yield and colour of the extracts obtained from defatted rapeseed meal. Values are mean of three replicates (n=3); Values with the same letter were not statistically different ($p$>0.05) (by Tukey test).

**Table 4.1** Central Composite design at various protein extraction conditions from rapeseed press-cake, defined through the independent variables along with the observed values of the dependent variables.

| Std Order | Uncoded and coded (between parentheses) values of independent variables | | | | Responses (dependent variables) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| . | Extraction time (h) ($X_A$) | Solvent:meal ratio (vol/wt) ($X_B$) | NaCl conc. (M) ($X_C$) | Sodium sulfite conc. (%) ($X_D$) | Relative protein yield (%) ($Y_1$) | Whiteness Index ($Y_2$) | Phytate content (mg Na-phytate equivalent/100g protein) ($Y_3$) | Emulsion capacity (%) ($Y_4$) | Emulsion stability (%) ($Y_5$) |
| *Factorial points* | | | | | | | | | |
| 1 | 2 (-1) | 15 (-1) | 0.05 (-1) | 0.1 (-1) | $19.8\pm0.53^{a}$ | $51.7\pm1.7^{a}$ | $1.2\pm0.12^{a}$ | $47.9\pm1.3^{a}$ | $50.4\pm1.3^{abf}$ |
| 2 | 4 (+1) | 15 (-1) | 0.05 (-1) | 0.1 (-1) | $24.4\pm0.25^{bc}$ | $44.6\pm0.28^{ab}$ | $0.95\pm0.12^{ab}$ | $49.0\pm1.4^{ac}$ | $50.2\pm1.2^{abf}$ |
| 3 | 2 (-1) | 25 (+1) | 0.05 (-1) | 0.1 (-1) | $25.3\pm0.05^{bc}$ | $66.5\pm2.4^{c}$ | $0.65\pm0.02^{bc}$ | $46.9\pm0.8^{a}$ | $50.9\pm1.6^{abf}$ |
| 4 | 4 (+1) | 25 (+1) | 0.05 (-1) | 0.1 (-1) | $23.7\pm0.31^{bcd}$ | $65.2\pm2.4^{c}$ | $0.52\pm0.05^{c}$ | $48.4\pm0.86^{ac}$ | $48.8\pm0.83^{ab}$ |
| 5 | 2 (-1) | 15 (-1) | 0.15 (+1) | 0.1 (-1) | $23.1\pm0.31^{cd}$ | $46.7\pm0.71^{ab}$ | $1.0\pm0.04^{ab}$ | $48.3\pm0.31^{a}$ | $49.0\pm1.4^{ab}$ |
| 6 | 4 (+1) | 15 (-1) | 0.15 (+1) | 0.1 (-1) | $25.5\pm1.2^{bc}$ | $50.8\pm1.7^{a}$ | $1.1\pm0.10^{abd}$ | $50.7\pm1.8^{ac}$ | $55.7\pm0.23^{c}$ |
| 7 | 2 (-1) | 25 (+1) | 0.15 (+1) | 0.1 (-1) | $25.7\pm0.16^{bc}$ | $57.4\pm1.2^{ad}$ | $0.66\pm0.04^{c}$ | $52.5\pm0.67^{b}$ | $44.3\pm1.1^{d}$ |
| 8 | 4 (+1) | 25 (+1) | 0.15 (+1) | 0.1 (-1) | $26.3\pm0.22^{b}$ | $66.5\pm0.52^{c}$ | $0.55\pm0.02^{c}$ | $51.4\pm1.6^{bc}$ | $47.9\pm1.3^{ae}$ |
| 9 | 2 (-1) | 15 (-1) | 0.05 (-1) | 0.3 (+1) | $19.8\pm0.50^{a}$ | $56.9\pm2.7^{a}$ | $1.1\pm0.06^{ab}$ | $48.5\pm0.67^{a}$ | $47.3\pm1.1^{ae}$ |
| 10 | 4 (+1) | 15 (-1) | 0.05 (-1) | 0.3 (+1) | $21.3\pm1.6^{ad}$ | $40.9\pm3.6^{b}$ | $0.71\pm0.01^{bc}$ | $51.3\pm1.3^{bc}$ | $45.2\pm1.4^{de}$ |
| 11 | 2 (-1) | 25 (+1) | 0.05 (-1) | 0.3 (+1) | $31.3\pm0.20^{f}$ | $64.5\pm1.3^{cd}$ | $0.46\pm0.08^{c}$ | $47.6\pm0.96^{a}$ | $49.3\pm0.31^{ab}$ |
| 12 | 4 (+1) | 25 (+1) | 0.05 (-1) | 0.3 (+1) | $23.9\pm1.3^{bc}$ | $61.6\pm1.2^{cd}$ | $0.56\pm0.05^{c}$ | $49.5\pm0.79^{abc}$ | $47.4\pm0.92^{ae}$ |
| 13 | 2 (-1) | 15 (-1) | 0.15 (+1) | 0.3 (+1) | $23.3\pm0.57^{ce}$ | $55.0\pm0.42^{a}$ | $0.82\pm0.10^{bc}$ | $47.1\pm0.59^{a}$ | $50.7\pm1.8^{abf}$ |
| 14 | 4 (+1) | 15 (-1) | 0.15 (+1) | 0.3 (+1) | $22.7\pm0.28^{ce}$ | $52.9\pm0.86^{a}$ | $0.83\pm0.14^{bc}$ | $47.4\pm0.92^{a}$ | $52.7\pm0.84^{f}$ |
| 15 | 2 (-1) | 25 (+1) | 0.15 (+1) | 0.3 (+1) | $33.4\pm0.88^{f}$ | $55.8\pm4.7^{a}$ | $0.53\pm0.01^{c}$ | $47.2\pm0.79^{a}$ | $48.6\pm1.2^{ab}$ |
| 16 | 4 (+1) | 25 (+1) | 0.15 (+1) | 0.3 (+1) | $24.5\pm0.00^{bc}$ | $63.8\pm4.7^{cd}$ | $0.57\pm0.10^{c}$ | $45.9\pm0.49^{a}$ | $50.0\pm0.00^{abf}$ |
| *Axial points* | | | | | | | | | |
| 17 | 1 ($-2^{4/4}$) | 20 (0) | 0.10 (0) | 0.2 (0) | $26.8\pm2.4^{bc}$ | $58.3\pm3.0^{a}$ | $0.75\pm0.15^{bc}$ | $47.4\pm0.49^{a}$ | $47.4\pm0.51^{ae}$ |
| 18 | 5 ($+2^{4/4}$) | 20 (0) | 0.10 (0) | 0.2 (0) | $26.5\pm0.90^{bc}$ | $59.4\pm1.6^{cd}$ | $0.75\pm0.03^{bc}$ | $48.4\pm1.5^{ac}$ | $49.4\pm0.86^{ab}$ |
| 19 | 3 (0) | 10 ($-2^{4/4}$) | 0.10 (0) | 0.2 (0) | $20.6\pm0.67^{ae}$ | $39.3\pm0.92^{b}$ | $1.6\pm0.07^{d}$ | $48.0\pm1.4^{a}$ | $51.3\pm0.14^{bf}$ |
| 20 | 3 (0) | 30 ($+2^{4/4}$) | 0.10 (0) | 0.2 (0) | $27.2\pm0.15^{b}$ | $66.2\pm1.3^{d}$ | $0.57\pm0.04^{c}$ | $49.0\pm0.08^{ac}$ | $50.4\pm0.55^{bf}$ |
| 21 | 3 (0) | 20 (0) | 0.00 ($-2^{4/4}$) | 0.2 (0) | $25.4\pm0.05^{bc}$ | $60.5\pm1.6^{cd}$ | $0.58\pm0.13^{c}$ | $47.9\pm1.3^{a}$ | $49.3\pm0.47^{ab}$ |
| 22 | 3 (0) | 20 (0) | 0.20 ($+2^{4/4}$) | 0.2 (0) | $25.9\pm1.2^{bc}$ | $54.8\pm4.5^{a}$ | $0.71\pm0.04^{bc}$ | $49.1\pm1.2^{ac}$ | $49.5\pm0.02^{ab}$ |

**Table 4.1** Central Composite design at various protein extraction conditions from rapeseed press-cake, defined through the independent variables along with the observed values of the dependent variables (*continued*)

| Std Order | Uncoded and coded (between parentheses) values of independent variables | | | | Responses (dependent variables) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Extraction time (h) ($X_A$) | Solvent:meal ratio (vol/wt) ($X_B$) | NaCl conc. (M) ($X_C$) | Sodium sulfite conc. (%) ($X_D$) | Relative protein yield (%) ($Y_1$) | Whiteness Index ($Y_2$) | Phytate content (mg Na-phytate equivalent/100g protein) ($Y_3$) | Emulsion capacity (%) ($Y_4$) | Emulsion stability (%) ($Y_5$) |
| 23 | 3 (0) | 20 (0) | 0.10 (0) | 0.0 ($-2^{4/4}$) | 24.3±0.52[bc] | 63.8±2.5[cd] | 0.80±0.14[bc] | 54.9±1.1[b] | 48.1±0.00[ab] |
| 24 | 3 (0) | 20 (0) | 0.10 (0) | 0.4 ($+2^{4/4}$) | 25.1±1.9[bc] | 60.7±3.2[cd] | 0.46±0.13[c] | 51.9±1.2[bc] | 48.7±0.62[ab] |
| *Centre points* | | | | | | | | | |
| 25 | 3 (0) | 20 (0) | 0.10 (0) | 0.2 (0) | 26.1±0.55[bc] | 59.2±1.8[cd] | 0.63±0.07[c] | 47.5±0.67[a] | 49.4±1.9[ab] |
| 26 | 3 (0) | 20 (0) | 0.10 (0) | 0.2 (0) | 26.4±1.7[bc] | 56.9±0.66[ad] | 0.75±0.10[bc] | 47.5±0.67[a] | 50.5±0.67[abf] |
| 27 | 3 (0) | 20 (0) | 0.10 (0) | 0.2 (0) | 26.7±1.2[b] | 58.2±2.2[ad] | 0.64±0.08[c] | 47.1±1.3[a] | 49.4±0.96[ab] |
| 28 | 3 (0) | 20 (0) | 0.10 (0) | 0.2 (0) | 25.3±1.4[bc] | 57.9±2.1[ad] | 0.70±0.05[bc] | 47.5±2.1[a] | 49.4±0.86[ab] |
| 29 | 3 (0) | 20 (0) | 0.10 (0) | 0.2 (0) | 26.5±0.74[bc] | 56.4±3.1[ad] | 0.59±0.02[c] | 47.7±0.84[a] | 48.3±0.27[ab] |
| 30 | 3 (0) | 20 (0) | 0.10 (0) | 0.2 (0) | 27.1±0.31[b] | 57.9±1.5[ad] | 0.62±0.14[c] | 47.3±0.94[a] | 48.2±0.26[ab] |
| 31 | 3 (0) | 20 (0) | 0.10 (0) | 0.2 (0) | 27.1±1.4[b] | 56.6±1.2[ad] | 0.65±0.21[c] | 47.6±1.6[a] | 49.7±0.42[abf] |

Duplicate set of each experimental run was performed and analyzed twice (i.e. n = 2x2 = 4). Values are means±standard deviation of n=4 analyses (subjected to Tukey test). Means with the same letter within one column were not statistically different ($p>0.05$). Each experimental value after the 3rd decimal place has been rounded off.

All factors were encoded ($X_A$—$X_D$), using -2 for the lowest level of a factor ($-\alpha$) and +2 for the respective maximum ($+\alpha$) with equidistant intermediate stages [$X_A = (X_t-3)/1$; $X_B = (X_{s/m}-20)/5$; $X_C = (X_{NaCl}-0.1)/0.05$; $X_D = (X_{Na_2SO_3} - 0.2)/0.1$].

**Table 4.2** Regression coefficients for the response surface models in terms of coded and uncoded units, along with the associated probability ($p$ value).

| Term | $Y_1$ (Relative protein yield) | | | $Y_2$ (Whiteness Index) | | | $Y_3$ (Phytate content) | | | $Y_4$ (Emulsion capacity) | | | $Y_5$ (Emulsion stability) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Coefficient | | $p$ | Coefficient | | $p$ | Coefficient | | $p$ | Coefficient | | $p$ | Coefficient | | $p$ |
| | Coded | Uncoded | | Coded | Uncoded | | Coded | Uncoded | | Coded | Uncoded | | Coded | Uncoded | |
| Constant | 26.5 | -18.9 | 0.00* | 57.6 | 28.6 | 0.00* | 0.65 | 4.6 | 0.00* | 47.5 | 43.2 | 0.00* | 49.3 | 54.1 | 0.00* |
| Extraction time | -0.43 | 9.3 | 0.02* | -0.25 | -12.7 | 0.47 | -0.03 | -0.37 | 0.08 | 0.39 | 2.9 | 0.02* | 0.48 | 1.7 | 0.00* |
| Solvent:meal | 2.0 | 2.2 | 0.00* | 6.5 | 3.6 | 0.00* | -0.22 | -0.25 | 0.00* | 0.04 | 0.02 | 0.81 | -0.66 | -0.38 | 0.00* |
| NaCl | 0.68 | 68.7 | 0.00* | -0.60 | -63.5 | 0.09 | 0.01 | -2.9 | 0.55 | 0.14 | 27.9 | 0.39 | 0.41 | -7.3 | 0.01* |
| Sodium sulfite | 0.33 | 20.9 | 0.07 | -0.18 | 37.2 | 0.61 | -0.07 | -2.1 | 0.00* | -0.70 | -17.3 | 0.00* | -0.21 | -28.8 | 0.19 |
| Extraction time x Extraction time | -0.05 | -0.05 | 0.74 | 0.05 | 0.05 | 0.87 | 0.02 | 0.02 | 0.14 | -0.03 | -0.03 | 0.85 | -0.22 | -0.22 | 0.14 |
| Solvent:meal x Solvent:meal | -0.74 | -0.03 | 0.00* | -1.5 | -0.06 | 0.00* | 0.11 | 0.00 | 0.00* | 0.12 | 0.01 | 0.44 | 0.40 | 0.02 | 0.01* |
| NaCl x NaCl | -0.30 | -119.6 | 0.07 | -0.25 | -100.2 | 0.44 | -0.01 | -2.4 | 0.68 | 0.12 | 49.1 | 0.41 | 0.04 | 14.4 | 0.80 |
| Sodium sulfite x Sodium sulfite | -0.55 | -54.9 | 0.00* | 0.90 | 90.0 | 0.01* | -0.01 | -0.89 | 0.53 | 1.3 | 134.4 | 0.00* | -0.23 | -22.5 | 0.12 |
| Extraction time x Solvent:meal | -1.6 | -0.31 | 0.00* | 2.1 | 0.43 | 0.00* | 0.03 | 0.01 | 0.13 | -0.36 | -0.07 | 0.08 | -0.33 | -0.07 | 0.09 |
| Extraction time x NaCl | -0.23 | -4.6 | 0.29 | 2.9 | 57.8 | 0.00* | 0.04 | 0.76 | 0.05* | -0.43 | -8.67 | 0.03* | 1.2 | 24.8 | 0.00* |
| Extraction time x Sodium sulfite | -1.3 | -13.4 | 0.00* | -1.1 | -11.1 | 0.01* | 0.01 | 0.14 | 0.46 | -0.01 | -0.06 | 0.98 | -0.55 | -5.5 | 0.01* |
| Solvent:meal x NaCl | -0.23 | -0.91 | 0.30 | -1.6 | -6.4 | 0.00* | 0.02 | 0.07 | 0.36 | 0.51 | 2.03 | 0.01* | -1.3 | -5.1 | 0.00* |
| Solvent:meal x Sodium sulfite | 1.1 | 2.2 | 0.00* | -1.37 | -2.7 | 0.00* | 0.03 | 0.07 | 0.08 | -0.46 | -0.93 | 0.02* | 0.80 | 1.60 | 0.00* |
| NaCl x Sodium sulfite | 0.02 | 3.8 | 0.93 | 0.64 | 128.7 | 0.14 | -0.00 | -0.44 | 0.91 | -1.2 | -247.3 | 0.00* | 1.0 | 201.6 | 0.00* |

* Significant at $p \leq 0.05$

**Table 4.3** Response surface models for extracting protein from defatted partially dephenolized rapeseed meal.

| **Response** | **Quadratic polynomial model (coefficients in uncoded units)** | ***p*** | **$R^2$** | **$R^2$ (predicted)** | **$R^2$ (adjusted)** | **Lack-of-Fit (*p* value)** | **RSEE (%)** |
|---|---|---|---|---|---|---|---|
| Relative protein yield (%) | $Y_1 = -18.947 + 9.302X_t + 2.161X_{s/m} + 68.673X_{NaCl} - 0.029X_{s/m}^2 - 54.927X_{Na_2SO_3}^2 - 0.314X_tX_{s/m} - 13.368X_tX_{Na_2SO_3} + 2.201X_{s/m}X_{Na_2SO_3}$ | 0.00 | 0.87 | 0.75 | 0.83 | 0.24 | 3.5 |
| Whiteness Index | $Y_2 = 28.621 + 3.562X_{s/m} - 0.059X_{s/m}^2 + 90.008X_{Na_2SO_3}^2 + 0.426X_tX_{s/m} + 57.761X_tX_{NaCl} - 11.070X_tN_{Na_2SO_3} - 6.372X_{s/m}X_{NaCl} - 2.731X_{s/m}X_{Na_2SO_3}$ | 0.00 | 0.91 | 0.83 | 0.89 | 0.09 | 2.9 |
| Phytate content (mg Na-phytate equivalent/100 g protein) | $Y_3 = 4.615 - 0.251X_{s/m} - 2.071X_{Na_2SO_3} + (4.239\times10^{-3})X_{s/m}^2 + 0.756X_tX_{NaCl}$ | 0.00 | 0.87 | 0.76 | 0.83 | 0.43 | 9.1 |
| Emulsion capacity (%) | $Y_4 = 43.190 + 2.864X_t - 17.325X_{Na_2SO_3} + 134.413X_{Na_2SO_3}^2 - 8.665X_tX_{NaCl} + 2.028X_{s/m}X_{NaCl} - 0.928X_{s/m}X_{Na_2SO_3} - 247.264X_{NaCl}X_{Na_2SO_3}$ | 0.00 | 0.79 | 0.59 | 0.72 | 0.16 | 1.7 |
| Emulsion stability (%) | $Y_5 = 54.126 + 1.701X_t - 0.377X_{s/m} - 7.309X_{NaCl} + 0.016X_{s/m}^2 + 24.809X_tX_{NaCl} - 5.452X_tX_{Na_2SO_3} - 5.108X_{s/m}X_{NaCl} + 1.597X_{s/m}X_{Na_2SO_3} + 201.6X_{NaCl}X_{Na_2SO_3}$ | 0.00 | 0.81 | 0.65 | 0.75 | 0.50 | 1.7 |

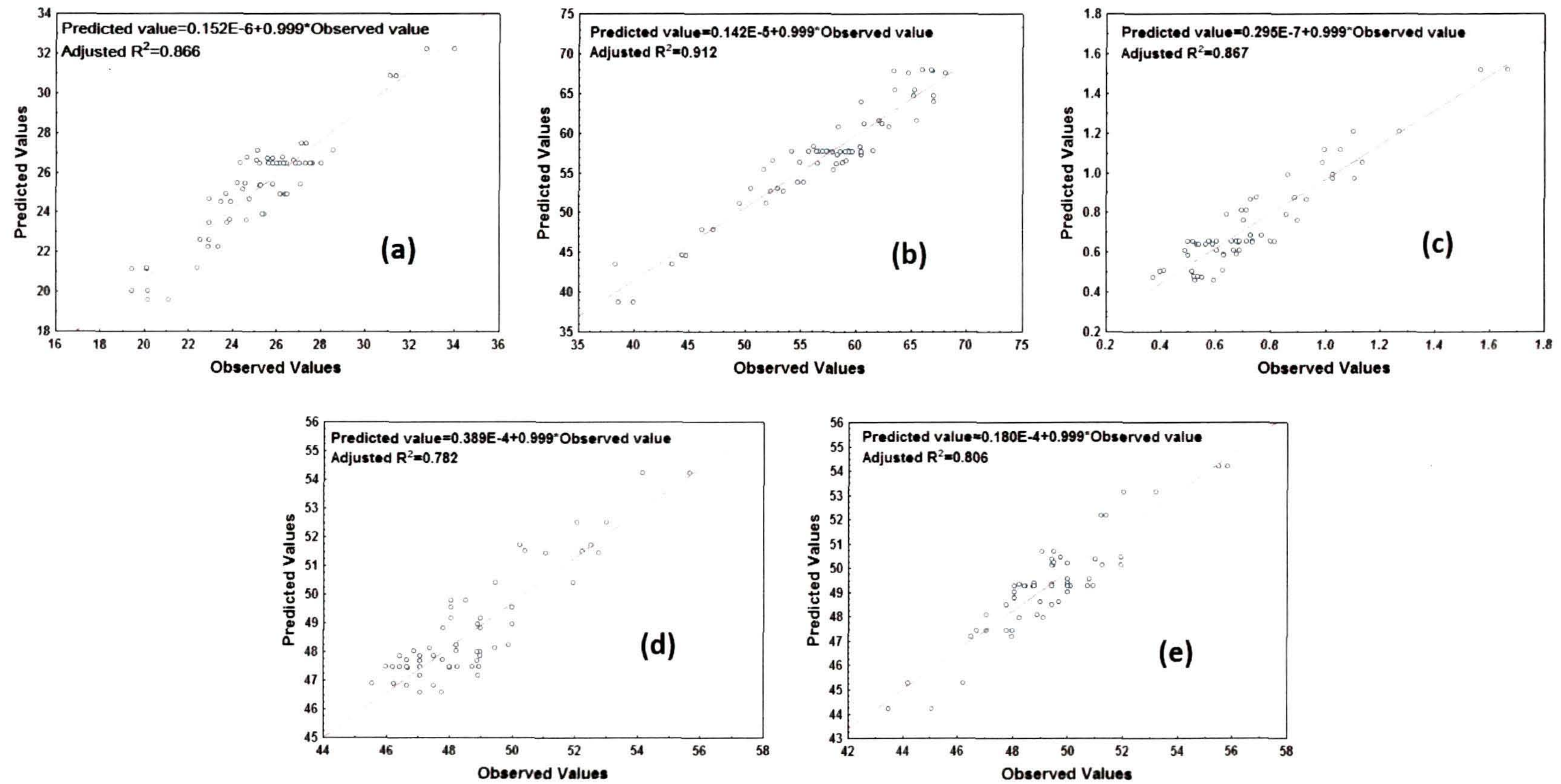



**Fig. 4.2** The fitted line plot indicating the closeness between observed response values and predicted response values for (a) Relative protein yield, (b) Whiteness Index, (c) phytate content, (d) Emulsion capacity, and (e) Emulsion stability.

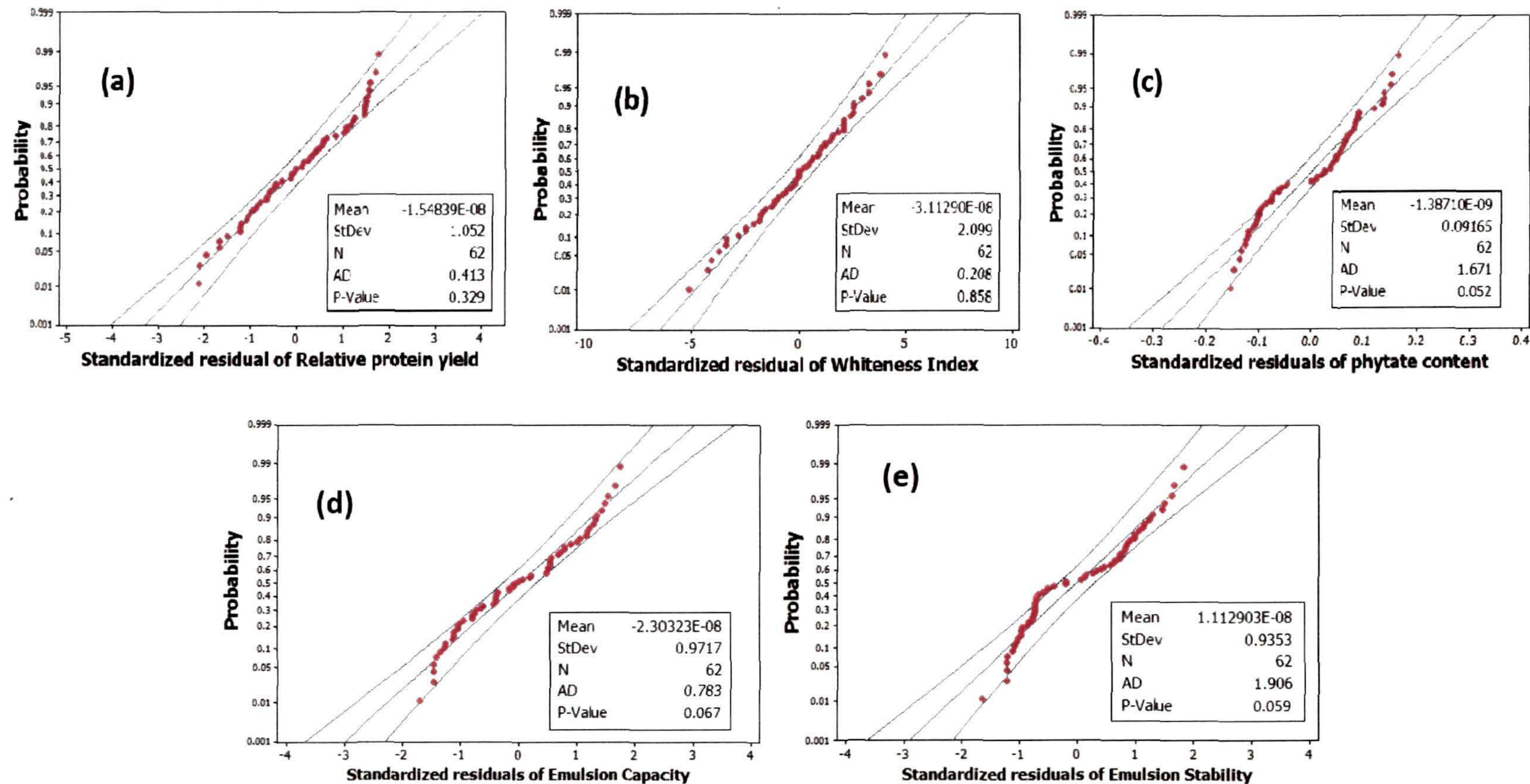



**Fig. 4.3** Normal Probability plots (by Anderson-Darling Normality test within 95% confidence interval) using standardized residuals of the dependent variable (a) Relative protein yield, (b) Whiteness Index, (c) phytate content, (d) Emulsion capacity, and (e) Emulsion stability.

### 4.3.2. Modeling the effects by Response Surface Regression Analysis (RSREG) and diagnostic checking of the fitted models

The CCD matrix and the response values obtained for the combination of 4-factors at 5-level each are given in **Table 4.1**. The estimated coefficients of each model are presented in **Table 4.2**. RSREG procedure was employed to fit the quadratic polynomial equation to the experimental data. To develop the fitted response surface model equations, all insignificant terms ($p$>0.05) were eliminated and the fitted models are shown in **Table 4.3**. The high values of coefficient of determination ($R^2$ and adjusted-$R^2$) (>0.8) suggest that the quadratic models can explain most of variabilities in the observed data, and thus can be considered as valid models. Good correlation existed between observed and predicted values (**Fig. 4.2**). All regression models were highly significant ($p \leq 0.000$) and $p$-values for lack-of-fit test were large ($p$>0.05), which prove that the models are adequate for predicting the responses. Only response $Y_4$ (Emulsion capacity) showed $R^2$ lesser than 0.8, which can be considered satisfactory for data of techno-functional properties.[31] So, accuracy of the models was further evaluated by RSEE[30] and by a normality test (Anderson-Darling normality test) for error terms,[32,33] using standardized residuals of all dependent variables. Smaller Anderson-Darling (AD) values along with $p$-values greater than 0.05, indicate that the distribution fits the data better. The error terms of all dependent variables had the normal distribution in the Anderson-Darling normality test within 95% prediction band (**Fig. 4.3**), meaning that these models are sufficiently accurate for predicting the relevant response(s). Additionally, a model can be considered acceptable if RSEE is <10%; this condition was also satisfied for all the responses (**Table 4.3**).

### 4.3.3. Effects of the extraction factors on the responses: Standardized Pareto chart analyses

Among the factors studied, the solvent:meal ratio had the highest impact on Relative protein yield (factor contribution=22.3%), Whiteness Index (35%) and phytate content (35% by linear term; 19.6% by quadratic term) of the recuperated protein (**Figs. 4.4a-c**). This factor had a more limited influence on emulsion stability (9.47% by linear

term; 6.23% by quadratic term) (**Fig. 4.4d**) and least on emulsion capacity (quadratic term contribution=2.28%; linear term contribution=0.72%) (**Fig. 4.4e**). Solvent:meal ratio (both linear and quadratic terms) showed a significant effect on all the evaluated parameters, except emulsion capacity. The yield increased profoundly ($p<0.05$) with increasing solvent:meal ratio (**Fig. 4.4f**). Solvent:meal ratio has been reported as one of the prime factor affecting protein yield from various plant sources. When the percentage of solvent in a solid-liquid reaction system increases, the reaction proceeds as a result of liquid diffusing, or otherwise penetrating into the interior of the reacting solid. The availability of more liquid increases the driving force of protein out of the meal,[34] and hence the yield increases. Within the experimental region, relative protein yield ranged from 19.4% to 34% (**Table 4.1**), which is much higher than the overall rapeseed protein yield (≈28%) reported by Manamperi et al.[17] Thus, we were successful in optimizing and increasing the protein yield from rapeseed meal better than those reported by earlier authors.

The greatest effect of solvent:meal ratio was on phytate content, which can be attributed to the higher solubility of phytates in water. Maga[35] considered phytates as an impurity in the isolation of protein and stated that when isolation of protein by means of isoelectric precipitation is used, a certain amount of phytates would also precipitate with the protein; extend of binding increases with decreasing pH, especially in low acid pH. Generally binding of phytate with protein at high alkaline pH is very less or negligible.[35] In the current investigation, the use of high alkaline pH might have resulted in lower presence of phytate in the resulting protein. During recuperation of protein, the added ammonium sulphate did not make the solution acidic and as such, less phytates from the extractant solution might have precipitated along with the protein. This recommends a clear advantage of using ammonium sulphate precipitation over isoelectric precipitation. Few workers[23] have suggested the use of salts like NaCl or $CaCl_2$ or both in extraction medium for reducing phytates from vegetable proteins. Although NaCl is being used in the current investigation, it failed to show any significant effect ($p>0.05$) on the phytate level, probably due to very low concentration of NaCl investigated herein. Higher

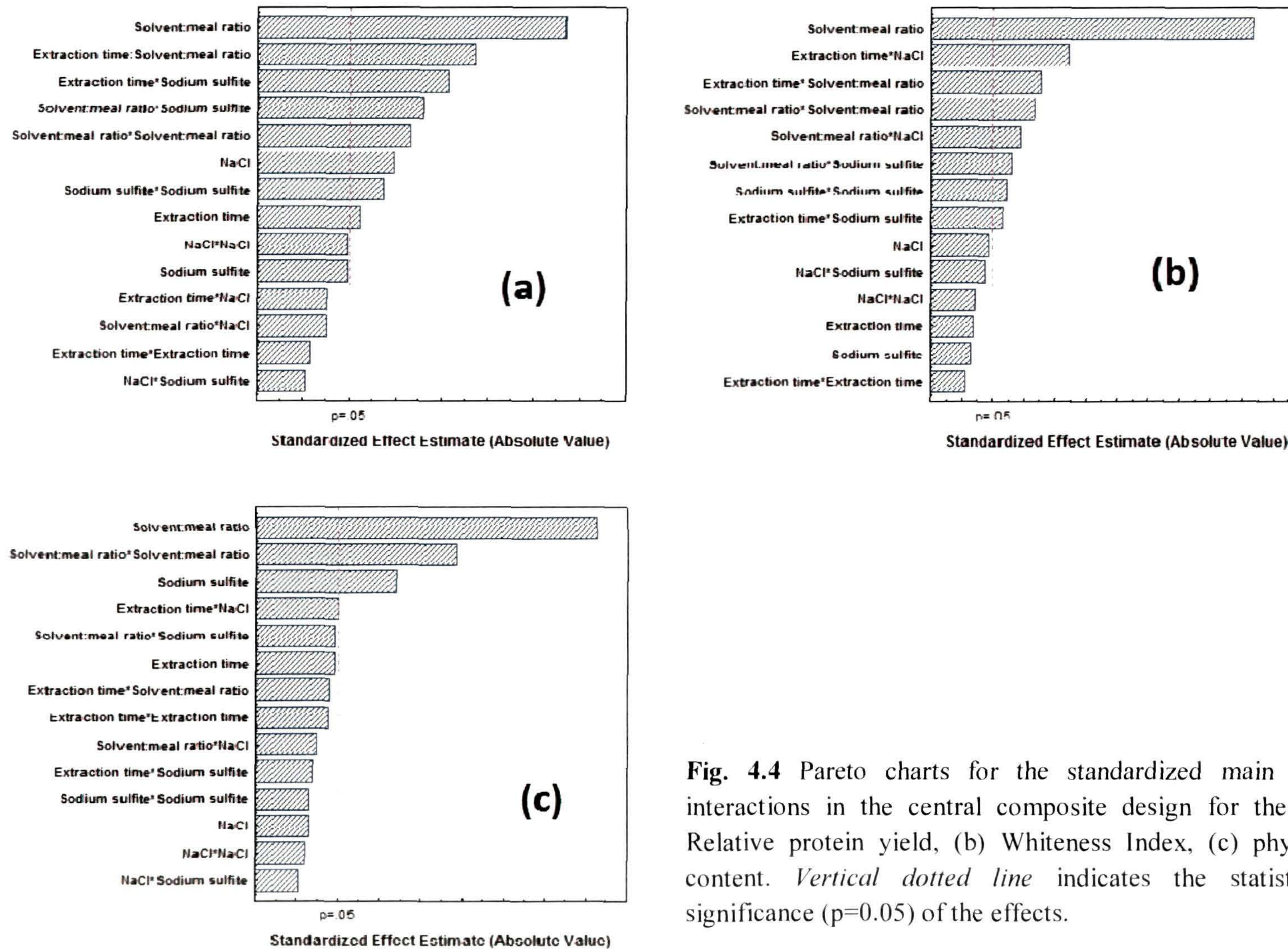
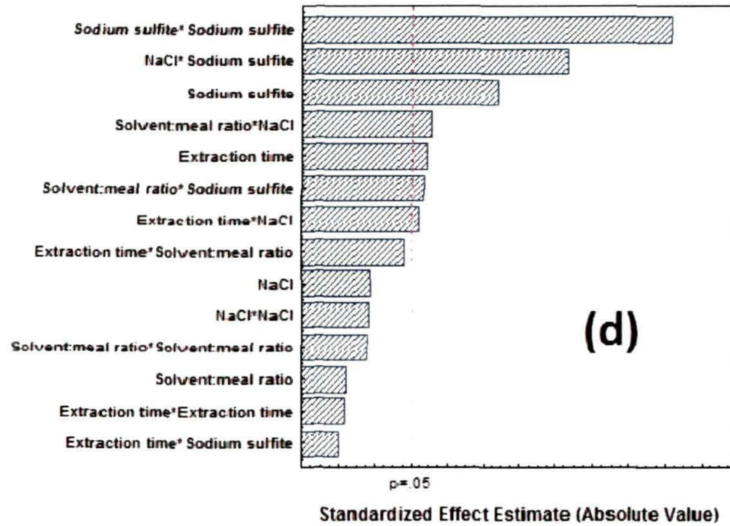
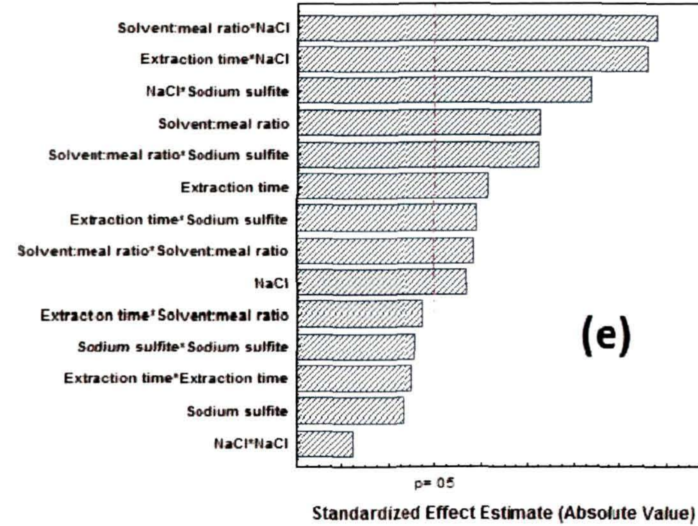



**Fig. 4.4** Pareto charts for the standardized main and interactions in the central composite design for the (a) Relative protein yield, (b) Whiteness Index, (c) phytate content. *Vertical dotted line* indicates the statistical significance (p=0.05) of the effects.



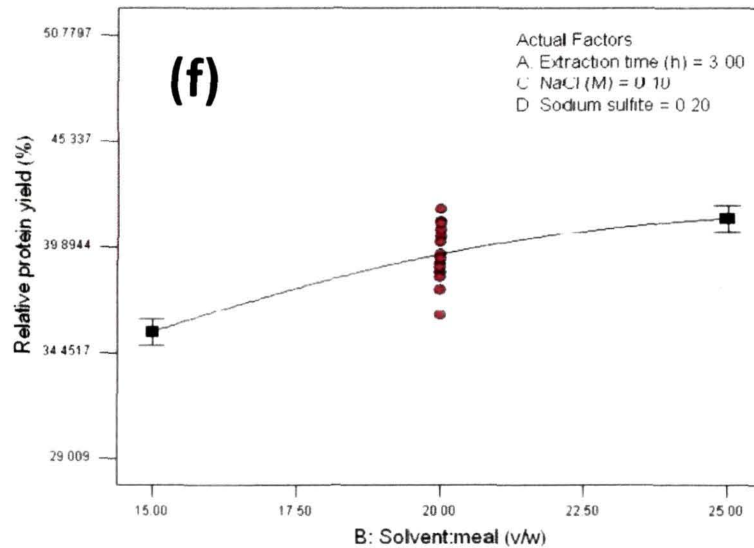




**Fig. 4.4** Pareto charts for the standardized main and interactions in the central composite design for the (d) Emulsion capacity, and (e) Emulsion stability. *Vertical dotted line* indicates the statistical significance (p=0.05) of the effects. (f) One factor plot showing the main effect of solvent:meal ratio on Relative protein yield. *(continued)*

concentration of NaCl may reduce the protein yield by causing salting out effect.[27] Hence, these parameters were not tested.

Presence of sodium sulfite in the extraction medium had the second greatest impact. This is evident from the high impact that this factor had on emulsion capacity (26.77% by quadratic term; 12.8% by linear term) and phytate content (12.21%) (**Figs. 4.4c-d**). The interaction term of sodium sulfite and solvent:meal ratio also showed good influence on relative protein yield (10.15%) and Whiteness Index (6.07%) (**Figs. 4.4a-b**). This is because sulphite helps in enhancing protein solubilisation[36,37] and also prevents oxidation of polyphenols, and thus limits reactions between proteins and oxidized polyphenols that are responsible for dark colour formation.[38]

The impact of NaCl was found to be comparatively low; but it significantly affected the yield (7.65%). Addition of salts enhances protein extractability due to the increase of ionic force promoted by the added NaCl mainly on globulin protein of oilseeds.[27] The predominant effect of salts can be seen on emulsion properties. $Na_2SO_3$ is known for its innate quality of breaking up certain disulfide linkages in protein molecules.[39,40] These smaller fragments of protein then easily orient themselves at the newly created interfaces during emulsification process. The role of interaction term is more evident on emulsification properties (**Figs. 4.4d-e**). For emulsion stability, the top three contributors were interaction terms (**Fig. 4.4e**), involving solvent:meal ratio*NaCl, extraction time*NaCl and sodium sulfite*NaCl; however their impact on the response was found to be comparatively low (15%, 14.6% and 11.8%, respectively). Incidentally, the presence of salts like NaCl in the extractant, favours the dissolution of globulin protein from the meal, which exhibit high surface activity and less rigid structural integrity, compared with the albumin. The arrangement of less rigid protein chain configurations in the film lamellae, introduce additional forces that help formation of stable emulsion So, it can be inferred that the presence of globulin constituent in protein isolate favours the formation and stability of emulsion, and this rationalization aptly explains the enhanced emulsion properties in presence of NaCl.

### 4.3.4. Condition for optimum responses

When various responses have to be considered at the same time, it is necessary to find optimal compromises between the total numbers of responses taken into account. So, in order to optimize five responses simultaneously, Derringer function or desirability function (d) was used because it is the most currently used multi-criteria methodology in optimization procedures. The optimization and individual desirability of each response variable was obtained by specifying the goals and boundaries (**Table 4.4**). The composite desirability was then combined with the individual desirability of all responses into a single measure by geometric mean.[31] The predicted responses and individual desirability are presented in **Table 4.4**. The behaviour of the predicted responses was generated from the optimized factors of extraction time of 1.85 h, solvent:meal ratio of 30:1 v/w, NaCl concentration of 0.0 M and sodium sulfite level of 0.4% (**Fig. 4.5a**). In order to make these parameters feasible in experimental runs, these observed optimum parameters were drawn to the nearest round figures of 2 h of extraction time, 30:1 v/w of solvent:meal ratio, NaCl concentration of 0.0 M and sodium sulfite level of 0.4%.[31] The behaviour of the predicted responses from a feasible experimental run at round figures of factor levels was also generated (**Fig. 4.5b**) and compared with **Fig. 4.5a.**[31] From these rounded figures of factors, the new predicted response values for Relative protein yield, Whiteness Index, *phytate content and emulsion stability slightly decreased, compared to those of the* original suggested optimal factors. The composite desirability (D) slightly reduced to 0.899 from 0.902 (**Fig. 4.5**). This is due to the described converse effects of several responses, i.e. unfeasible conformance to all requirements.[27]

**Table 4.4** Multi-Response optimization and individual desirability obtained by Derringer function.

| Response | Goal | Lower | Target | Upper | Predicted responses | Desirability |
|---|---|---|---|---|---|---|
| $Y_1$ (Relative protein yield; %) | Maximum | 19.4 | 34.0 | 34.0 | 35.2 | 1.0 |
| $Y_2$ (Whiteness Index) | Maximum | 38.4 | 68.2 | 68.1 | 71. 1 | 1.0 |
| $Y_3$ (Phytate content; mg /100g protein) | Minimum | 0.37 | 0.37 | 1. 7 | 0.54 | 0.87 |
| $Y_4$ (Emulsion capacity; %) | Maximum | 45.6 | 55.8 | 55.8 | 52.6 | 0.69 |
| $Y_5$ (Emulsion stability; %) | Maximum | 43.5 | 55.8 | 55.8 | 55.9 | 1.0 |

### 4.3.5. Verification of predicted values

To verify these predicted results, extraction experiments were conducted at the feasible optimal condition (i.e., extraction time of 2 h, solvent:meal ratio of 30:1 v/w, NaCl concentration of 0.0 M and sodium sulfite level of 0.4%). The observed experimental values (mean of 4 measurements) were compared to the predicted values (**Table 4.5**). The predicted values could realistically be achieved within a 95% confidence interval of experimental values or at least within 99% confidence interval, an observation similar to that of Pickardt et al.[27] The obtained experimental values for the responses, in the new feasible condition, were quite close to the predicted optimum values and are in reasonable agreement within the said confidence intervals for these optimized conditions. The closeness between the experimental and predicted values of the quality parameters also indicated the suitability of the corresponding polynomial models. Thus, we were successful in developing an extraction procedure that can produce rapeseed protein, with high yield, acceptable whiteness (**Fig. 4.6**), improved emulsion properties and reduced level of harmful phytates, better than those reported by earlier authors.

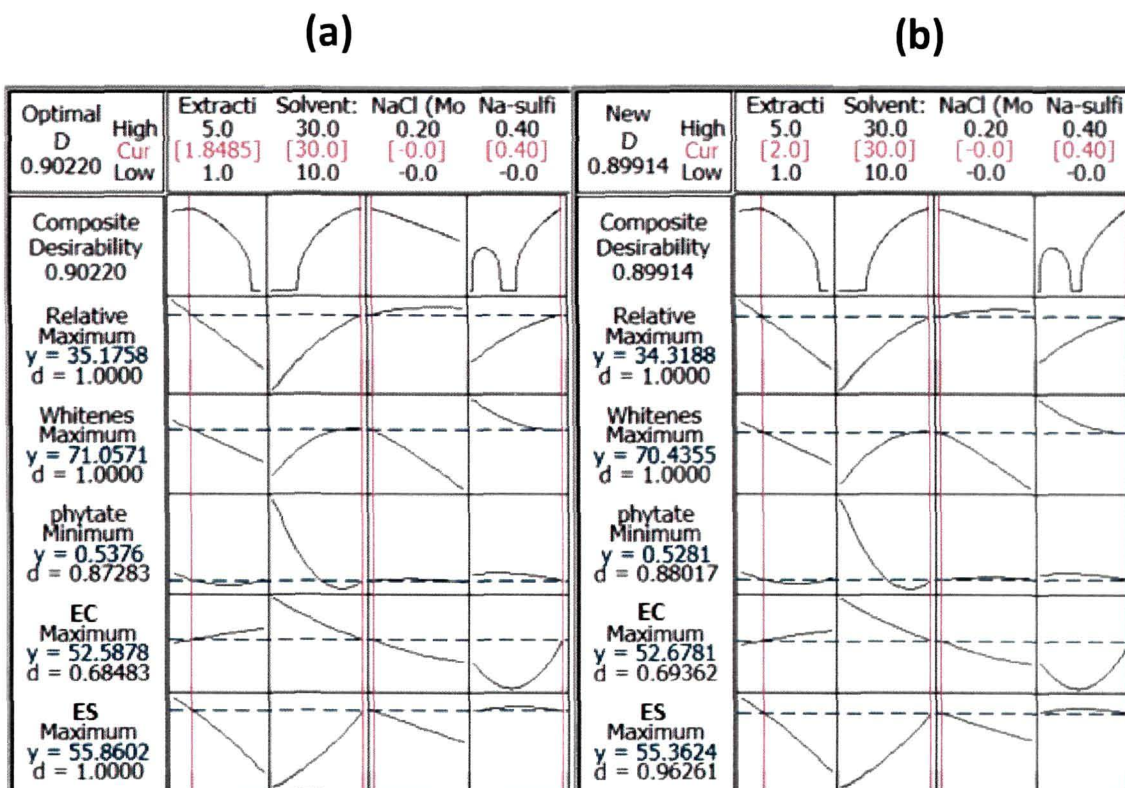



**Fig. 4.5** Overall optimum conditions and response behaviour predicted from (a) the observed optimum condition and (b) feasible experimental condition. (EC = emulsion capacity; ES = emulsion stability)

## 4.4. Conclusion

In this work, multi-response surface methodology along with desirability function was successfully employed to model and optimize the conditions to obtain rapeseed protein with improved yield, whiteness, emulsification properties (emulsion capacity and emulsion stability) and reduced level of residual phytates. The derived optimal conditions were extraction time of 2 h, solvent:meal ratio of 30:1 v/w, NaCl concentration of 0.0 M and sodium sulfite level of 0.4%. Predicted values under the feasible optimum conditions were experimentally verified to be in general agreement (95% or 99% confidence interval). The outcomes of this study can prove productive for food industries.

**Table 4.5** Confirmatory trials of the optimal conditions by comparison of experimental and predicted values at observed optimum and feasible condition.

| Response | Predicted value from optimum condition | Predicted value from new feasible condition | Observed experimental value* | Confidence Interval (95%) | Confidence Interval (99%) |
|---|---|---|---|---|---|
| $Y_1$ (Relative protein yield; %) | 35.2 | 34.3 | 48.2±2.00 | (43.1, 49.3) | (30.2, 62.2) |
| $Y_2$ (Whiteness Index) | 71. 1 | 70.4 | 75.0±3.7 | (66.4, 78.3) | (41.9, 102.8) |
| $Y_3$ (Phytate content; mg /100g protein) | 0.54 | 0.53 | 0.23±0.03 | (0.22, 0.33) | (0.00, 0.55) |
| $Y_4$ (Emulsion capacity; %) | 52.6 | 52.7 | 49.8±1.00 | (47.6, 50.7) | (41.4, 57.0) |
| $Y_5$ (Emulsion stability; %) | 55.9 | 55.4 | 53.8±3.1 | (44.8, 57.1) | (19.2, 82.7) |

*Each experimental value represents the means±standard deviation from four replicates (n=4).

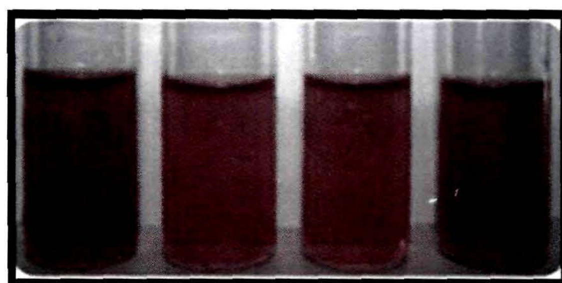


**Fig. 4.6** Colour of the protein solution (aqueous extract) extracted at optimum condition from defatted, partially dephenolized meal (extraction done at pH 11).

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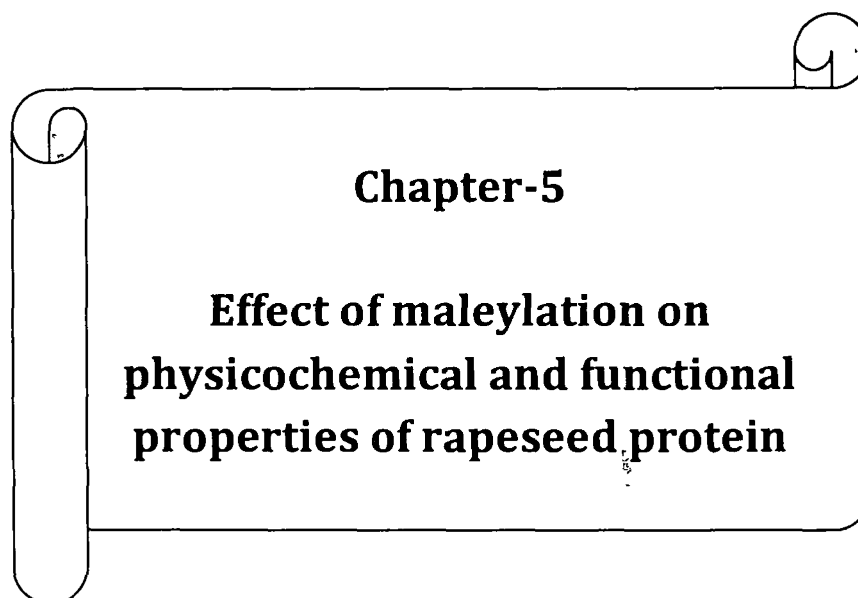

Chapter-5
Effect of maleylation on physicochemical and functional properties of rapeseed protein

## 5.1. Introduction

The need for multiple functional food ingredients and fabricated foods has increased the pressure on food industries and researchers to develop different modification techniques to enhance and diversify protein functionalities.[1] A roadblock to the large-scale use of rapeseed press-cake proteins is their unacceptable colour and poor techno-functional properties.[2] For the above-stated reason, numerous investigations through chemical (succinylation, acetylation, phosphorylation and sulfamidation) or enzymatic modification of rapeseed proteins have been undertaken;[3-6] however, proteolysates from the latter method are frequently bitter and lack satisfactory foam/emulsion stability.[7] Acylation by succinylation or acetylation has been widely used to improve the functional properties of food proteins. References on maleylation of plant proteins are very scarce; the only reported study was that of Lawal and Dawodu,[8] where the authors studied the functional properties of maleylated African locust bean protein. To date, maleylation of rapeseed proteins has not been reported. Understanding the structural changes in modified proteins is imperative for interpreting their changed surface properties. So, this chapter examines the physicochemical and functional changes in rapeseed protein after progressive maleylation, while also taking into consideration the changes in cytotoxicity and *in-vitro* digestibility of this protein following modification.

## 5.2. Materials and methods

Rapeseed press-cake was obtained from Assam Khadi & Village Industries Board, Guwahati, India. Detoxification of the meal and subsequent protein extraction from it, were performed according to the earlier Chapter-4.

### 5.2.1. Maleylation of rapeseed protein

Acylation was carried out according to the protocol of Lawal and Dawodu.[8] Protein slurry (25% w/v in distilled water) was adjusted to pH 9 using 1 N NaOH and cooled in an ice-bath. Maleic anhydride (MA) was added in small increments at the levels of 0.1, 0.2, 0.4, 0.6 and 0.8 g/g of protein, during which pH was maintained at 8.5-9.0 with constant stirring. The reaction was considered to be complete when the pH remained

constant. Following exhaustive dialysis against water for 48 h at 4 °C, the suspensions were freeze-dried to obtain maleylated proteins (coded as 0.1MA, 0.2MA, 0.4MA, 0.6MA and 0.8MA, respectively). Rapeseed protein without anhydride treatment was regarded as the control.

**5.2.2. Extent of chemical modification (maleylation)**

Trinitrobenzene sulphonic acid (TNBS) method for free amino groups estimation was used for determining modification level.[3] One millilitre each of 0.05 M $Na_2HPO_4$ and 0.1% TNBS were added to 1 ml of protein solution (1% w/v in 0.05M NaCl, pH 9.2), which was then allowed to react at 60 °C water-bath for 2 h. To stop the reaction, 1 ml of 10% SDS was added, followed by 0.5 ml of 1 N HCl. Absorbance at 335 nm was read against reagent only blank (omitting protein). Absorbance of the control protein was set equal to 100%. Degree of maleylation was calculated by the percent decrease in absorbance relative to that of the control, because fewer amino groups were able to react with TNBS reagent.

**5.2.3. Free sulfhydryl group (SH) and disulfide bond (S-S)** were assayed by the method of Tang et al. and Deng et al.,[9,10] as described in Chapter-3.

**5.2.4. Surface hydrophobicity ($S_o$)** was measured by SDS binding method,[11,12] given in Chapter-3.

**5.2.5. Surface tension** measurement of aqueous protein solution (7 mg/ml) was conducted by applying the platinum ring method (6 cm diameter) with the aid of a tensiometer (Jencon, Sl. No. 315, Kolkata-28, India) after calibrating the instrument with water (72.0 mN/m), the medium used for dispersing protein.[13]

**5.2.6. Intrinsic fluorescence spectra** were recorded in a Perkin Elmer LS55 fluorescence spectrophotometer (Perkin Elmer Corp., USA) using 2mg/ml protein solution and background calibrated with Milli-Q water. The solution was excited at 290 nm to minimize the contribution of tyrosine residues.

**5.2.7. Fourier transform infrared (FTIR) spectroscopy** was carried out on solid samples by KBr pelleting method using Perkin Elmer Spectrum-100 model. Secondary structure quantitations were performed by the Gaussian curve-fitting of the spectrum in the Amide I region.

**5.2.8. Molecular weight (MW) determination by Gel Permeation Chromatography (GPC)**

Lyophilized protein (4 mg) was dissolved in 0.5 ml of phosphate buffer (pH 11), vortexed and then mixed with 0.5 ml of 0.05 M NaCl, so that the final solution had a pH of 9.2.[14] The solution was centrifuged for 15 min at 8586 x g (4 °C), and the supernatant were filtered through a 0.22 μm cellulose membrane filter and analyzed by a Waters GPC system (515 HPLC Pump), equipped with a Waters HSPgel™ AQ 3.0 column (6.0 x 150 mm) and a Waters 2489 UV detector. Following chromatographic conditions were applied: injection volume=20 μl; isocratic flow rate=0.3 ml/min; elution solvent=Milli-Q water containing 0.3 M NaCl; and run time=15 min. The UV absorbance was recorded at 280 nm and the spectra were processed using Empower 2 software (Waters, Milford, MA). A standard curve was obtained using a Gel Filtration Kit for protein MWs (MWGF70-1KT, SLBB8609, Sigma-Aldrich, USA), which contains aprotinin (6.5 kDa), cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), and bovine serum (66 kDa).

**5.2.9. *In-vitro* digestibility of protein** was determined as described by Groninger and Miller[15] after incorporating modification from Zhong et al.[16] Twenty milligrams of pepsin was added to 700 mg of protein suspension having pH 1.5-2.0 (adjusted with HCl) and maintained at 37 °C water-bath shaker. After 2 h, pH was raised to 7.0 with 1 N NaOH to stop the reaction. When peptic digestion was finished, the protein hydrolyzate was further digested by adding trypsin (20 mg trypsin to 700 mg of protein suspension, pH 7.0-8.0 borate buffer with 0.1 M $CaCl_2$). After 2 h of shaking (37 °C), the reaction was stopped by adding 20% trichloroacetic acid, centrifuged at 4293 x g for 20 min. Nitrogen content released by enzyme in the supernatant was directly measured by Kjeldahl method (Nx6.25).[17] Casein, a protein usually employed as standard in studies on human feeding, was used as reference.[18]

$$\% \text{ enzymic digestion} = \frac{\text{Nitrogen released by enzyme}}{\text{Total nitrogen of undigested protein}} \times 100\% \quad (1)$$

**5.2.10. Colour characteristics** were determined with Hunter Lab colorimeter (Ultrascan, VIS-Hunter Associates Lab., USA) and were expressed as L (lightness), a (redness/greenness), b (yellowness/blueness) colour units. Whiteness was calculated as follows:[19]

$$\text{Degree of Whiteness} = 100 - \left[(100 - L)^2 + a^2 + b^2\right]^{1/2} \quad (2)$$

**5.2.11. Cytotoxicity assay**

Human Embryonic Kidney (HEK-293T) and Mouse embryonic fibroblasts (MEF) cells were grown in 24-well plates using complete culture media (10% Fetal bovine serum (FBS) + 45% high glucose Dulbecco's Modified Eagle's Medium (DMEM) + 45% Nutrient F10 + 1% antibiotic-antimycotic mix) at 37 °C with 5% $CO_2$ and 85% relative humidity. Trypan blue dye exclusion test was performed to check viability of cultured HEK cells upon treatments with the protein samples for 48 h. Proteins were solubilised in incomplete culture medium (DMEM and Nutrient F10 (1:1 w/w)). Different concentration of protein samples (for HEK: 0.1, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/ml; for MEF: 1.0, 2.0, 3.0 and 4.0 mg/ml) were added to 40% confluent cells and grown in $CO_2$ incubator at 37 °C with 5% $CO_2$ and 85% relative humidity for 48 h. After the incubation period, cells were harvested by 0.25% trypsin EDTA (Gybco Life technologies, UK) treatment followed by neutralization using complete culture medium. Cell suspensions were centrifuged at 200g for 5 min at room temperature. Supernatant was discarded and the cell pellet was re-suspended in 100 μl of complete culture medium. Trypan blue (0.4%) and cell suspension in the ratio of 1:1 (v/v) was prepared and incubated for 8 min, followed by counting of live and dead cells using a haemocytometer chamber in a phase contract bright field microscope. Percentage of viable cells was calculated as

$$\text{Cell survivality (\%)} = \frac{\text{Number of live cells}}{\text{Number of live cells} + \text{Number of dead cells}} \times 100 \quad (3)$$

**5.2.12. Protein solubility (PS)** was determined by dispersing 1% (w/v) freeze-dried protein powder in distilled water.[20] Suspension was agitated for 2 h at room temperature before centrifuging at 4293 x g for 20 min (4 °C).

$$\% \text{ Solubility} = \frac{\text{Soluble protein content in supernatant}}{\text{Total protein in freeze-dried sample}} \times 100\% \qquad (4)$$

**5.2.13. Emulsion capacity (EC), emulsion stability (ES), foaming capacity (FC) and foam stability (FS)** were studied by the methodology of Hassan et al.,[21] with slight modification. Oil-in-water (o/w) emulsions were prepared by blending soybean oil with aqueous protein solution (7 mg/ml) at a ratio of 3:7 (v/v). The mixture was sonicated in an ultrasonic water bath (Labotec Inc., UK) for 10 min to form emulsion.

**5.2.14. Emulsion microstructure by fluorescent microscopy**

Emulsion (1 ml) was stained with one drop of florescence dye Rhodamine B (0.025% dissolved in water) and gently mixed by inversion. A 15 μl aliquot of the resulting emulsion was added to 1 ml of distilled water, stirred gently, and then a single drop was poured onto a glass-slide and observed via fluorescent microscope (LEICA DM 3000, Power: ebq 50 qc, USA) attached with a LEICA DFC 450C camera. The 568 nm laser line was used for excitation, inducing a fluorescent emission of Rhodamine B, detected between 600 and 700 nm.[22] Average droplet size (μm) was measured by ImageJ® software and represented by $d_{32}$.[23]

$$d_{32} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2} \qquad (5)$$

where $n_i$ is the number of droplets of diameter $d_i$

**5.2.15. Protein surface load ($\Gamma_s$)**, expressed as mg protein per $m^2$ fat surface area, was calculated by relating the adsorbed protein to the specific surface area of the emulsion.[13,24] Freshly prepared emulsions (as described earlier) were centrifuged for 30 min at 4293 x g (4 °C) to accelerate creaming, and the lower serum (aqueous) phase (unadsorbed protein) was withdrawn carefully using a syringe. Protein concentration in

the serum ($C_{serum}$) and that in the initial solution prior to emulsification ($C_{initial}$) was determined by Lowry method using BSA as standard. $\Phi$ is the volume of oil phase used for emulsification (=0.3).

$$\Gamma_s = \frac{d_{32}(C_{initial} - C_{serum})(1-\Phi)}{6\Phi} \quad (6)$$

### 5.2.16. Mayonnaise preparation

The recipe for making mayonnaise was taken from Aluko and McIntosh,[25] who posited that unhydrolyzed canola proteins could only be incorporated into mayonnaise up to a maximum 15% (w/w) substitution of egg yolk without emulsion breakdown. So, following their work, egg yolk was partially substituted by unmodified or modified proteins at levels of 15%, which were accordingly referred to as mayo_control, mayo_0.1MA, mayo_0.2MA, mayo_0.4MA, mayo_0.6MA and mayo_0.8MA. Mayonnaise with 100% egg yolk (mayo_100EY), without any added protein, was used for comparison.

**5.2.16a Texture Profile Analysis** of mayonnaise were carried out with the TA.XT2i Texture Analyser (Stable Micro Systems Ltd, UK) with a 5 kg load cell in a Back extrusion cell with 35 mm diameter compression disc, according to the protocol of Liu et al.[26]

**5.2.16b Sensory evaluation** was conducted on the mayonnaise samples after one-day storage at room temperature, according to the procedure mentioned by Liu et al.[26] Sensory characteristics: appearance, colour, odour, texture, taste, and overall acceptability were evaluated by 15 semi-trained panelists on 5-point hedonic scale, 1=the least, the lowest; 5=the most, the highest. The panelists were also asked to give additional comments on the products, if any.[27] All mayonnaise samples were coded and orders of serving were randomized. Water was provided between samples to cleanse the palate.

**5.2.16c Storage stability of mayonnaise** was performed at room temperature by allowing the samples to stand quiescently for 21 days, during which, the extent of creaming was characterized by creaming index (CI, %).[28]

$$CI = \frac{H_S}{H_E} \times 100\% \qquad (7)$$

where, $H_S$ is the height of serum layer, and $H_E$ is the total height of emulsion.

**5.2.16d Mayonnaise structural visualization by Scanning Electron Microscope (SEM)**

For cross-linking and fixation of the emulsion, the methodology of Rathna et al.[29] was followed with slight modification. To 5 ml of the emulsion that was stored for 21 days, a 20% glutaraldehyde solution (1 ml) was added dropwise under mild agitation for cross-linking the protein network. The cross-linked sample was smeared gently on a glass cover-slip, which was then immersed in acetone for 1 h. Prior to SEM observation, samples were mounted on stubs with double-sided adhesive tape, followed by coating the samples with a thin layer of gold. Finally, micrograph of the sample was obtained using a JSM-6390LV SEM (JEOL, Japan) at an accelerating voltage of 15 kV.

**5.2.17. Statistical analyses**

All analyses were performed in triplicate and the mean value was calculated. Analysis of variance and separation of means (by Tukey's HSD test) were performed by SPSS software (version 16.0, SPSS Inc., Chicago) and considered significantly different at $p<0.05$.

***5.3. Results and discussion***

**5.3.1. Physicochemical properties of maleylated rapeseed proteins**

Incremental levels of addition of MA increased the extent of acylation from ≈29.3-62.9% of the ϵ-amino groups (**Fig. 5.1A**). These values were comparatively lower than those reported earlier on maleylated locust bean protein,[8] which can be attributed to

the differences in physicochemical nature of locust bean protein and rapeseed protein. Protein acylation reactions follow the carbonyl addition pathway (**Inset of Fig. 5.1A**) and the rate of reaction depends on the rate of nucleophilic attack: the $\pi$-electrons on the double bond of MA hinder the nucleophilic attack of the amino group.[8] This may be the cause for lower level of modification in this study compared to those obtained by succinylation of canola 12S protein.[3] Even at the highest level of anhydride-to-protein ratio, all free amino groups were not maleylated. Such incomplete acylation is common for storage proteins of oilseeds,[17] which may be due to spatial steric hindrance of some amino groups in protein.[30]

Free SH group of the control was found to be 20.2 μM/g, which is comparatively close to the value (≈25 μM/g) reported for native rapeseed protein by He et al.[31] In relation to the control, maleylation resulted in remarkable decreases in SH and S-S content ($p<0.05$). This is because of the possible interaction of the acyl moiety with SH groups, as acylation is possible on all nucleophilic groups of amino acid residues, e.g. $\epsilon$-amino groups of lysine, hydroxyl groups of serine and threonine, sulfhydryl group of cysteine, phenol group of tyrosine, and imidazole group of histidine.[7] This observation corroborates with the findings of Groninger and Miller[15] where one-third and one-fourth of the SH groups of fish proteins were found to react with succinic and acetic anhydrides, respectively. Moreover, the reactivity of the sulfhydryl groups in acylated samples towards Ellman's reagent could be impeded in presence of bulky maleyl groups, thus giving a delutionary decrease in SH content as compared to the control. Apparently, the content of free SH in the maleylated samples under study presented a gradual rising trend with the increasing level of modification (**Fig. 5.1B**). This suggests either deacylation of SH in presence of high MA[15] or acylation-induced exposure of inaccessible thiol groups initially buried within the hydrophobic interior (mainly by protein unfolding and/or subsequent dissociation) has occurred. It should be pointed out that higher degree of unfolding and dissociation of protein molecules intensifies hydrophobic interactions, which in-turn favours the generation of S-S bonds.[32] This might account for the overall marginal rise in S-S linkage as-well. Reduced availability of free thiol groups or the

presence of negative maleyl groups in modified proteins probably gave rise to fewer disulfide bonds, as opposed to that of the control.

$S_0$ increased progressively up to 0.4MA, but decreased thereafter (**Fig. 5.1C**). This observation is similar to that of Matemu et al.[1] The increased hydrophobic nature can be related to the gradual structural unfolding or exposure of initially buried hydrophobic clusters by progressive dissociation of protein molecules into its constituent subunits. Confirmation of this explanation is given by FTIR, GPC and intrinsic fluorescence of these samples. Occurrence of high $S_0$ is thought to be responsible in enhancing protein-protein interaction or S-S content. This assumption coincides well with FTIR data. Despite the presence of high amount of randomness and protein dissociation in 0.6MA and 0.8MA, the ulterior drop in hydrophobicity after exhaustive acylation may be due to the dense distribution of negatively charged maleyl residues, inhibiting Coomassie Blue dye (probe used for $S_0$ measurement) from approaching and binding to the exposed hydrophobic moieties on the protein surfaces.[33] Similar behavior has been reported also for 1-anilino-8-naphthalenesulfonic acid (fluorescence probe for $S_0$ measurement) in modified soy protein hydrolyzates[34] and acylated kidney bean protein.[35]

Equilibrium surface tension of concentrated protein solutions are often around 45 mN/m.[8] Oilseed protein isolates are known for their high $S_0$.[23] Consequently, it may explain the good adsorptive behavior of the unmodified rapeseed protein at the air-water interface and its rather low surface tension at equilibrium ($\approx$40 mN/m) (**Fig. 5.1C**), which in-turn was found to be much lower than that reported for BSA (57.7 mN/m).[6] Highly surface-active molecules were obtained by maleylation, the surface tension decreasing up to 36.9, 36 and 35.2 mN/m for 0.1MA, 0.2MA and 0.4MA, respectively. These values are close to those mentioned for acylated rapeseed peptides at air-water interface.[6] The values indicate that introduction of maleyl groups facilitated flexibility of the protein molecule by exposing previously buried hydrophobic portions to the interface, thus enhancing lowering of surface tension[8] and such samples are expected to show improved foamability. Samples with moderate level of maleylation were slightly more effective in reducing the interfacial tension compared to highly acylated ones. This can be credited to

their respective $S_0$; nevertheless, the lower $S_0$ of 0.6MA and 0.8MA derivatives was probably offset by their high solubility, explaining their low surface tension values with respect to that of the control.

$\lambda_{max}$ of the fluorescence emission spectrum of the unmodified sample was determined to be 344.2 nm (**Fig. 5.1D**), which is close to the reported value of 340 nm for 12S canola protein.[3] Except 0.8MA, maleylation resulted in a red shift of $\lambda_{max}$ as compared to the control, whose extent varied depending on the level of anhydride used. Gruener and Ismond[3] and Schwenke et al.[5] observed similar shifts in fluorescence emission after succinylation and phosphorylation of canola/rapeseed protein, respectively. Red shift indicates the exposure of tryptophan (Trp) residues from the interior of protein molecule to the polar solvent/environment. This can be deduced from the conformational perturbations like partial unfolding, progressive dissociation or expansion of the native oligomeric structure,[1] a conclusion in accordance with the greater random coil feature and dissociated protein subunits as suggested by FTIR and GPC results, respectively. Some refolding mechanism or protein aggregation through hydrophobic interactions might have occurred in 0.8MA leading to less exposure of Trp groups to the polar solvent,[4] as evident from the blue shift of its $\lambda_{max}$. Maleylation also induced a strong quenching of the fluorescence, which proceeded with increasing extent of modification. Based on the interpretation of Schwenke et al.,[5] decrease in fluorescence intensity was probably due to the attached maleyl groups and partly due to the aggregate formation at higher modification level. Kim and Kinsella[36] reported that the decrease in fluorescence intensity of soy glycinin above 50% succinylation indicated re-association of the unfolded polypeptides to occlude certain Trp residues.

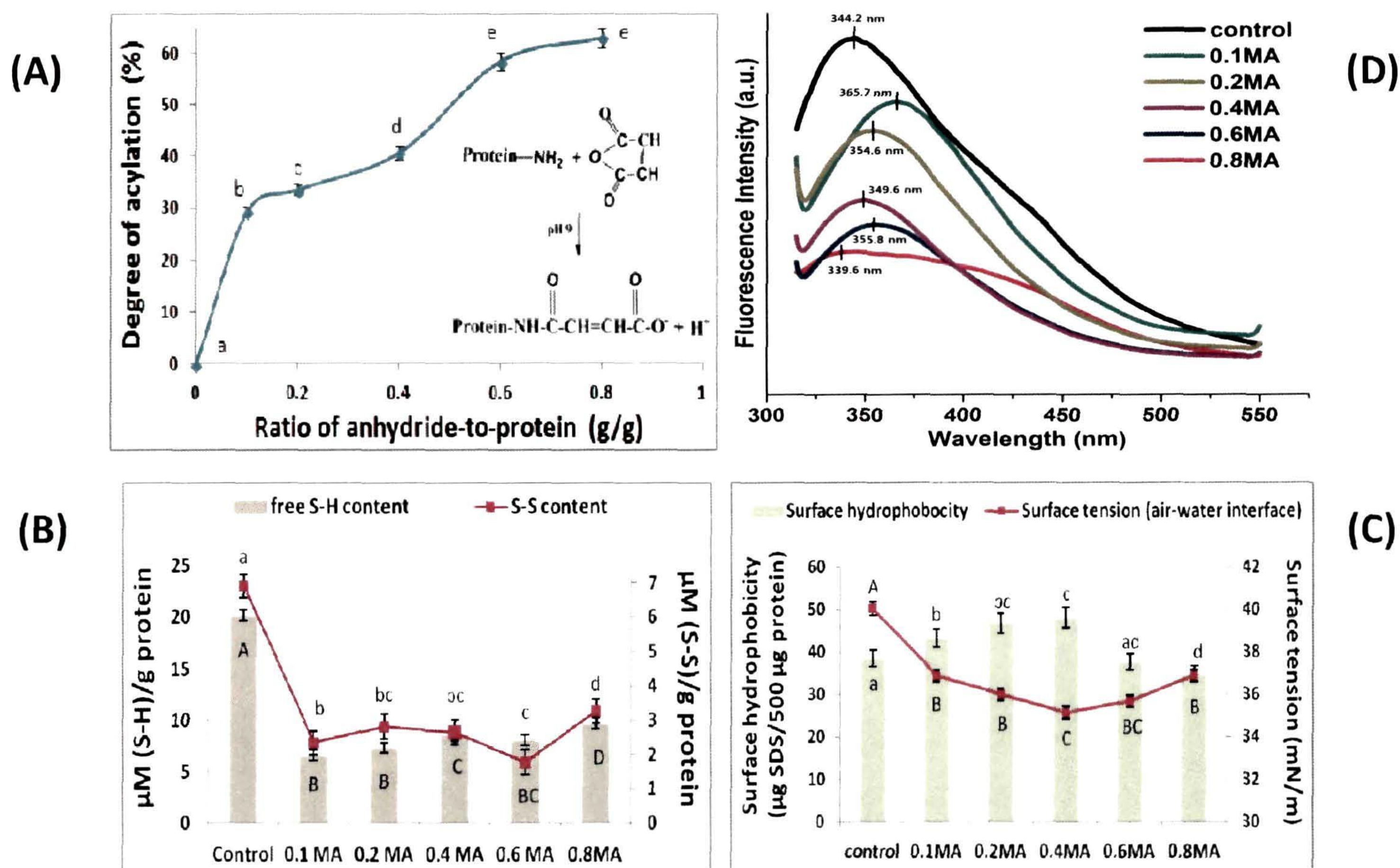



**Fig. 5.1** (A) Effect of various concentration of MA on degree of acylation of rapeseed protein (Inset showing the reaction scheme of MA with ϵ-amino group of protein); (B) Free sulfhydryl and disulfide content; (C) surface hydrophobicity and surface tension of unmodified and modified proteins; and (D) Comparative representation of emission fluorescence spectra of different proteins (Excitation at 290 nm). Bars or points with different letters in each type of analysis indicate significant difference ($p<0.05$).

Visual inspection of the FTIR profile revealed subtle changes among the samples, as they had nearly identical infrared spectra (**Fig. 5.2**). The band observed at 1401 $cm^{-1}$ (attributable to C=O bond of ionized carboxyl ($COO^-$) group) became more pronounced after modification with respect to the control. This is because acylation introduces additional carbonyl groups.[33] With an increasing extent of maleylation, a sharp peak at ≈861 $cm^{-1}$ and a shoulder at ≈1185 $cm^{-1}$, implying the presence of CH bend of alkene (or ring substitution) and acyl C-O, respectively, became more prominent especially above 0.4MA. Such signs gave obvious indication to the introduction of maleyl groups in the protein backbone. Besides aromatic C-H bending, the peak at 861 $cm^{-1}$ may also indicate the presence of indole ring, possibly by the exposure of buried Trp or tyrosine residues.[37] Dissociated protein subunits in the acylated samples (≥0.4MA), as evident from their GPC chromatograms, most likely revealed the buried Trp residues. These results follow the trend observed in intrinsic fluorescence emission spectra and points to the susceptibility of the polypeptide chains to substantial disruption upon exhaustive acylation. Infrared spectral changes in Amide I region were used for the conformational analysis of the secondary structure of proteins (**Fig. 5.3**). Successive maleylation caused a drastic loss in α-helix content with a concomitant rise in β-structures and random coils (**Table 5.1**). These results are in good agreement with published data on modified 12S and 2S rapeseed proteins[4] and BSA.[38] Achouri and Zhang[34] also found a pronounced tendency of soy protein hydrolysates towards randomness with increasing modification level. It is feasible to believe that under high electrostatic repulsion and steric hindrance of the bulky negatively-charged acyl groups, most α-helical chains might have unraveled, leading to random coils, and then some of these unraveled chains realigned into β-conformations,[39] facilitated by hydrophobic interactions.

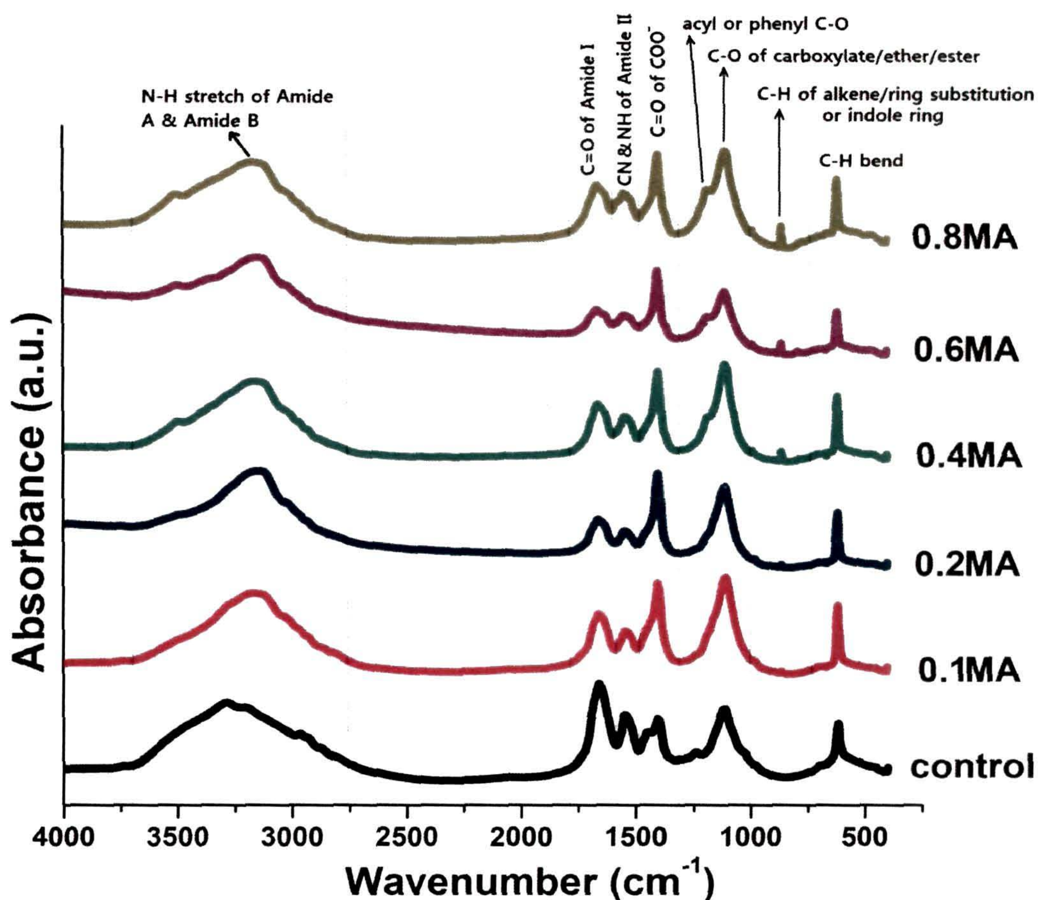



**Fig. 5.2** FTIR spectra of unmodified and modified rapeseed proteins.

**Table 5.1** Estimated secondary protein conformations obtained in the Infrared (Amide I) spectral region of unmodified and modified rapeseed proteins.

| Proteins | Area (%) | | |
|---|---|---|---|
| | **α-helix** | **β-structure (β-sheet+ β-turn)** | **Random coil** |
| Control | 27.33±0.4$^{a}$ | 41.24±0.65$^{a}$ | 31.43±0.18$^{a}$ |
| 0.1 MA | 21.2±0.5$^{b}$ | 49.89±0.97$^{b}$ | 28.91±0.23$^{a}$ |
| 0.2 MA | 20.16±0.25$^{b}$ | 43.93±0.41$^{ac}$ | 35.91±0.68$^{b}$ |
| 0.4 MA | 15.63±0.3$^{c}$ | 42.79±0.35$^{c}$ | 41.58±0.62$^{c}$ |
| 0.6 MA | 11.17±0.41$^{d}$ | 57.38±0.57$^{d}$ | 31.45±0.37$^{a}$ |
| 0.8 MA | 7.65±0.42$^{e}$ | 54.79±0.62$^{e}$ | 37.56±0.55$^{b}$ |

Values are mean±SD (n=2). Means with the same superscript letter within one column were not statistically different ($p$>0.05).

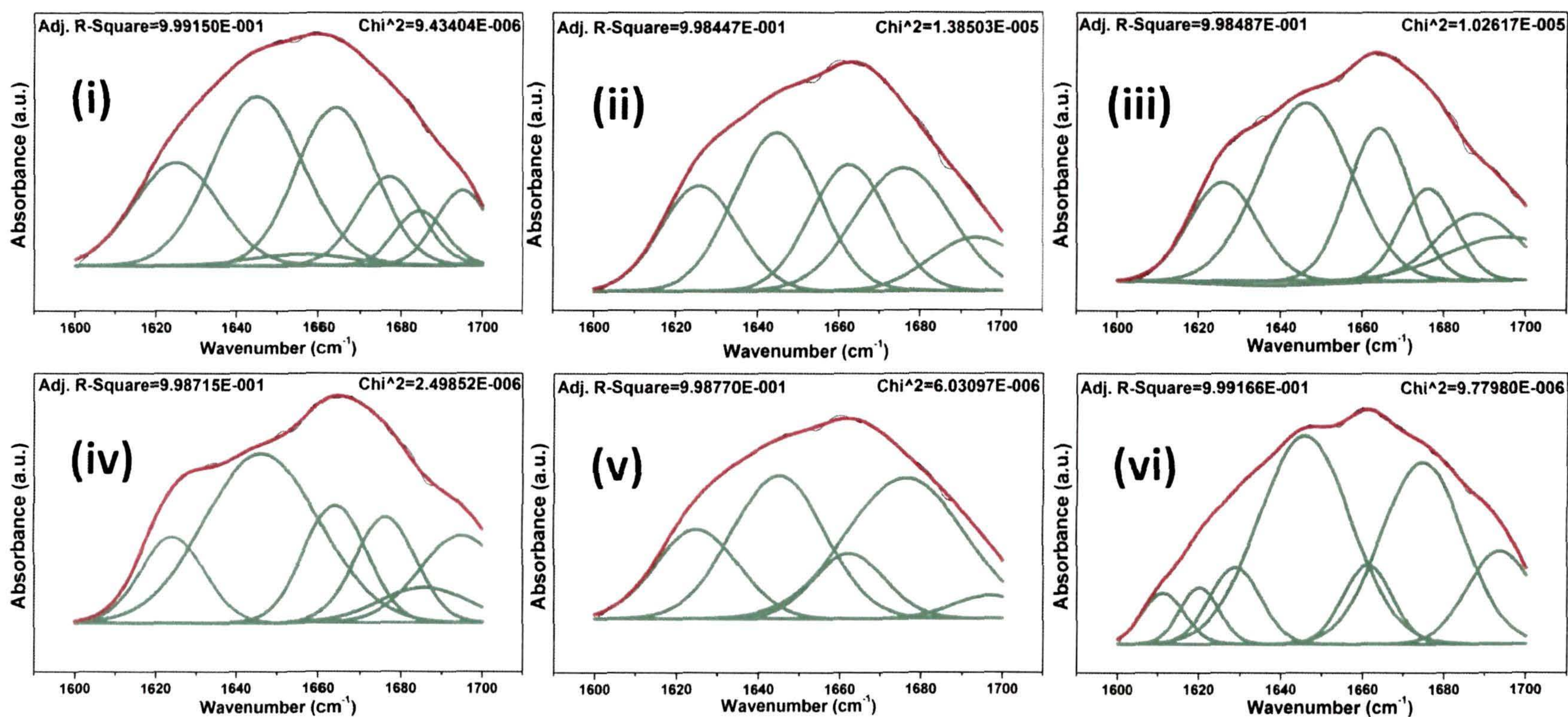



**Fig. 5.3** Curve-fitted Amide I bands of (i) control, (ii) 0.1MA, (iii) 0.2MA, (iv) 0.4MA, (v) 0.6MA, and (vi) 0.8MA. The red line represents the protein IR spectra, which coincide with the results of the Gaussian curve-fitting. Green lines are the individual Gaussian bands fitted to the spectra. Spectral correlation coefficients between the original IR spectra and the Gaussian curve-fitting were calculated (adjusted $R^2$) and error of the estimate is represented as Chi-square value ($\chi^2$).

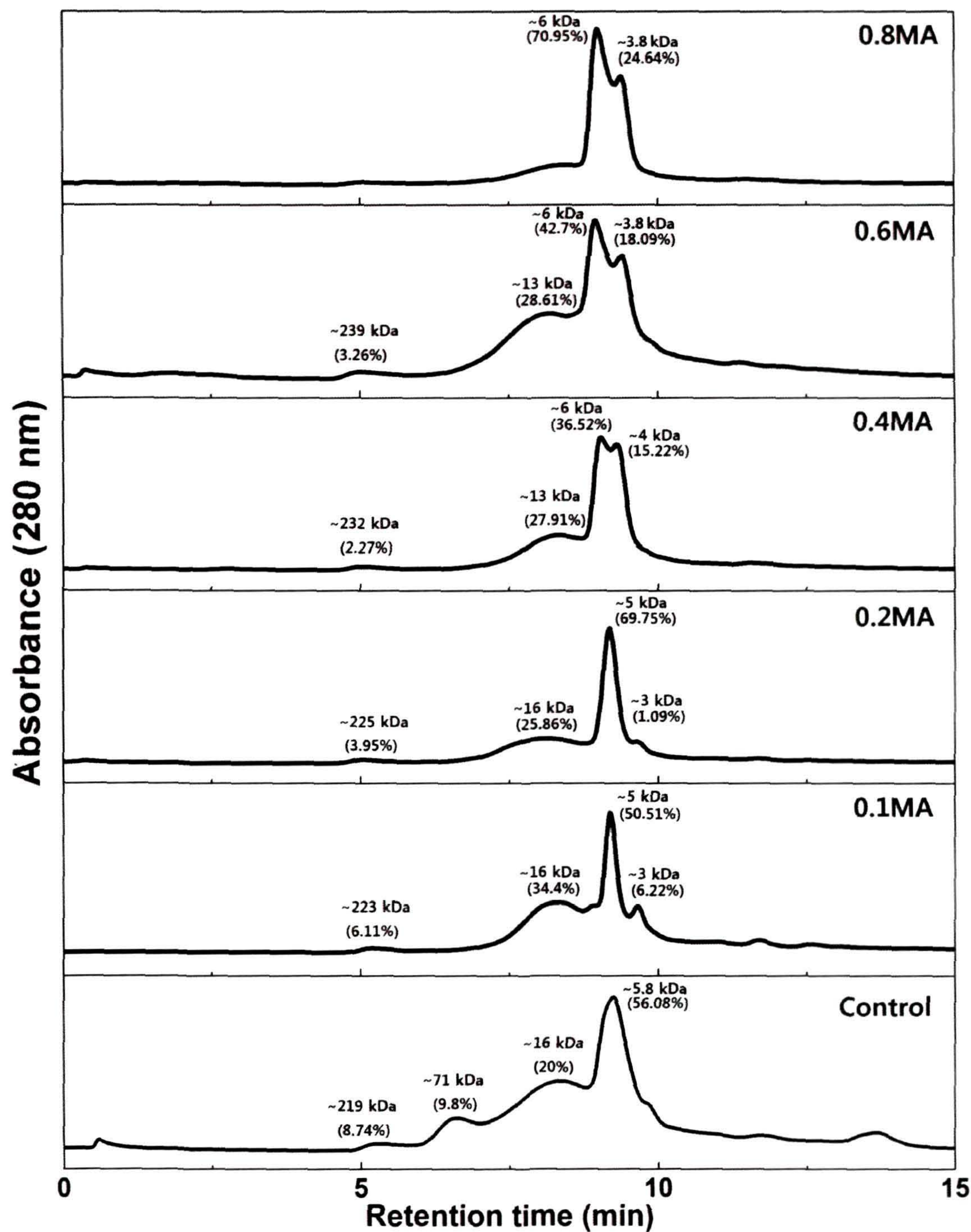


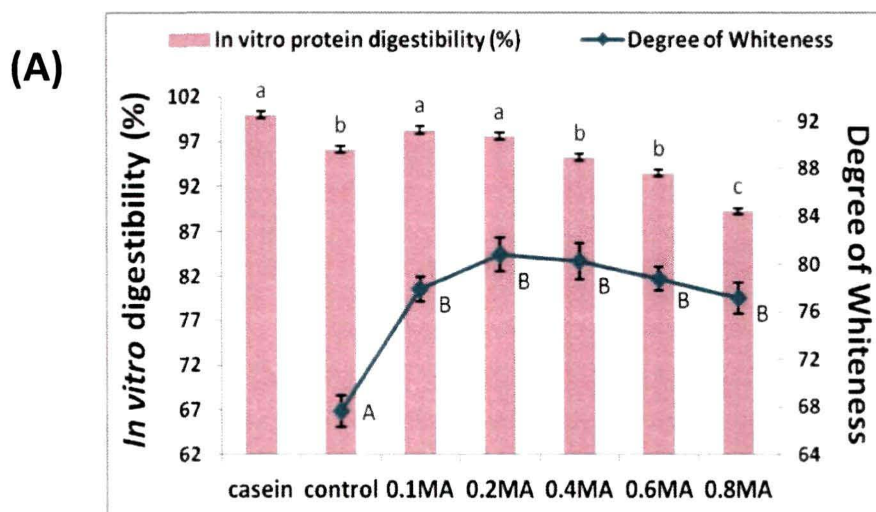

**Fig. 5.4** Gel Permeation Chromatograms of unmodified and modified proteins.

The control sample was mainly composed of 4 components, with estimated MW of 219, 71, 16 and 5.8 kDa, respectively (**Fig. 5.4**). These correspond well with the values determined by earlier authors.[40-43] Lower MW fractions (16 and 5.8 kDa) were the most abundant, possibly due to the reducing effect of sodium sulfite used in protein extraction protocol (cleaves disulfide linkages to form smaller polypeptides).[41] High MW minor fraction (ranging from 219 to 239 kDa) probably represented cruciferin,[31] whose amount reduced successively till 0.4MA and finally disappeared at 0.8MA. This is because cruciferin is highly susceptible to dissociation upon acylation.[3,5] Attachment of negatively-charged succinyl group or phosphorous oxychloride has been found to induce stepwise dissociation of oligomeric protein to its constituent subunits.[5,34] Analogous structural changes ought to be expected in case of MA treatment. At 0.1MA, the peaks having MW of 16, 5 and 3 kDa appeared. The 5 kDa peak, major component of 0.1MA, was even more predominant at 0.2MA. The 16 and 5 kDa moieties decreased with progressive acylation in favour of higher and lower MW fractions. Furthermore, the decrease in 16 and 3 kDa peaks with acylation led to its complete diminution at 0.4MA along with the emergence of new peaks having MW of 13, 6 and 4 kDa. These observations suggest that repulsive forces between maleyl groups provoked the dissociation of protein and subsequently caused aggregation between unfolded proteins or dissociated low MW subunits via hydrophobic interactions and other weak bonds.[44] Such dissociation followed by re-aggregation has been reported in a number of oilseed proteins. In addition, there is also a possibility that introduction of acyl groups may slightly expand the subunits, making them to change their hydrodynamic radii, so that their characteristic peaks would shift or appear at different retention time.[32] Upon exhaustive acylation ($\geq$0.6MA), the relative proportion of 6 and 3.8 kDa fractions were distinctly increased at the expense of 13 kDa peak which finally disappeared at 0.8MA. Similar phenomenon was observed during succinylation of fababean proteins, where 11S oligomer formed 7S and 3S components and finally created only 3S monomer at the highest modification level.[45] Thus, GPC results seem to reflect the formation of both products of dissociation and aggregation after maleylation.

*In-vitro* digestibility of protein was determined using casein as a reference (100% digestibility). The control presented a reduced digestibility of 96.1%, which is attributable to the residual polyphenols present in it (**Fig. 5.5A**). Factually, in a number of polyphenol-protein models, phenolics (mainly tannins) have been shown to reduce protein digestibility.[18] Maleylation treatment at 0.1MA and 0.2MA increased digestibility ($p<0.05$), which may be due to subunits dissociation and subsequent unfolding of polypeptide chains, making them more accessible to enzyme attack.[20] Further increase in acylation level reduced the digestibility; however, the differences were trivial in comparison to the control ($p>0.05$), except 0.8MA. This is due to the inability of the enzymes to hydrolyze peptide bonds that involve derivatized or acylated amino groups.[15] Though exhaustive acylation results in unfolding and dissociation of proteins, high charge accumulation may actuate unusual conformational perturbations in the molecule, making it hard for the enzymes to act on the requisite active sites for hydrolysis to occur. Overall the current results are partly in agreement with those of Achouri and Zhang[34] and Yin et al.,[30] and the values are high enough to assure that there is not any adverse effect of maleylation on digestibility.

Unmodified rapeseed protein displayed a light-brownish colour and gritty texture. Maleylation greatly lightened its colour to off-white or chalk-white, with exceedingly fluffy texture (**Fig. 5.5B**). It has been observed earlier that rapeseed/canola proteins portraying off-white colour in dry powder state, show brown colour of different intensities up on dissolution in water,[46] which is noticeable in the control. So following the previous protocol, colorimetric evaluation of protein was performed by scanning their aqueous solution (5% w/v). The control had the lowest whiteness value (**Fig. 5.5A**), due to its brown colouration caused by polyphenols, that can become oxidized during alkaline protein extraction from the meal. All the acylated counterparts had significantly higher whiteness values than the control ($p<0.05$). The observation is congruent with the reports of Franzen and Kinsella,[7] Gruener and Ismond,[2] and Wanasundara and Shahidi.[17] This dramatic improvement in colour can be explained with the blocking off the nucleophilic amino groups of protein with acyl groups, which can other react with the electrophilic o-quinone (oxidation product of polyphenols).[47] The pH condition (pH 8-9) used for

acylation improves the reaction selectivity of acyl group towards ϵ-amino groups (most reactive due to its low $pK_a$ and low steric hindrance),[20,38] and thus cause a significant decrease in the content of colour-forming quinones. There was no significant difference in the whiteness among the acylated proteins ($p>0.05$).



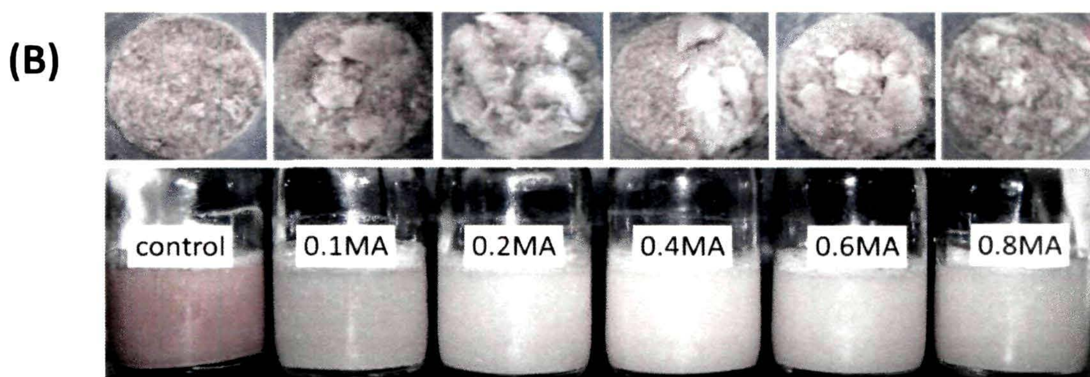




**Fig. 5.5** (A) *In-vitro* digestibility and Degree of Whiteness; (B) Photograph showing unmodified or modified freeze-dried proteins (above) and their respective aqueous solution (5% w/v) (below). Bars or points with different letters in each type of analysis indicate significant difference ($p<0.05$).

Compared to 0.6MA and 0.8MA, the physicochemical and functional properties (discussed later) of 0.1MA, 0.2MA and 0.4MA were found to be satisfactory. So, these samples were selected for cytotoxicity assay and compared with that of the control. Results from Trypan blue dye exclusion test indicated that the tested protein samples do not pose severe cytotoxicity to the HEK and MEF cells (**Fig. 5.6**). Cell survivality was found to be higher than 90% and 80% for HEK and MEF cell-lines, respectively, even in the presence of high protein concentration (3 mg/ml); however, further rise in protein level, especially control and 0.4MA, these values were slightly reduced to 84% and 68% in HEK and MEF, respectively. Although plant polyphenols are usually regarded as valuable natural antioxidant, not all phenolics present in foods are beneficial and some may be of anti-nutritional concern. The presence of high level of phenolics (6.4-18.4 mg/g rapeseed flour) or tannins (0.2-3% of defatted rapeseed meal)[48] or their oxidized forms in rapeseed protein products have been identified as potent inhibitors of enzymes and ions (especially iron) in the gastrointestinal lumen. So, in accordance with the notion that the presence of residual tannins and phenolic compounds in the control sample may trigger cell binding/clumping by interacting with available proteins on cell surfaces, as is also seen in hazelnut meal protein;[49] dramatic morphological changes became obvious at ≥2 mg/ml, with prominent cell clustering (**Figs. 5.7-5.8**). It is noteworthy to mention that the *in-vitro* cytotoxic/antiproliferative tests reported in the literature generally use very low concentration of the test material (in μg/ml), whereas in the current study, isolates were tested at much higher scale (in mg/ml). Therefore, a point is possibly reached as the concentration increases where unadsorbed protein molecules begin to accumulate. In case of 0.4MA, the reduction is presumed to be because of the high negative charge density of the molecule. Nevertheless, 0.1MA and 0.2MA were found to be non-toxic to the cells, with the survivality values as high as 92% in HEK and ≈80% in MEF, even at very high concentration (4 mg/ml). Cell viability results of this study are comparable or marginally better than those reported for micro- and nanoemulsions prepared with modified starch.[50]

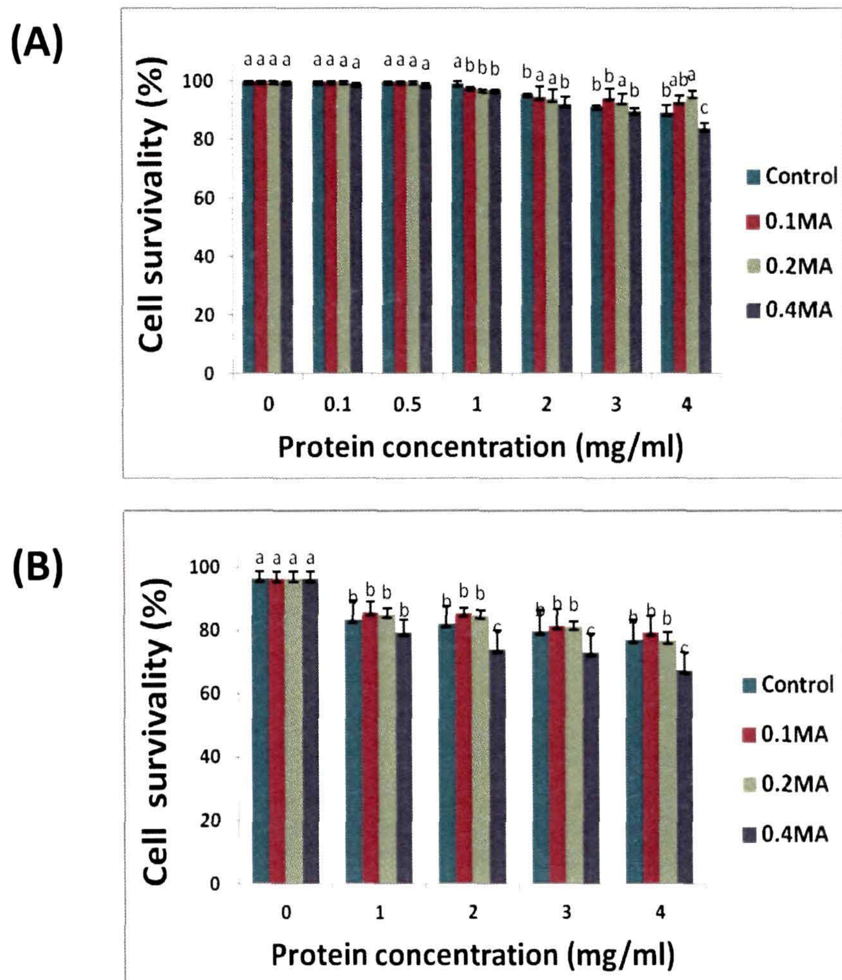



**Fig. 5.6** Cytotoxic effect of unmodified and modified rapeseed proteins on (A) HEK (B) MEF cells. Bars with different letters indicate significant difference ($p<0.05$).

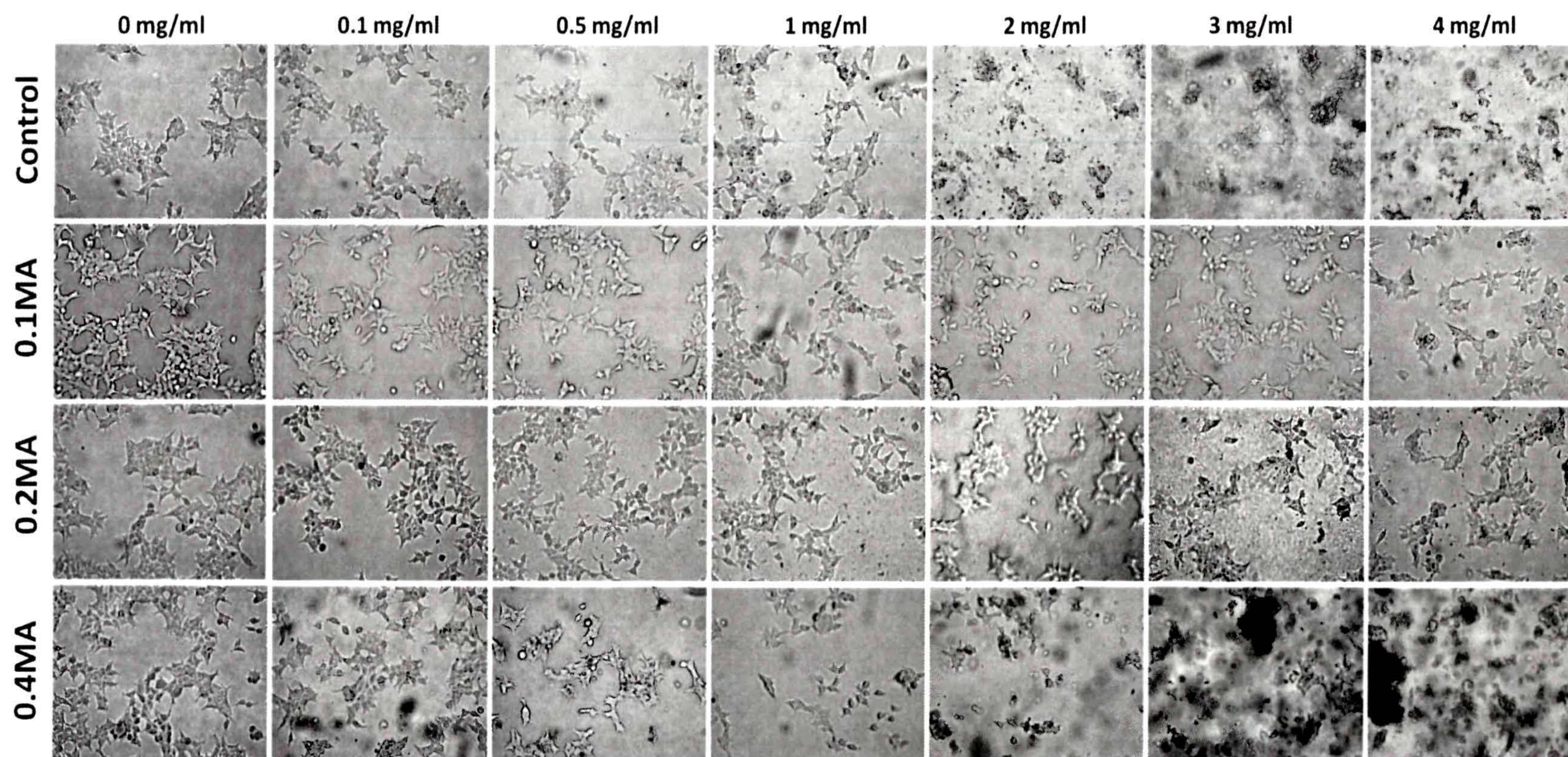



**Fig. 5.7** Photomicrographs of HEK cells following treatment with varying concentrations of unmodified and modified rapeseed proteins.

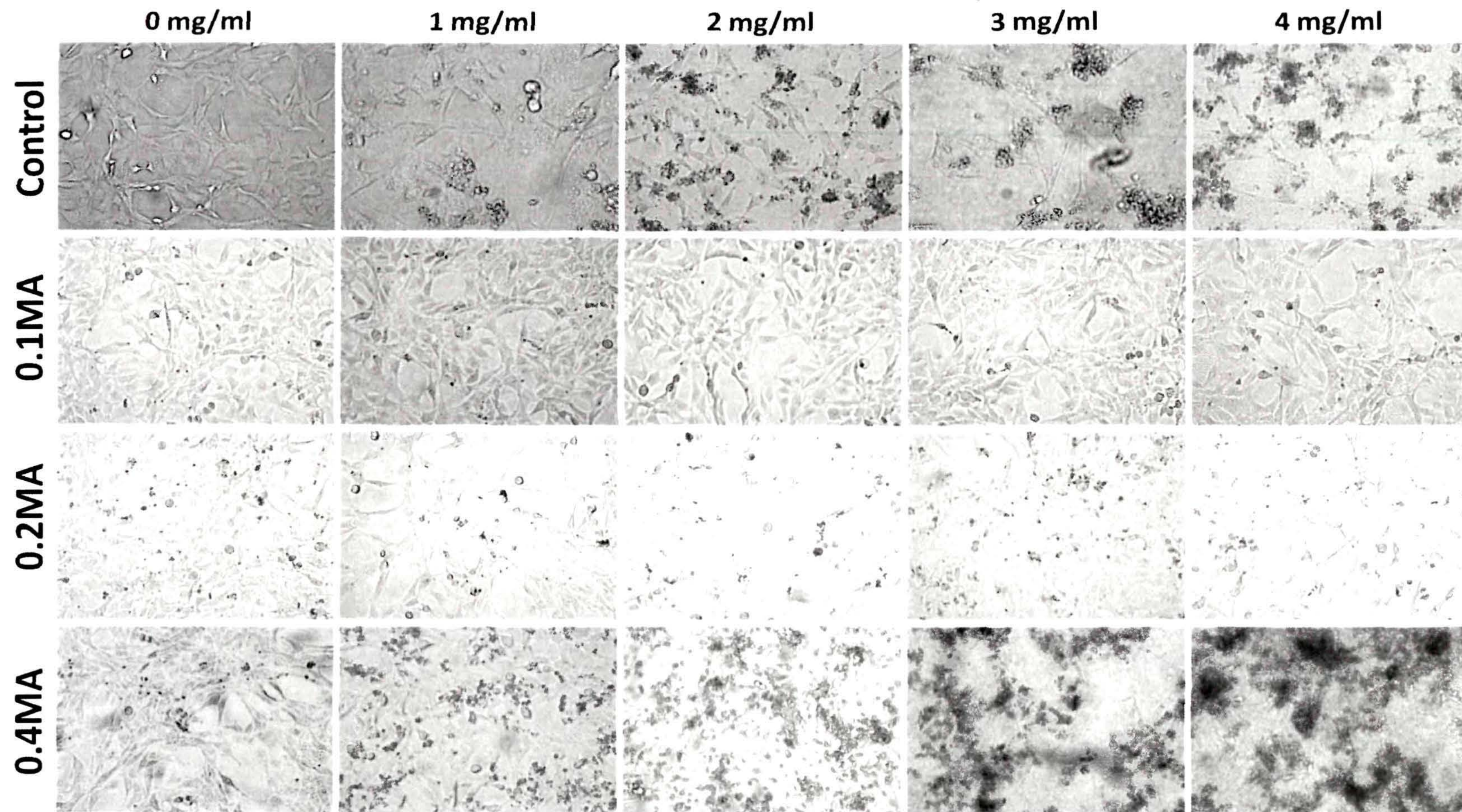



**Fig. 5.8** Photomicrographs of MEF cells following treatment with varying concentrations of unmodified and modified rapeseed proteins.

### 5.3.2. Functional properties of maleylated rapeseed proteins

PS (at pH 7) was remarkably increased by maleylation (1.2-1.7 fold) (**Fig. 5.9A**). After acylation, the cationic amino groups on protein are converted to net negative charge, which causes repulsion among the maleyl carboxyl and the native carboxyl groups. Resulting electrostatic repulsion promotes protein unfolding and penetration of water molecules is physically easier because of the expanded/loosened state of the polypeptides.[7] This result lends credence to similar observation by Lawal and Dawodu,[8] Gruener and Ismond[3] and Yin et al.[30] Pronounced improvement in PS of 0.6MA and 0.8MA is mainly attributed to increased protein dissociation into low MW subunits, which results in better protein-water interactions.[51]

Increases in EC observed following maleylation (1.3-1.8 fold) may be a reflection of the increased solubility and molecular flexibility due to unfolding and consequent exposition of the reactive hydrophobic clusters on protein surface (**Fig. 5.9B**). These developments facilitate re-arrangement of the lipophilic and hydrophilic residues of protein at the interface, improving EC. Moreover, decrease in size due to partial dissociation may enable the protein to diffuse/migrate more quickly to the oil-water interface; especially causing enhancement in EC of highly acylated derivatives, despite their low $S_0$. These observations are in line with the earlier reports.[2,8] Likewise, ES of acylated derivatives increased over the control till 0.2MA (1.5 fold), after which it reduced gradually (**Fig. 5.9B**). Improvement in ES at low level of maleylation can be due to the low net charge of the acylated protein layer formed around oil droplets, which increases electrical potential (of the ionized interfacial film) and imparts coalescence stability.[51] On the contrary, decrease in ES above 0.2MA can be due to low MW subunits in such acylated counterparts, which may be insufficient to form thicker interfacial films resulting in less stable emulsion.[1] It is noteworthy that initial increase in PS by acylation facilitated enhanced interaction between oil and aqueous phases; however, as the solubility is increased, probably a point was reached where further increase in solubility (by modification) led to accumulation of proteins in the aqueous phase instead of being at the interface, thereby deteriorating ES. Similarly, in case of casein-stabilised emulsions,

the presence of excess protein in the continuous aqueous phase (due to high solubility) proved deleterious, since the micelles induce depletion flocculation.[52] It can also be assumed that high negative charge density maintains the molecule in a highly unfolded state and inhibits re-arrangement processes at the droplet surface by repulsive effect.[53]

Unlike emulsification, FC was nearly unaffected till 0.4MA ($p>0.05$), and then decreased up to a 3.4 fold at the highest level of modification ($p<0.05$) (**Fig. 5.9B**). The pattern here is congruent to the observations on surface tension (**Fig. 5.1C**). The same structural alterations of the protein molecules that are important for emulsification are also believed to be responsible for foamability; however dispersion interactions between proteins and oil-water interface are always attractive whereas that between proteins and air-water interface are generally repulsive, because of this difference proteins are adsorbed much more readily at the oil-water interface than at the air-water interface.[54] Thus foamability of a protein may not be analogous to its emulsion behavior. At low acylation level, the proteins possess relatively low net charge, because of which they are not strongly repelled, a development that reduced the effect of drainage of protein from foam lamellae and contribute to the formation of stable molecular layers to encapsulate air particles, leading to enhanced foaming.[51] Apart from this factor, increase in FC can also be related to enhanced solubility and high $S_0$ of such acylated samples compared to the control, enabling them to unfold more easily (flexibility) at the interface.[2] In highly acylated proteins, excessive repulsion due to high charge density maintains the molecule in a highly unfolded state and inhibits re-arrangement process at the interface, leading to deterioration in surface functionality.[53] Similarly, decrease in FS (**Fig. 5.9B**) following modification is reasonably due to repulsive effect of maleyl groups that limited protein-protein interactions, hindering the formation of a continuous network around air bubbles.[8] Similar behaviour has been reported for acylated mung bean isolates.[20] Therefore, maleylated rapeseed protein may not be suitable in food systems that require high FS such as cake and ice cream.

Maleylated proteins afforded smaller emulsion droplets when compared to unmodified one (**Fig. 5.9C**); the decrease in $d_{32}$ increases interfacial area and hence the

EC. This is a reflection of increased solubility and looser structure of modified proteins. So, the faster the molecules anchor at the interface, the smaller the average droplet size will be.[6] It is feasible to believe that the electrostatic repulsion between droplets, due to negative charge of maleylated proteins, is expected to prevent inter-droplet protein–protein interaction, therefore preventing flocculation. This is evident from the typical smooth surface of the microspheres fabricated by most of the samples under study (**Fig. 5.10A**). In sharp contrast, emulsion droplets stabilized by 0.6MA and 0.8MA showed rugged surfaces. The latter observation is because of the creation of numerous small droplets which appeared to be highly flocculated. This may indicate that such acylated samples contained some low molecular weight fractions that were very surface active, enabling the formation of small droplets, but the content of emulsifier (protein) present at the newly created oil-water interface was not sufficient for full coverage of droplet surface,[28] therefore, bridging flocculation of droplets became possible. This in-turn is caused by low $S_0$ and excessive charge density, preventing intra-molecular protein–protein interaction. Poor ES of such samples can be anticipated from their flocculated droplets. As Rhodamine-B stains the protein component, individual droplets encapsulated by protein and/or regions rich in protein appear as red patches, which seems to be much lesser in **Figs. 5.10A(v)-5.10A(vi)** compared to other samples. This observation also helps in drawing the inference that proteins from 0.6MA and 0.8MA are less available at the interface. A comparison of the fluorescent image of the fresh emulsion (**Fig. 5.10A**) and that of the emulsion stored for 24 h (**Fig. 5.10B**) reveals significant amount of ruptured/collapsed droplets, because of which their $d_{32}$ values could not be calculated at t=24 h. Both Ostwald ripening (shrinkage of small bubbles with eventual disappearance, and expansion of large bubbles) and coalescence (merging of two or more droplets to form larger one) phenomena seems relevant for the systems considered. In fact, the analysis of micrographs of highly acylated samples evidenced drastic reduction in the number of droplets. This is because small MW surfactants possess poor surface dilatational elasticity compared with the larger ones and, therefore, thinning and breakage

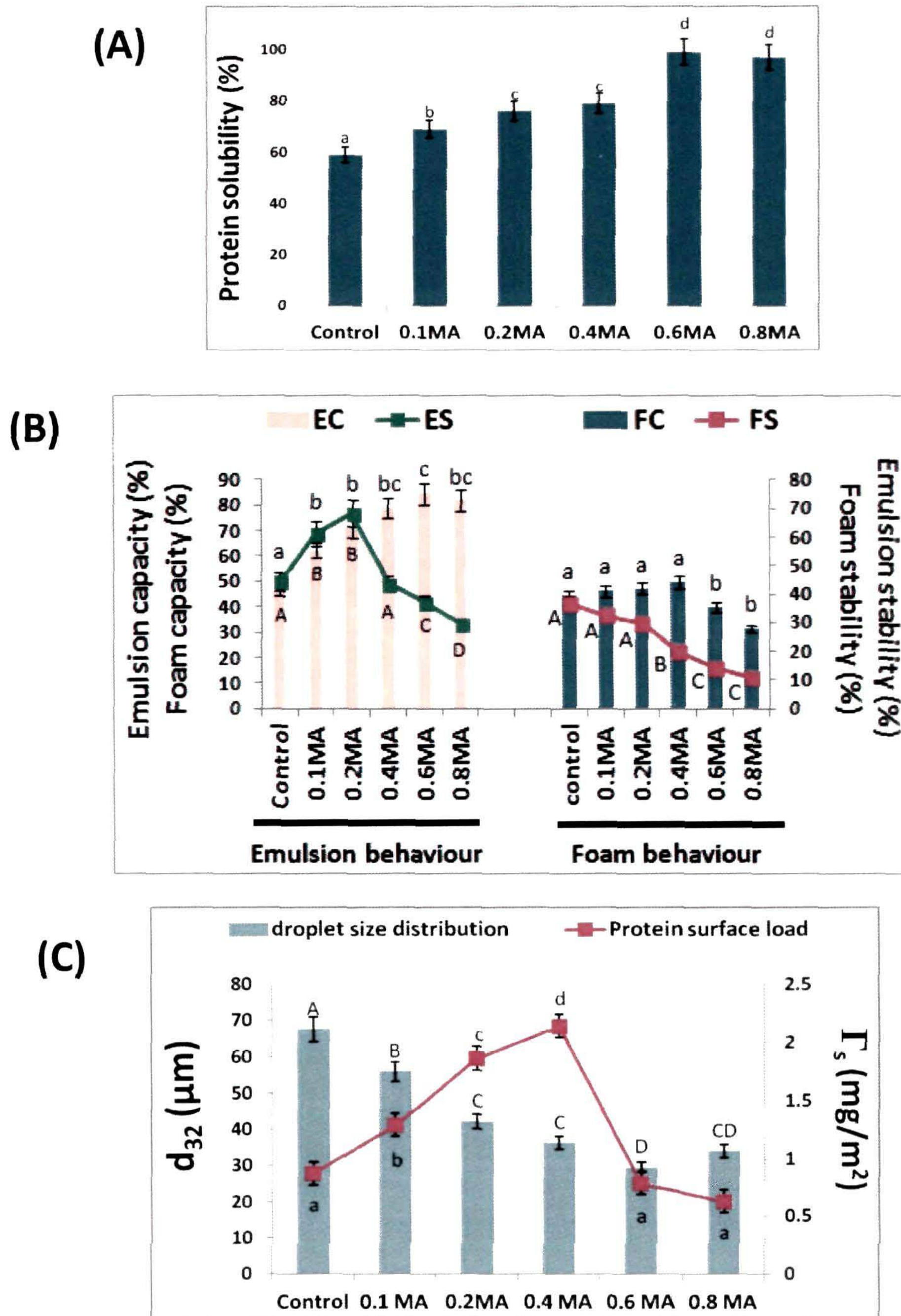



**Fig. 5.9** (A) Solubility (at pH 7) of the unmodified and modified rapeseed proteins; (B) Impact of different proteins on Emulsion Capacity (EC), Foaming Capacity (FC), Emulsion Stability (ES) and Foam Stability (FS); (C) Average droplet-size ($d_{32}$) and protein surface load ($\Gamma_s$) of o/w emulsions stored at 25 °C for t=0 h. Bars or points with different letters in each type of analysis indicate significant difference ($p<0.05$).

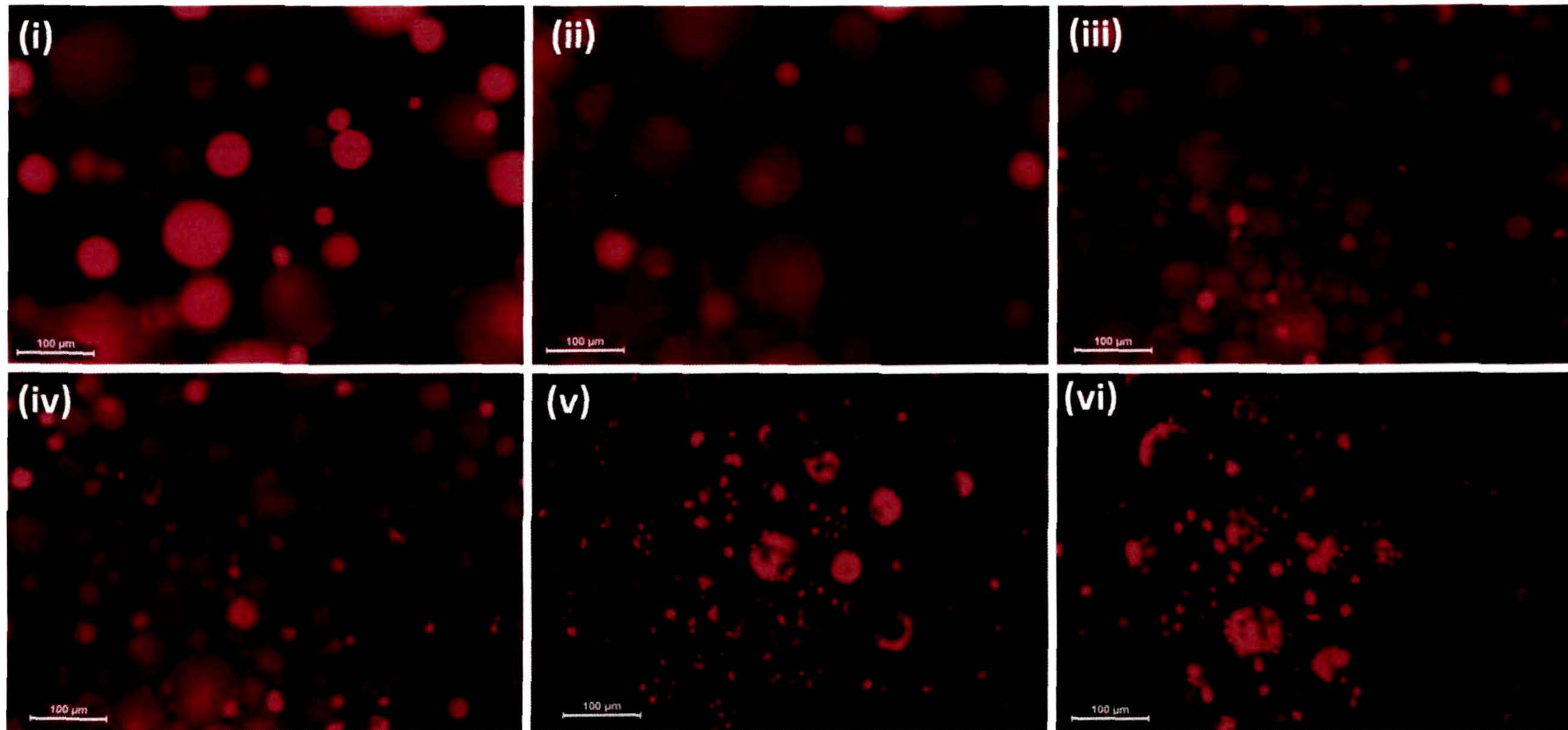



**Fig. 5.10A** Fluorescence micrographs of the o/w emulsion stabilized by (i) control, (ii) 0.1MA, (iii) 0.2MA, (iv) 0.4MA, (v) 0.6MA, and (vi) 0.8MA stored at 25 °C for t=0 h (Scale bar represents 100 µm).

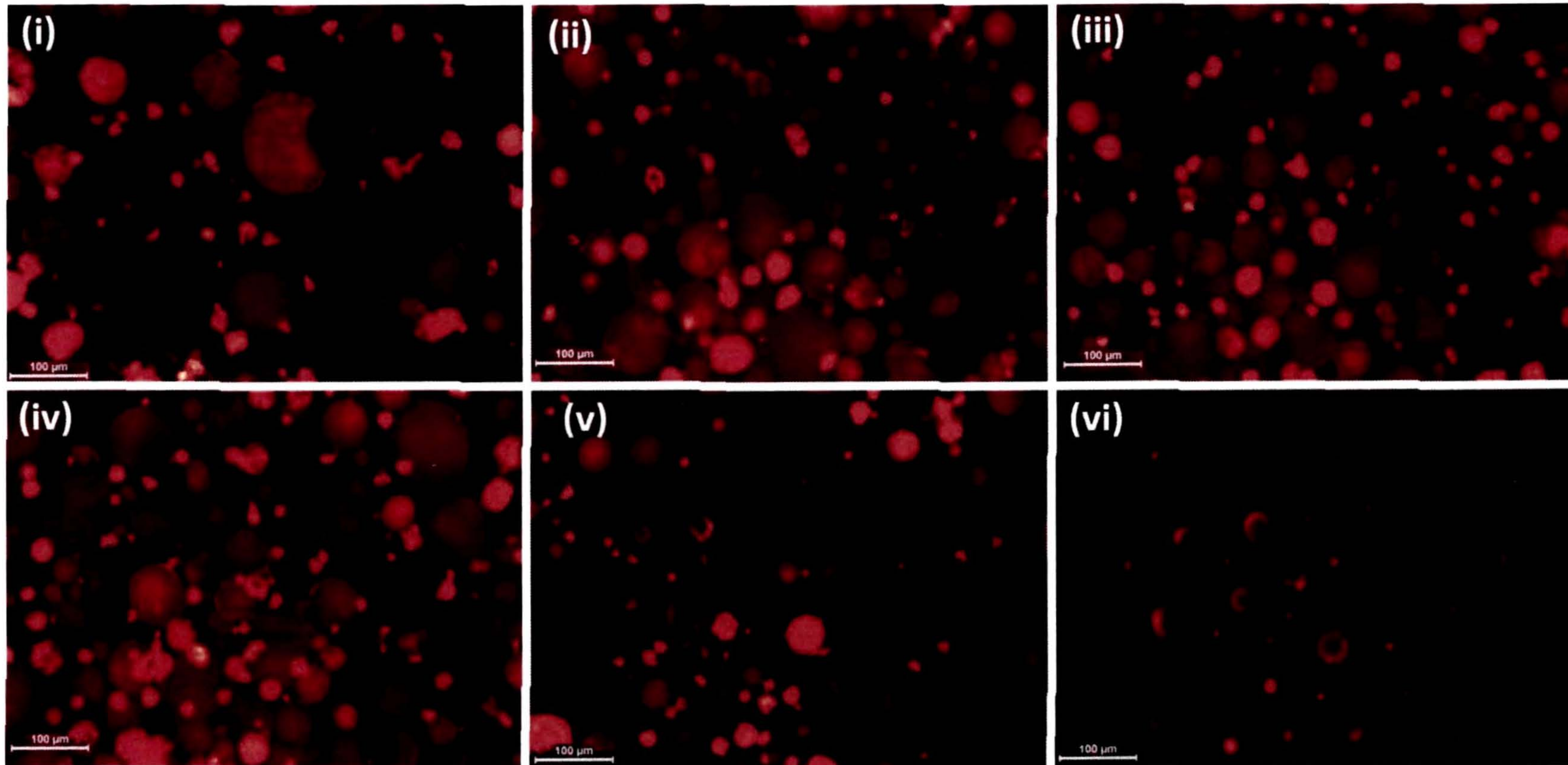



**Fig. 5.10B** Fluorescence micrographs of the o/w emulsion stabilized by (i) control, (ii) 0.1MA, (iii) 0.2MA, (iv) 0.4MA, (v) 0.6MA, and (vi) 0.8MA stored at 25 °C for t=24 h (Scale bar represents 100 μm).

of the film lamella occurs at a faster rate in foams/emulsions stabilized by small surfactants.[54]

Effectiveness of a protein as an emulsifier also depends strongly on the protein surface load at saturation ($\Gamma_s$). $\Gamma_s$ for most food proteins have been reported in the range of 1-10 mg/m$^2$.[28] Our result is comparable with that of legume protein[13] and suggests that maleylation increased $\Gamma_s$ till 0.4MA and then lessened it with further modification (**Fig. 5.9C**). A possible explanation could be sought in light of much higher flexibility of the modified protein molecules, compared with the native one which enables them to adsorb at a much higher rate at the newly formed interfaces during emulsification. Native proteins seem to possess a much more globular and rigid structure and thus have a slow conformational change at the interface.[13] High $\Gamma_s$ may also point to the formation of protein layers around fat globules that renders the emulsion more stable and resistant to coalescence. If the molecules are highly charged, it becomes increasingly difficult for more molecules to adsorb to the surface because of the electrical potential established by previously adsorbed molecules, creating an energy barrier to further adsorption.[13] This might be the possible cause for the reduction of $\Gamma_s$ in 0.6MA and 0.8MA. Although highly acylated samples adsorbed at a lower degree, their reduced molecular sizes afforded rapid mobility/spreading, leading to the creation of a film around oil droplets faster,[13] hence higher EC. In the same way, protein hydrolyzates or peptides often increases emulsifying activity index and produces smaller oil droplets than the intact protein, but produces very unstable emulsions and foams.[54]

### 5.3.3. Colour, texture and sensory characteristics of mayonnaise supplemented with maleylated rapeseed proteins

Mayonnaise has been chosen as a model food in order to study the potential of acylated proteins as a replacement for egg proteins. The first encounter with food products is often visual and is known to affect subsequent willingness to accept a product. So, colour parameters of freshly prepared mayonnaises were analyzed by Hunter colorimeter. Hunter-L, Hunter-a and Hunter-b values of the mayonnaises prepared with acylated proteins were similar to those of the mayo_100EY with no significant difference

between them ($p>0.05$) (**Table 5.2**). This result was expected since the degree of whiteness of the acylated protein derivatives were quite comparable with each other ($p>0.05$) (**Fig. 5.5A**). Mayo_control showed slightly inferior appearance because of its darker colour, as evident from the least Hunter-L (indicator of the reduction of lightness) and Hunter-b (indicator of the reduction of yellowness) values in addition to having the highest Hunter-a (indicator of the tendency towards redness) characteristic. Based on these data, it can be presumed that maleylated proteins can be used in formulation of mayonnaise with acceptable colour properties. The obtained results were better than those reported by Aluko and McIntosh[25] in case of hydrolyzed canola protein-substituted mayonnaise.

The creamy texture is a key part of mayonnaise, which is the second most important feature that the consumers look for. Despite the distinct differences in their conformations and functional properties (particularly EC), texture parameters of the mayonnaises prepared with different maleylated proteins were found to be either slightly lower or rather similar to that of Mayo_100EY. This discrepancy is contributed by the difference in oil content used in different analytical methods: in o/w emulsion, the ratio of oil:water was 30:70 (v/v), where the hydrophilic properties of the proteins are more important; on the contrary, the oil content in the mayonnaise formulation was very high (70%), where the emulsifier's lipophilic property play a crucial role.[27] Moreover, addition of low level of acylated protein (15%) into mayonnaise, as partial replacement of the major stabilizer (egg yolk, 85%), probably was not sufficient enough to make a discernible change on textural properties. Nonetheless, consistency of Mayo_0.2MA represented the highest value, which is ascribable to the entrapment of air droplets in the protein gel-like three-dimensional structure with oil granules (discussed later). Residual polyphenols in unmodified rapeseed protein adversely affected the textural properties of Mayo_control, which is in good agreement with the postulation of Mattia et al.[55] Concerning the mayonnaises generated with highly acylated proteins, their poor textural features can be explained by their weak resistance to phase separation (discussed later). It is well documented in the literature that low MW proteins/peptides confer poor stabilizing effect on the surface layers.[6,54] Therefore, in order to make a product with a

**Table 5.2** Characterization of mayonnaise supplemented with unmodified and modified rapeseed proteins.

| Properties | Mayo_100EY | Mayo_control | Mayo_0.1MA | Mayo_0.2MA | Mayo_0.4MA | Mayo_0.6MA | Mayo_0.8MA |
|---|---|---|---|---|---|---|---|
| ***Hunter colour parameters*** | | | | | | | |
| Hunter-L | 78.9±0.03$^{a}$ | 70.5±0.12$^{b}$ | 78.5±0.18$^{a}$ | 77.9±0.17$^{a}$ | 76.5±0.11$^{a}$ | 79.3±0.23$^{a}$ | 79.5±0.51$^{a}$ |
| Hunter-a | 1.3±0.09$^{a}$ | 2.0±0.02$^{b}$ | 1.4±0.03$^{a}$ | 1.1±0.03$^{a}$ | 1.5±0.05$^{a}$ | 1.8±0.12$^{a}$ | 1.3±0.99$^{a}$ |
| Hunter-b | 19.4±0.95$^{a}$ | 14.7±1.1$^{b}$ | 18.9±0.93$^{a}$ | 19.0±0.87$^{a}$ | 20.7±0.82$^{a}$ | 19.7±0.77$^{a}$ | 19.2±0.31$^{a}$ |
| ***Texture properties*** | | | | | | | |
| Firmness (g) | 216.4±1.1$^{a}$ | 211.4±0.58$^{b}$ | 212.5±1.09$^{b}$ | 213.4±0.53$^{b}$ | 212.0±1.4$^{b}$ | 212.4±1.9$^{b}$ | 210.7±1.1$^{b}$ |
| Consistency (g sec) | 2282.5±1.5$^{a}$ | 2236.3±3.7$^{b}$ | 2274.5±2.5$^{c}$ | 2327.7±2.3$^{d}$ | 2269.6±1.8$^{c}$ | 2253.6±2.1$^{e}$ | 2246.1±2.6$^{f}$ |
| Cohesiveness (g) | -115.5±2.7$^{a}$ | -110.5±0.63$^{b}$ | -111.0±3.1$^{a}$ | -110.5±3.4$^{a}$ | -110.9±2.3$^{a}$ | -109.7±2.0$^{b}$ | -96.7±3.3$^{b}$ |
| Index of viscosity (g sec) | 102.1±1.3$^{a}$ | 102.3±1.8$^{a}$ | 102.6±1.7$^{a}$ | 102.6±1.0$^{a}$ | 101.0±2.0$^{a}$ | 97.5±1.6$^{b}$ | 99.6±2.3$^{b}$ |
| ***Sensory attributes*** | | | | | | | |
| Appearance | 3.5±1.2$^{a}$ | 2.1±0.9$^{b}$ | 3.3±1.0$^{a}$ | 3.5±1.3$^{a}$ | 3.2±1.4$^{a}$ | 2.2±1.1$^{b}$ | 1.5±0.8$^{c}$ |
| Colour | 4.1±1.1$^{a}$ | 1.1±0.6$^{b}$ | 3.9±0.9$^{a}$ | 3.9±1.2$^{a}$ | 3.9±1.7$^{a}$ | 2.8±0.6$^{c}$ | 2.6±1.0$^{a}$ |
| Odour | 2.6±1.02$^{a}$ | 2.5±1.4$^{a}$ | 2.6±1.0$^{a}$ | 2.7±1.3$^{a}$ | 2.6±1.2$^{a}$ | 2.5±0.9$^{a}$ | 2.5±0.9$^{a}$ |
| Texture | 3.5±0.9$^{a}$ | 3.5±1.0$^{a}$ | 3.5±1.1$^{a}$ | 3.7±1.0$^{a}$ | 3.2±1.4$^{a}$ | 2.7±1.2$^{b}$ | 2.0±0.7$^{b}$ |
| Taste | 3.8±0.2$^{a}$ | 3.3±0.2$^{b}$ | 3.8±0.4$^{a}$ | 4.0±0.7$^{a}$ | 3.8±1.0$^{b}$ | 3.0±0.3$^{c}$ | 2.3±0.4$^{c}$ |
| Overall acceptability | 4.0±1.3$^{a}$ | 3.8±0.5$^{b}$ | 3.9±1.0$^{a}$ | 3.9±1.5$^{a}$ | 3.9±1.0$^{a}$ | 3.1±0.9$^{b}$ | 2.5±0.8$^{c}$ |
| ***Creaming Index (CI)*** | | | | | | | |
| At Day 0 (%) | nd | nd | nd | nd | nd | 4.8±1.7$^{a}$ | 19.0±1.4$^{b}$ |
| At Day 21 (%) | 1.6±0.94$^{a}$ | 12.0±2.6$^{b}$ | nd | nd | 7.4±1.9c | 14.8±3.1$^{b}$ | 35.2±4.1$^{d}$ |

nd= Not detectable. Values are means±SD of 3 replicates (n=3), except for sensory data, where n=2. Means with the same superscript letter within one row were not statistically different ($p$>0.05).

texture close to that of traditional mayonnaise, moderately maleylated proteins seem to be more favoured than their non-acylated or highly acylated counterparts.

To achieve mayonnaise with appropriate consumer acceptability, sensory evaluation of the samples was performed and the scores are shown in **Table 5.2**. Mayo_control obtained the lowest colour score which is solely due to its dark colour, imparted by the polyphenols of unmodified rapeseed protein. Similar observation was noted by Aluko and McIntosh[25] as the level of canola protein incorporation was increased in mayonnaise. This factor was also responsible for its low appearance and overall acceptability score. Mayo_0.6MA and Mayo_0.8MA also suffered very low sensory scores, mainly because of their rapid phase separation (**Fig. 5.11**). The major negative comments related to these products outlined the watery texture of serum phase and the oily mouth-coating of the separated cream phase as-well. The overall acceptability of Mayo_0.1MA, Mayo_0.2MA and Mayo_0.4MA were ranked almost the same as Mayo_100EY, with Mayo_0.2MA giving the best result among the three samples. Maleylated proteins appear to offer potential role in mayonnaise to replace conventional egg proteins. No bean or bitter flavour or off-odors were detected in any of the products so obtained.

### 5.3.4. Stability test of mayonnaise

Creaming is one of the most common instability mechanisms in oil-in-water emulsion (such as mayonnaise) that lead to macroscopic phase separation into cream and serum layers.[23] As such, storage stabilities of mayonnaise formed by the test samples were investigated over a storage period of 21 days at room temperature with respect to creaming index, which is presented in **Table 5.2**. Within the considered time window, 0.1MA and 0.2MA were very effective in generating stable mayonnaise with no detectable creaming, whereas Mayo_0.4MA creamed marginally (**Fig. 5.11**). It can be noticed that CI of Mayo_100EY is higher than that exhibited by Mayo_0.1MA and Mayo_0.2MA after 21 days of quiescent storage, and this suggests that the mayonnaise formed with the maleylated protein (with the best result given by 0.1MA and 0.2MA) together with egg yolk are more stable than that stabilized with egg proteins alone. This

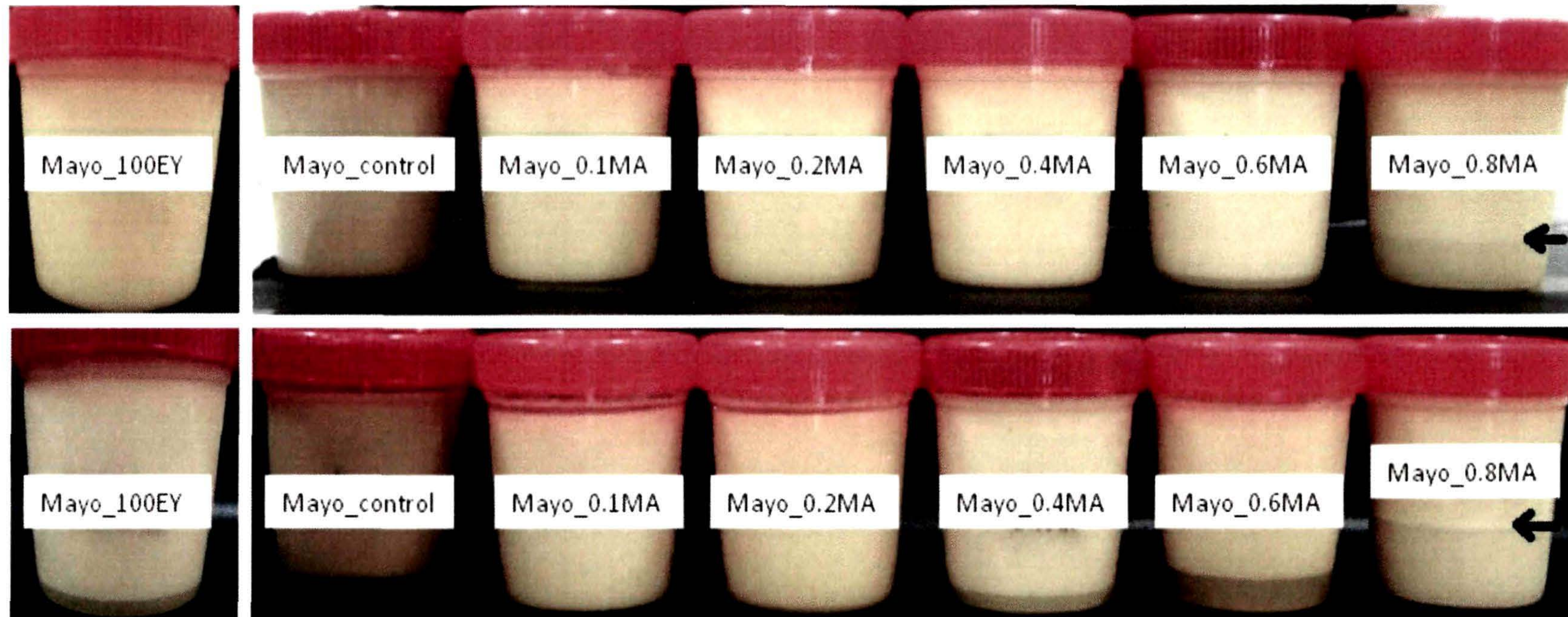



**Fig. 5.11** Photograph showing the phase separation in mayonnaise supplemented with different proteins, quiescently stored at room temperature on day 0 (above) and day 21 (below).

behavior can be ascribed to the generation of sufficient repulsive effect between the oil droplets by acylated proteins to avoid them from coalescing; however, very strong repulsions may lead to rapid phase separation, where the emulsifier fails to cover the oil-water interface thoroughly.[23] The latter cause obviously reinforced the high CI value in Mayo_0.8MA. Moreover, amelioration of the surface activity due to possible interaction between egg proteins and low maleylated rapeseed proteins (with favoured state of conformational changes) may also corroborate such a result, because lysozyme has been shown to form binary or ternary electrostatic complexes with the other proteins and thus its adsorption to the interface is facilitated by the adsorption of other proteins.[56]

As incorporation of 0.4MA, 0.6MA and 0.8MA into mayonnaise have been found to mar the product, especially in terms of CI; on the other hand, Mayo_0.1MA and Mayo_0.2MA did not show any detectable sign of creaming even after storing for 21 days, so the microstructure of Mayo_0.1MA and Mayo_0.2MA were compared with those of Mayo_100EY and Mayo_control for evaluating their colloidal difference. Mayo_100EY exhibited large-sized spherical moieties, possibly representing fat particles, dispersed unevenly in a protein network (**Fig. 5.12A**). The use of high concentration of glutaraldehyde during sample preparation for SEM analysis might have caused strong protein-protein cross-linking of the film lamellae[57] around the oil droplets and thus maintained the globular morphology of dispersed fat during topography viewing of the mayonnaise. In Mayo_0.1MA, a protein matrix is visible with irregular forms of fat globules surrounding an open space, which probably represented the air droplets entrapped by vigorous mixing during mayonnaise preparation (**Fig. 5.12B**). Similar observation was also noted for Mayo_0.2MA, where the open spaces gave the appearance of a concave cavity whose peripheral wall seems to be made up of stacked spherical fat globules, held onto each other by compact protein network structures; however the globules were much smaller in diameter (**Fig. 5.12C**). Unlike Mayo_0.1MA and Mayo_0.2MA, the fat globules in Mayo_control did not distribute uniformly to form a discrete spot of big open space; rather they appeared to be embedded in thick layers of protein (**Fig. 5.12D**). The sample was also observed to own fewer fat globules and shallow dents in protein layers (marked by arrows). All these facets imply that 0.1MA

and 0.2MA-stabilized mayonnaises tended to have a smooth and homogeneous texture due to the amelioration of the package density of molecules at the interface, along with retention of large air droplets (which possibly formed big open spaces or dents in protein network) that consequently slowed down oil droplets movement, retarding droplet coalescence, flocculation, and creaming.

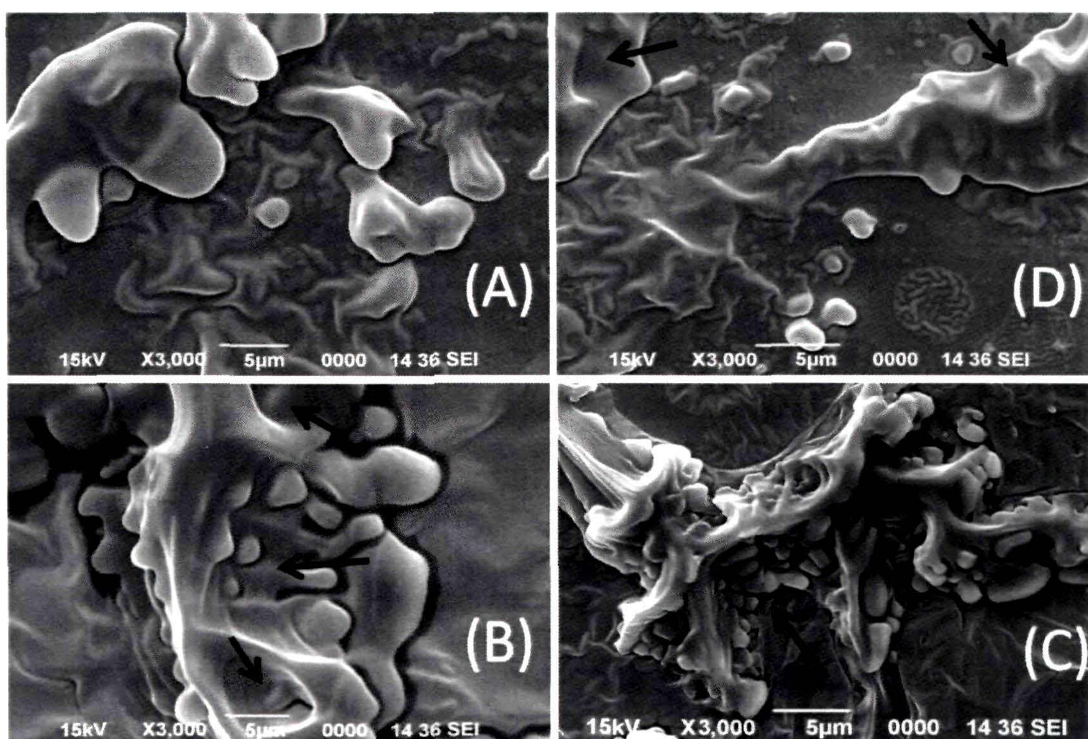



**Fig. 5.12** SEM images of the emulsions stored at room temperature for 21 days: (A) Mayo_100EY, (B) Mayo_0.1MA, (C) Mayo_0.2MA, and (D) Mayo_control.

## 5.4. Conclusion

There are good relationships between physicochemical and functional properties of acylated proteins. Acylation increased whiteness value and dissociation of proteins, but reduced free sulfhydryl and disulfide content ($p<0.05$). Intrinsic fluorescence emission and FTIR spectra revealed distinct perturbations in maleylated proteins' tertiary

and secondary conformations. Increase in surface hydrophobicity, foaming capacity, emulsion stability, protein surface load at oil-water interface and decrease in surface tension at air-water interface, occurred till moderate level of modification. While maleylation impaired foam stability, protein solubility and emulsion capacity were markedly ameliorated ($p<0.05$), which were concomitant with decreased droplet size distribution ($d_{32}$). Overall, maleylation appeared to be effective in enhancing several surface functionalities of rapeseed protein. *In-vitro* digestibility and cytotoxicity tests suggested no severe ill-effects of modified proteins, especially at low degrees of maleylation. Improved whiteness, together with good digestibility and biocompatibility, of maleylated proteins allow reappraising the value of underutilized oilseed-cakes as a source of proteins useful for the food industry. The study showed good potential of maleylated proteins as functional food ingredient. A detailed evaluation of the digestibility and nutritional quality of maleylated proteins require more elaborate *in-vivo* tests to support these findings.

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# Chapter-6

# Synthesis of carbon nanoparticle from oil-and-protein spent meal and its application in fabricating rapeseed protein-based nanocomposite film

## 6.1. Introduction

Currently, fluorescent nanoparticles (NPs) (also known as quantum dots, QDs due to their typical sizes below 10 nm), a young smart member of the nano-material family is gaining attention, especially in the field of optoelectronics, bio-imaging, biomedicines, etc. Unfortunately, application of NPs in food[1-5] and its packaging materials is still in its infancy. The reason behind this seems to be the raised toxicity concern and environmental hazard of the fluorescent semiconductor QDs, which are based on metallic elements and heavy metals (elements from the periodic groups II-IV, III-V and IV-VI). Alternatively, fluorescent carbon NPs (called fluorescent carbon-dots or FCDs or C-dots or simply CNPs) can become an exciting option, because they are greener for the environment and show aqueous dispersibility, numerous possible applications in nano-biotechnology, and far less toxic to living organisms. Recently, Sk et al.[6] reported the presence of FCDs in regular carbohydrate-based food items such as bread, jaggery, sugar caramel, corn-flakes and biscuits, and also showed their biocompatibility. This novel discovery alleviated the misapprehension that all fluorescent NPs are toxic and revealed that humans have been consuming fluorescent nano-materials in the form of food caramels for centuries, and thus they can be considered safe.

Recently, procurement of novel C-dots from greener sources such as egg,[7] orange juice,[8] banana juice,[9] soy-milk,[10] glucose, sucrose, starch,[11] citric acid,[12] chitosan,[13] etc. has been the centre of attraction. Although they are the current state-of-the-art, most of these methods suffer to some degree from certain drawbacks such as the requirement of sophisticated and expensive equipments such as laser beam or microwave or high-power autoclave; neat chemicals as precursors for synthesis, or even multi-steps of operation along with surface passivation to improve the water-solubility and photoluminescent properties of C-dots. Moreover, use of such food/feed or other edible stuffs as raw materials for FCD preparation is expensive for bulk use, and in the long-run would create undue strains on the food resource system and food security scheme. This is a major drawback to the commercial synthesis of NPs from these edible resources, which is

unlikely to be extended in the near future. This motivated us to search for a simple economical technique for the production of FCDs from industrial or agricultural by-products. This is a challenging but worthy concept as the use of waste materials is one of the most attractive options to reduce the raw material cost and also seems benign from an ecological point of view. In this context, very few articles are available. Lu et al.[14] reported such an endeavor from pomelo peel and the resulting CNP was used for the detection of mercury ions in water. Wang et al.[15] prepared C-dots from egg-shell membrane and used it for designing a fluorescent probe for glutathione detection. In a closely related work, FCDs were prepared from used coffee grounds,[16] and their practicality in cell imaging and detection of angiotensin I and insulin was assessed. Most reported C-dots have been prepared for bioimaging which limits their application in other fields. As such, it has become urgent to develop effective routes to create functional C-dots as-well-as expand their applications.[10] Explicitly the role of FCD in food packaging domain is unexplored to date.

Industrially, mechanical pressing of the oilseeds produces the oil (main product) and the press-cake (by-product). Due to the high quantity of protein, the oil-cake or meal is being used mainly as animal feed. Recently, owing to the high nutritive value of the meal protein, techniques are now being devised to harness it. After the extraction of meal protein, the residual fibrous waste material (protein-spent meal) is discarded and is not extensively used in industries. At present there are very few possibilities for the utilization of this waste; usually the residue is disposed as landfill and hence, is as an "end-of-pipe" waste. In this chapter, the synthesis of green fluorescent quantum-sized carbonaceous NPs from 'oil-and-protein spent' rapeseed meal by a facile hydrothermal process has been reported. These NPs are referred as 'carbogenic' because of their high oxygen content along with carbon.[12]

Undeniably, the most active area of food nanoscience research and development is 'packaging'. This is likely connected to the fact that the public has been shown in some studies to be more willing to embrace nanotechnology in "out-of-food" applications than

those in which NPs are directly added to food.[17] So, the practicability of the synthesized FCDs for making green-fluorescent edible biopackaging material was investigated in this study, thereby expanding the potential application of C-dots. Synthetic plastic packaging is beginning to be replaced by biodegradable ones because of environmental concerns. Films made of carbohydrates and protein are long and empirically been used to make food-grade biodegradable packaging materials. A recent approach to this technology involves the use of vegetable protein from different types of oil-cakes such as cottonseed,[18] pumpkin seed,[19] soybean,[20] and rapeseed/canola.[21] In this milieu, edible films using rapeseed protein were developed in the current investigation, because this biopolymer can be obtained as value-added product from the under-utilized meal. As such, the study was extended to evaluate the effect of such a nano-additive on the physicochemical properties of rapeseed protein film.

Mostly, the consumers have to rely on holograms and other displays on the packets to segregate authentic products from their duplicate inferior counterparts. Often, fluorescent dyes are incorporated into the packaging to help customers easily detect authentic products (e.g. Erythrosine); however, many of these dyes have been shown to be cytotoxic to a variety of mammalian cell types.[22] The unique "green" photoluminescence property of the here-in synthesized carbogenic NPs, along with high antioxidative potential and hemocompatibility, offer a solution to this problem. It is envisioned that FCD-incorporated edible films would not only be useful for quality control to ensure that consumers are able to purchase authenticated products, but would also improve the oxidative stability of the produce/commodity. To justify this possibility, further study was conducted to see the effect of FCD-protein composite film on the oxidative shelf-life of an oil sample. For simplicity, the preparative protocol along with the summarization of the complete investigation undertaken in this chapter is depicted pictorially in **Fig. 6.1**.

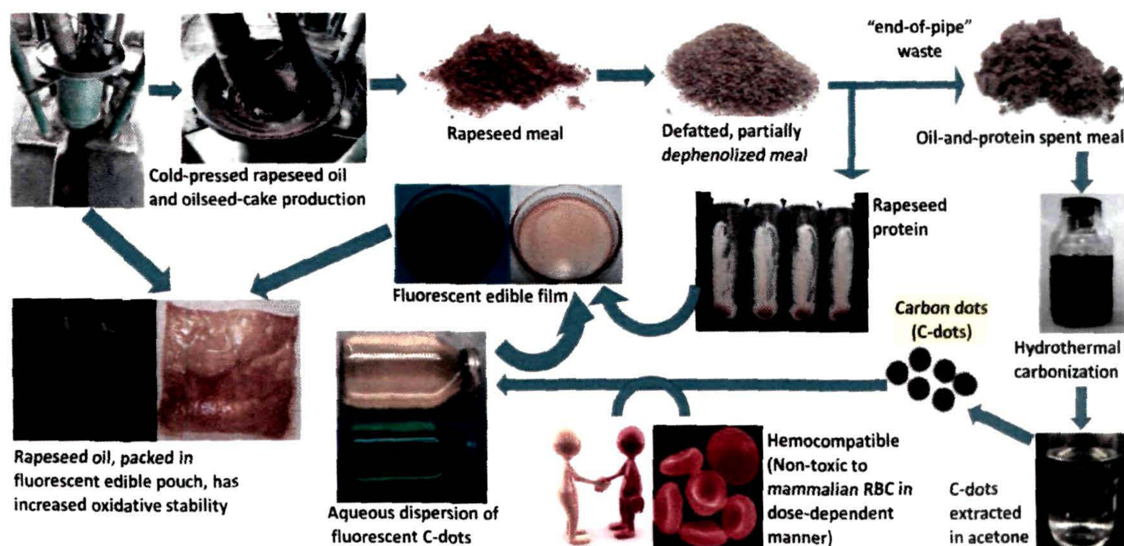


**Fig. 6.1** Steps in the preparation of fluorescent carbon-dots and its application.

## 6.2. Materials and methods

### 6.2.1. Chemicals

All solvents and reagents were obtained from E. Merck® (India), of either high-performance liquid chromatography (HPLC) grade or analytical reagent grade, and were used without further purification.

### 6.2.2. Materials and sample preparation

Cold-pressed rapeseed oil and press-cake were obtained from Assam Khadi & Village Industries Board, Guwahati, India. Press-cake was defatted and then partially detoxified prior to protein extraction according to the procedure mentioned in Chapter-4, and then stored at -20 °C until use.

### 6.2.3. Protein extraction from detoxified meal and recovery of oil-and-protein spent meal

Aqueous suspension of detoxified meal was prepared with water (30:1 v/w), followed by the addition of 0.0 M NaCl and 0.4% sodium sulfite. The pH of the

suspension was adjusted to 11±0.1 with 1 N NaOH solution; the suspension was mixed (213 x g) for 2 h at 25 °C in the orbital shaker (Sartorius Stedin Biotech, CERTOMAT® IS), followed by centrifugation (SIGMA 3-18K Centrifuge) at 7,513 x g for 20 min at 4 °C. The solid residue (oil-and-protein-spent meal) was vacuum-dried (Lab companion model OV-12, Jeiotech Co., Korea) for 48 h at 40 °C, ground to pass through a sieve having 250 μm pores, and then stored at -20 °C for further use. On the other hand, the protein-rich supernatant was filtered through Whatman filter no. 41 and then ammonium sulfate was added up to 85% saturation. The mixture was kept in an ice bath for 3 h with gentle stirring and then centrifuged at 10,733 x g for 20 min at 4 °C. The obtained protein precipitate was re-dispersed in Milli-Q water (Millipore Water Purification System, Model-Elix, USA), neutralized to pH 7, dialyzed against water at 4 °C and finally freeze-dried, which was subsequently used for making the composite film. Proximate analyses of the spent meal were performed by the methods mentioned in Chapter-2.

### 6.2.4. Synthesis of C-dots from oil-and-protein spent meal

FCDs were synthesized by hydrothermal carbonization (HTC) of spent meal using the reported protocols[8,23] with slight modification. In a typical procedure, oil-and-protein spent meal was dispersed in 1N NaOH solution (solvent) such that a definite solvent:meal ratio was attained as per the design matrix. Then the suspension was refluxed in an oil-bath under magnetic stirring, at each of the indicated temperature and heating duration mentioned in the central composite design (CCD). After the reaction is over, the resultant black solution was allowed to cool down and centrifuged at 3220 x g for 10 min to separate out the unreacted residue. The brownish supernatant was washed with dichloromethane to remove the unreacted organic moieties. Subsequently, the aqueous phase was mixed with acetone (water:acetone ratio=1:3 v/v) and the acetonic extract was finally reduced to dryness under vacuum. The residue was dispersed in Milli-Q water, dialyzed against water (using 1 kDa membrane, Himedia, India) and finally dried under vacuum at 40 °C to obtain FCD, whose weight is recorded.

### 6.2.5. Characterization of C-dots

High resolution transmission electron microscopy, HRTEM (JEOL, JEMCXII) images along with the selected-area electron diffraction (SAED) pattern were obtained at an accelerating voltage of 200 kV, and the sample was prepared by drop casting 2 μl of NP solution (0.25 mg/ml) on a 300 mesh carbon coated copper grids and subsequent air drying before analysis.

X-ray Diffraction (XRD) measurement was carried out by thin film mode of powdered sample using Rigaku Miniflex model (Japan), operated at 30 kV voltages and a current of 15 mA with Cu Ka radiation source ($\lambda$=1.54 Å) at a scan rate of $5^0$ (2$\theta$) $min^{-1}$ over the range of 10–70°.

Thermogravimetric analysis (TGA) curves were collected on a TG 50 model (Shimadzu, Japan). The samples were combusted under nitrogen flow (10 ml/min, to avoid thermo-oxidative reactions) at the temperatures ranging from 25 to 600 °C, at a rate of 3 °C/min.

Fourier transform infrared (FTIR) spectra were obtained on a FTIR Nicolet Magna 5PC spectrometer (Impact-410, Madison, USA), coupled to a PC with Omnic analysis software and having DTGS (Deuterated Triglycine Sulfate) detector and Nernst Filament as the IR light source. The sample was ground with KBr powder to form well-defined pellets for IR measurement, with 32 scans from 4000 to 400 $cm^{-1}$ at a resolution of 4 $cm^{-1}$.

Micrograph of the sample was obtained using a JSM-6390LV scanning electron microscope (SEM; JEOL, Japan) at an accelerating voltage of 15 kV. Prior to SEM observation, samples were mounted on stubs with double-sided adhesive tape, followed by coating the samples with a thin layer of gold. For determining the elemental composition and purity, sample was prepared on a carbon-coated copper grid and kept under vacuum desiccation for 3 h before loading them onto a specimen holder. Elemental

analysis on single particles was carried out using electron dispersive X-ray spectroscopy (EDX, JSM-6390LV) attachment equipped with SEM.

$^{1}H$- and $^{13}C$-Nuclear Magnetic Resonance (NMR) spectra were detected at 400 MHz by a JEOL NMR system (Japan), using the inbuilt DELTA (δ) software (version-G4.3.6, Japan) provided by the manufacturer.

Ultraviolet-visible (UV-vis) absorption spectrum of aqueous NP solution (0.1 mg/ml) was recorded on a UV-vis spectrophotometer (CECIL 7400, 7000 Series, Aquarius).

Photoluminescence (PL) spectrum of aqueous NP solution (0.1 mg/ml) was measured on a photoluminescent spectrophotometer (Model LS 55, Perkin Elmer, Singapore PTE Ltd., Singapore). Illumination of the aqueous NP dispersion for detecting its fluorescence property was done inside a UV cabinet (BD-198 model, Test Master, Kolkata, India).

Quantum yield (QY) of C-dots was measured in reference to quinine sulphate in 0.1M $H_2SO_4$ (QY = 58% at 354 nm excitation).[6,24] The formula used for QY is as follows:

$$(QY)_{Sm} = (QY)_{St} \times [(PL\ area/OD)_{Sm}/(PL\ area/OD)_{St}] \times \eta^2_{Sm}/\eta^2_{St} \quad (1)$$

where Sm indicates the sample, St indicates the standard, $\eta$ is the refractive index of the solvent, and PL area and OD are the fluorescence area and absorbance value, respectively. For aqueous solutions $\eta_{sm}/\eta_{st}=1$ was chosen.[6]

Antioxidant activity was measured using the modified 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical method.[25] Briefly, 3 ml of sample solution was mixed with 1 ml of 1 mM methanolic solution of DPPH. The mixture was then vortexed and incubated in the dark at ambient temperature for 30 min. The absorbance was measured at 517 nm in a UV-vis spectrophotometer. In case of FCDs or ascorbic acid (as standard), the amount was varied from 10-180 μg per ml of reaction mixture;[26] whereas for film samples, 25 mg of each film was dissolved in 3 ml of Milli-Q water.[25] The DPPH scavenging percentage was calculated using the following formula:

Scavenging activity (%) = $\{(A_D - A_S) \times 100\}/A_D$ (2)

where, $A_D$ and $A_S$ are the absorbance of the DPPH solution and the standard/sample, respectively.

Hemolytic activity assay was performed according to the reported procedure.[27] Briefly, fresh goat blood from a slaughter-house was collected in a centrifuge tube containing anti-coagulant, tri-sodium citrate (3.2%), and was centrifuged at 3220 x g for 10 min. The supernatant was discarded and only the red blood corpuscles (RBCs or erythrocytes) were collected. RBCs were further washed three times with phosphate buffer solution (PBS, pH=7.4). A 10% (v/v) suspension of erythrocytes in PBS was prepared and 1.9 ml of this erythrocyte solution was placed in a 2 ml centrifuge tube and 0.1 ml of FCD in PBS was added to it. The tubes were then incubated for 1 h at 37 °C. For comparison, Triton X-100 (0.2%) and PBS were taken as the positive and negative controls, respectively. After incubation, the tubes were subjected to centrifugation at 3220 x g for 10 min, and finally absorbance of the supernatant was taken at 570 nm in a UV-visible spectrophotometer.

Cell labeling with FCDs was done by the procedure of Chandra et al.[28] Erythrocytes enriched fraction was centrifuged twice (3220 x g, 15 min) at 4 °C to remove the residual plasma and buffy coat. RBCs were washed 3 times with PBS (pH 7.4) and re-suspended in the same buffer to make a cell concentration of ≈10% (w/v) as stock. Then, cell labeling was carried out by mixing different aliquot of the above RBC stock suspension with different volumes of FCD (30.4, 45.5, 60.8 and 75.8 μl) from its stock solution (660 mg/ml) for making up the final concentration of FCDs as 20, 30, 40 and 50 mg/ml by addition of PBS in each set of experiment. Afterward, the erythrocytes were incubated for 4 h at room temperature, centrifuged (3220 x g, 15 min) at 4 °C and then washed thrice with PBS. Finally, 20 μl of the cell suspension were taken and used to prepare a smear on glass slides, which was viewed under a fluorescent microscope (LEICA DM 3000, Power: ebq 50 qc, USA) attached with a LEICA DFC 450C camera.

### 6.2.6. Preparation of rapeseed protein-FCD composite film and its application for oil packaging

Rapeseed protein film solution was prepared by using the methodology of Cho and Rhee.[20] Freeze-dried rapeseed protein powder (8 g) and glycerol (4 g) were dissolved in 100 ml of Milli-Q water. The suspension was heated on a hot-plate magnetic stirrer for 10 min at 80 °C until a homogeneous clear solution was obtained. FCDs (20 or 30 mg) were added to the mixture and the blending was further continued for 15 min at 80 °C. The mixture (40 ml each) was cast onto glass petriplates and then dried in an oven at 35 °C for 48 h. Films were conditioned in an environmental chamber (Plant growth chamber HB 303DH, K&K Scientific Supplier, India) set at 25 °C with 50% relative humidity (RH) for 48 h. The composite films with 20 and 30 mg of FCDs per 100 ml of film-forming solution were accordingly coded as F1 and F2, respectively. Film without FCDs served as control and was designated as F0.

Films were drawn into small rectangular sachets/pouches (5 cm x 3.5 cm), each of which was filled with 4 ml of cold-pressed rapeseed oil, and then sealed after the headspace had been flushed with nitrogen gas. Oil containing sachets were stored for 28 days in the environmental chamber (25 °C, 50% RH), and the oil samples were analyzed after every 7 days of interval. Oil samples packed in F0, F1 and F2 were accordingly labeled as S0 (control), S1 and S2, respectively.

### 6.2.7. Characterization of the composite films

Film thickness was measured at 5 random positions with a micrometer (Mitutoyo Corp., Japan). The mean thickness was used to calculate the mechanical and barrier properties of film.

For moisture content determination, film samples were weighed into aluminum pans and dried at 105 °C in an oven for 24 h (until the equilibrium weight). The weight loss of the sample was determined, from which the moisture content was calculated.

Water vapour permeability (WVP) was determined gravimetrically following the standard method of the American Society for Testing and Materials (ASTM).[25] Conditioned film samples were sealed to glass cups (4.7 cm diameter) containing water. The film-covered cups were placed in the environmental chamber (25 °C, 75% RH). Cups were weighed periodically using an analytical balance (Denver Instrument, Bohemia, USA), until steady state was reached (±0.0001 g). Once the steady state was reached, water vapour transmission rate (WVTR, ng/$m^2$ s) of the film (Eq. 3) was determined from the slope obtained from the regression analysis of moisture weight gain ($\Delta w$) transferred through the film area (A) during a definite time ($\Delta t$). WVTR was then used to calculate WVP using Eq. (4).

$$WVTR = \Delta w / A(\Delta t) \quad (3)$$

$$WVP = WVTR(x/\Delta p) \quad (4)$$

where x is the average thickness of the film and $\Delta p$ is the partial water vapor pressure between the two sides of the film (i.e., 3167-2385 = 782 Pa)[29]

Oxygen permeability of the films was determined by the wet chemical procedure of Ayranci and Tunc.[30] Tensile strength (TS, in MPa) and Elongation at break (EB, %) of the film was measured according to ASTM D-882-91.[31] For evaluating puncture strength (given as force in Newton (N)), film was punctured with a 2 mm probe in a Texture Analyzer (TA HD Plus, Stable Micro Systems, UK).

Color intensity of the film was measured using a Hunter Lab Colorimeter (Ultrascan, VIS-Hunter Associates Lab., USA), fitted with a large area port (2.5 cm diameter aperture). The instrument (including 65°/0° geometry, D25 optical sensor, 10° observer, specular light) was calibrated using white and black reference tiles provided by the manufacturer.

For determining opacity, film absorbance was measured at 600 nm using UV-vis spectrophotometer.[25] The sample was cut into a rectangle piece and directly placed in

spectrophotometer test cell, using an empty test cell as the reference. The opacity index of the film was calculated by following equation

$$Opacity = Abs_{600}/x \quad (5)$$

where $Abs_{600}$ is the absorbance at 600 nm and x is the average film thickness (mm).

### 6.2.8. Analytical tests for oil sample

Free fatty acid (FFA) and peroxide value (PV) were determined according to the method of Chaijan et al.[32] and expressed as g/100 g lipid and milliequivalents (meq) of free iodine/kg of lipid, respectively.

Conjugated diene (CD) was estimated from the absorbance value at 233 nm using cyclohexane as the solvent blank.[33]

For thiobarbituric acid-reactive substances (TBARS) *assay*,[32,34,35] oil sample (0.5 g) was mixed with 2.5 ml of a solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25N HCl. The mixture was heated in a boiling water bath (95–100 °C) for 10 min to develop a pink colour, cooled under running tap water and then centrifuged (3600g) at 25 °C for 20 min. Absorbance value of the supernatant at 532 nm was expressed as the result ($A_{532}$).

### 6.2.9. Statistical analysis

Applying Response surface methodology (RSM) with a $(2)^3$-CCD matrix, hydrothermal carbonization (HTC) was optimized with 3 combinations of independent variables, namely time ($X_1$), temperature ($X_2$) and solvent:meal ratio ($X_3$). The yield of FCD (Y) was considered as the response. To have an idea of the amount of NPs which can be extracted from the spent meal, we analyzed the amount of particles obtained from 1 g spent meal under each experimental run. The selected variables were coded at five levels (−1.682, 1, 0, 1, and +1.682) (**Table 6.1**), and their real values were chosen based on preliminary experiments. The variables were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (6)$$

where $x_i$ is the dimensionless coded value of an independent variable, $X_i$ is the real value of an independent variable, $X_0$ is the real value of an independent variable ( $X_i$) at the centre point and $\Delta X_i$ is the step change value. The complete $(2)^3$-CCD matrix consisted of 20 experimental runs, including six replications at the centre points (0, 0, 0) to estimate the pure experimental error, and is summarized in (**Table 6.2**).

The system behavior towards the response was determined by a second-order polynomial equation, based on the equation below:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (7)$$

where $Y$ is the predicted value for the response, $\beta_0$ is the offset term, $\beta_i$ is the linear effect coefficient, $\beta_{ii}$ is the squared effect coefficient and $\beta_{ij}$ is the interaction effect. $x_i x_j$ represents the interaction between different coded values, where $i$ is one parameter and $j$ is other.

All analyses were performed in triplicate, unless otherwise indicated, and the mean value was calculated. Separation of means were carried out by Tukey test, using SPSS software (version 16.0, SPSS Inc., Chicago, USA) and considered significantly different at $p<0.05$. Analysis of variance (ANOVA) was applied to assess the adequacy (by lack-of-fit test) and statistical significance of the developed model at a confidence level of 95% (transgression probability, $p<0.05$). Response surface plots for any two independent variables were drawn while fixing the remaining one at coded zero level, for gaining a perspective on the interaction between the variables and how they influence the response, and also to find the optimum condition.

**Table 6.1** Independent variables and their levels for production of FCD from spent meal by HTC process.

| Independent variables | Symbols | Range and levels | | | | |
|---|---|---|---|---|---|---|
| | | **-1.682** | **-1** | **0** | **+1** | **+1.682** |
| Time (h) | $X_1$ | 2.636 | 4 | 6 | 8 | 9.364 |
| Temperature (°C) | $X_2$ | 126.36 | 140 | 160 | 180 | 193.64 |
| Solvent:meal ratio (v/w) | $X_3$ | 131.8:1 | 200:1 | 300:1 | 400:1 | 468.2:1 |

### 6.3. Results and discussion

The total mass recovery of spent meal was about 61.02±1.7% solid of starting meal (detoxified meal). This value was slightly higher than those reported for yellow mustard and canola meal.[36,37] C-dots were synthesized by HTC of spent residue, which mainly contains carbohydrates, crude fiber and insoluble structural protein (**Table 6.2**). These act as the sources of carbon precursors.[38] Till date, very few authors have reported the possible schematic route for the formation of C-dots from carbon precursors, as the formed intermediates and the final material structures are complex and a clear scheme has not yet been reported.

**Table 6.2** Proximate composition of spent meal (% dry basis).

| Parameters | Mean±SD[§] |
|---|---|
| Crude protein | 8.83±0.94 |
| Crude fiber | 18.09±1.07 |
| Residual oil | <0.01 |
| Total ash | 5.92±0.21 |
| Moisture content | 11.52±0.81 |
| Total carbohydrate (by difference) | 55.63±0.72 |

[§]Mean ± standard deviation of three replicates

### 6.3.1. Optimal production of FCD using RSM

The regression equation of the generated model describing the yield (Y) as a function of the uncoded factor levels, is shown in Eq. (8)

$$\begin{aligned} Y = 93.19 + 0.38X_1 - 1.09X_2 - 0.03X_3 - 1.47X_1^2 - 1.41X_2^2 \\ -0.86X_3^2 - 1.78X_1X_2 - 1.21X_1X_3 - 0.08X_2X_3 \end{aligned} \tag{8}$$

The high coefficient of determination, $R^2$=91.29% (goodness-of-fit test) advocates a good correlation between observed and predicted values, which is also evident from **Table 6.3**. It also indicates that at least 91% of the variability in the response could be explained by the derived second-order polynomial equation. Model was highly significant ($p<0.05$) and p-value for lack-of-fit test was large ($p>0.05$) (**Table 6.4**). This in-turn ascertains the validity of the model and is adequate for predicting the response within the conditions investigated here-in.

**Fig. 6.2a** shows the Pareto chart of the individual, quadratic and interactive effect of the chosen independent variables ($X_1$, $X_2$ and $X_3$) on the response (Y). The chart includes a vertical line at the critical value for $p=0.05$. An effect that exceeds the vertical line can be considered significant ($p<0.05$) and those which falls below this line are taken as non-significant ($p>0.05$). The need for the use of RSM during NP synthesis is reconfirmed by the Pareto chart; linear term of either temperature or time was found to be non-significant on the yield ($p<0.05$); whereas the interaction term of time and temperature ($X_1$*$X_2$), followed by their quadratic terms ($X_1^2$ and $X_2^2$) had the most significant effect. Thus, among the 3 factors, time and temperature of HTC process had significant effect ($p<0.05$) on the yield; whereas, that of solvent:meal ratio was non-significant ($p>0.05$). This partly corroborates with the postulations of Ray et al.[23]

Generally, lower heating temperature in HTC mandates the need of long processing time. This is in line with the observation of **Fig. 6.2b**. High temperature seems to produce increased yield in a shorter span of time, which seems judicious for practical

relevance.[39] Likewise, **Fig. 6.2c** shows that higher yield is attainable by use of long processing time. From **Fig. 6.2d**, it is clearly apparent that high temperature and large solvent:meal ratio can harbor marked improvement in the yield. Solvent volume ($X_3$) is not a factor frequently studied for its relationship with NP formation; nevertheless, some authors[23] reported the use of high solvent volume during CNP synthesis such as water:carbon soot=600:1 ml/g. Excessively large solvent volume may add to the processing cost of waste water and handling problem during recuperation of NPs.

**Table 6.3** Central composite design and response (dependent variable) for FCD production from spent meal by HTC.

| **Run no.** | **Coded levels of independent variable** | | | **Response (Y)** | |
|---|---|---|---|---|---|
| | **$X_1$** | **$X_2$** | **$X_3$** | **Actual§** | **Predicted** |
| ***Factorial points*** | | | | | |
| **1** | -1 | -1 | -1 | 85.33±0.63ª | 87.12 |
| **2** | 1 | -1 | -1 | 94.30±0.92ᶜᵈ | 93.86 |
| **3** | -1 | 1 | -1 | 87.47±1.11ª | 88.66 |
| **4** | 1 | 1 | -1 | 88.56±0.27ᵃᵇ | 88.27 |
| **5** | -1 | -1 | 1 | 89.02±0.53ᵇᶜ | 89.64 |
| **6** | 1 | -1 | 1 | 92.39±1.89ᶜ | 91.54 |
| **7** | -1 | 1 | 1 | 90.10±0.36ᶜ | 90.87 |
| **8** | 1 | 1 | 1 | 87.10±0.71ª | 85.64 |
| ***Axial points*** | | | | | |
| **9** | -1.682 | 0 | 0 | 90.84±0.33ᶜ | 88.40 |
| **10** | 1.682 | 0 | 0 | 87.72±0.29ª | 89.68 |
| **11** | 0 | -1.682 | 0 | 91.54±1.47ᶜ | 91.04 |
| **12** | 0 | 1.682 | 0 | 87.34±0.69ᵃᵇ | 87.37 |
| **13** | 0 | 0 | -1.682 | 91.97±0.13ᶜ | 90.80 |
| **14** | 0 | 0 | 1.682 | 90.01±0.52ᵇᶜ | 90.71 |
| ***Center points*** | | | | | |
| **15** | 0 | 0 | 0 | 93.09±0.82ᶜ | 93.19 |
| **16** | 0 | 0 | 0 | 92.99±0.45ᶜ | 93.19 |
| **17** | 0 | 0 | 0 | 92.07±1.22ᶜ | 93.19 |
| **18** | 0 | 0 | 0 | 93.72±0.93ᶜ | 93.19 |
| **19** | 0 | 0 | 0 | 95.21±1.64ᵈ | 93.19 |
| **20** | 0 | 0 | 0 | 91.98±0.70ᵇᶜ | 93.19 |

§Values are mean±standard deviation of n=3 analyses (subjected to Tukey test). Values with the same letter within one column were not statistically different (*p*>0.05).

**Table 6.4** ANOVA of regression model built for FCD yield.

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| **Regression** | 9 | 114.982 | 114.982 | 12.776 | 4.61 | 0.013 |
| **Linear** | 3 | 18.199 | 18.199 | 6.066 | 2.19 | 0.152 |
| **Square** | 3 | 59.562 | 59.562 | 19.854 | 7.16 | 0.007 |
| **Interaction** | 3 | 37.221 | 37.221 | 12.407 | 4.48 | 0.031 |
| **Residual Error** | 10 | 27.715 | 27.715 | 2.772 | | |
| **Lack-of-Fit** | 5 | 20.596 | 20.596 | 4.119 | 2.89 | 0.134 |
| **Pure Error** | 5 | 7.120 | 7.120 | 1.424 | | |
| **Total** | 19 | 142.697 | | | | |

SS, sum of squares; *df*, degree of freedom; MS, mean squares; *p*, transgression probability

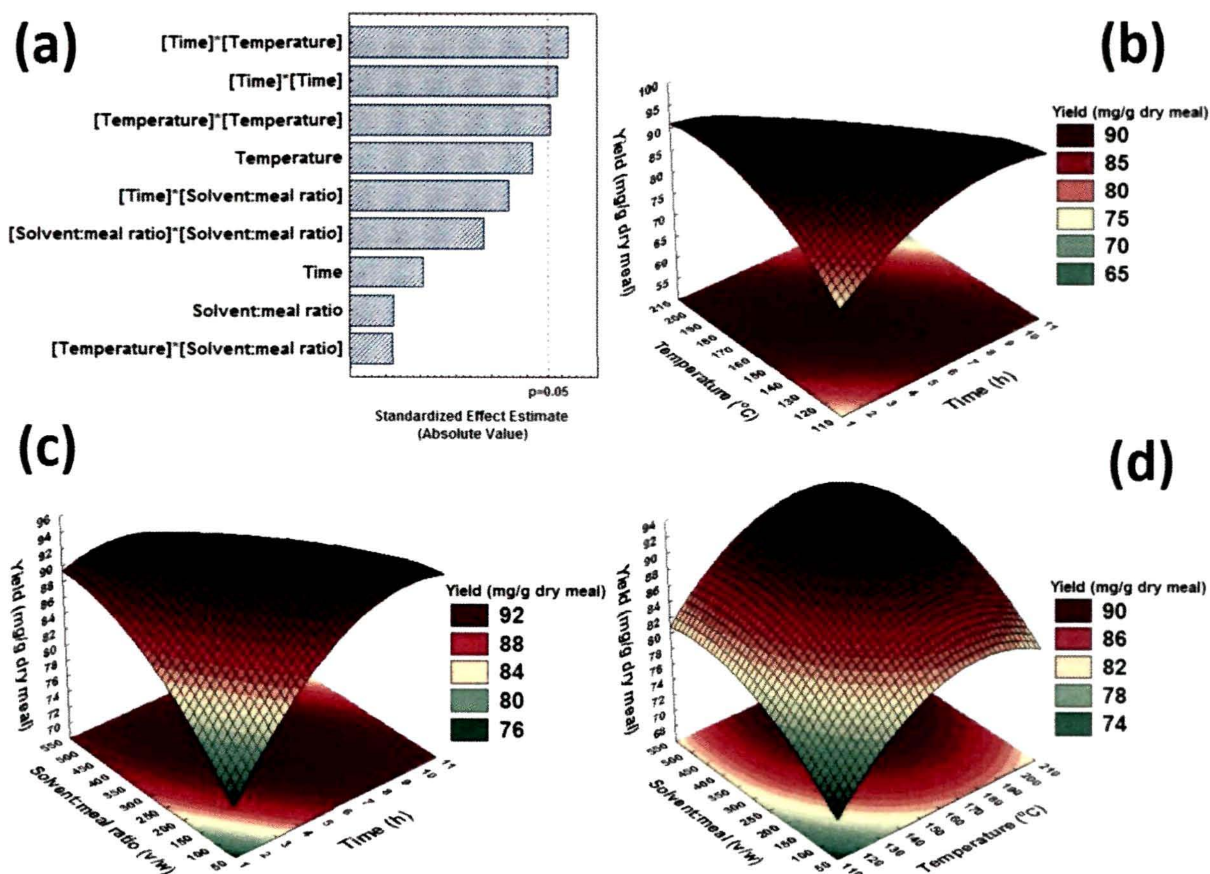


**Fig. 6.2** (a) Pareto chart showing the effect of independent variables on the yield of FCD; (b) Yield of FCD as a function of time and temperature; (c) Yield of FCD as a function of time and solvent:meal ratio; (d) Yield of FCD as a function of temperature and solvent:meal ratio.

Based on the regression equation and response surfaces, the predicted optimal condition is 2.6 h of refluxing time, temperature of 179.2 °C and a solvent:meal ratio of 462.8:1 (v/w). In order to make these parameters feasible in experimental run, the optimum parameters were drawn to the nearest round figures[40] of 3 h of heating time, temperature of 180 °C, and solvent:meal ratio of 463:1 (v/w). To verify the predicted values, additional experiments were conducted at the feasible optimum condition. The predicted yield at the optimum condition was 98.39 mg/g dry meal and the real value obtained by experimentation was found to be 95.73±0.9 mg/g dry meal. The closeness between the experimental and predicted values indicates the suitability of the model for prediction and shows a substantial improvement in the process yield, which is important from a commercial point of view.

### 6.3.2. Characterization of FCD

The formation of C-dots was confirmed by TEM measurement (**Fig. 6.3a**). HRTEM images clearly revealed that the NPs are spherical in shape; their diameters mainly lay in the range of 4.71-10.77 nm with maximum population at 7.66 nm. It can be seen that there are holes or crevices on the surface of C-dots, giving them an appearance of a puffer-like hollow ball (**Fig. 6.3b**). The average diameter of these surface holes was calculated to be 0.42 nm (approx.). SEM image (**Fig. 6.3c**) indicates that dried FCD powder is an aggregate of small carbon grains hundreds of nanometers in size. The entities appear to be non-homogeneous in size and shape. This asymmetry is probably due to the effect of drying, because of which the NPs formed clusters of varying sizes and shapes.[23]

The absence of any discernible lattice fringes in HRTEM image suggests an amorphous nature of the as-prepared NPs. Concomitantly, SAED pattern of the C-dots showed diffused rings (**Fig. 6.3d**), revealing an amorphous carbon phase,[7] which agrees well with the HRTEM analysis. Furthermore, the XRD pattern (**Fig. 6.3e**) presented a broad hump-like peak near 25.8° which is attributed to amorphous carbon.[11]

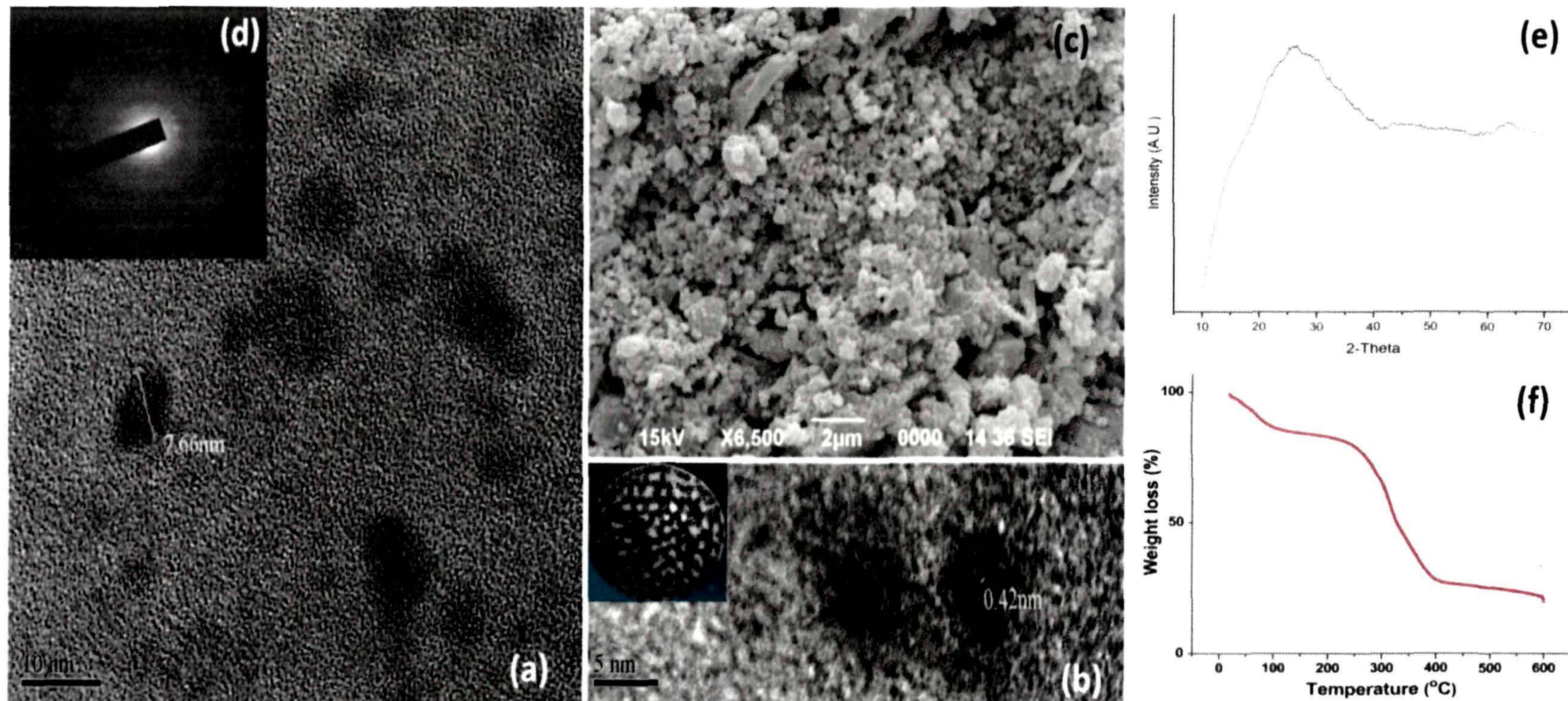


**Fig. 6.3** (a) TEM image of FCD thus formed; (b) The corresponding HRTEM image of two nanoparticles. The inset shows the pictorial representation of the morphology of one nanoparticle; (c) SEM image of the clusters of nanoparticles obtained after drying; (d) SAED image; (e) XRD pattern; and (f) TGA curve of FCDs.

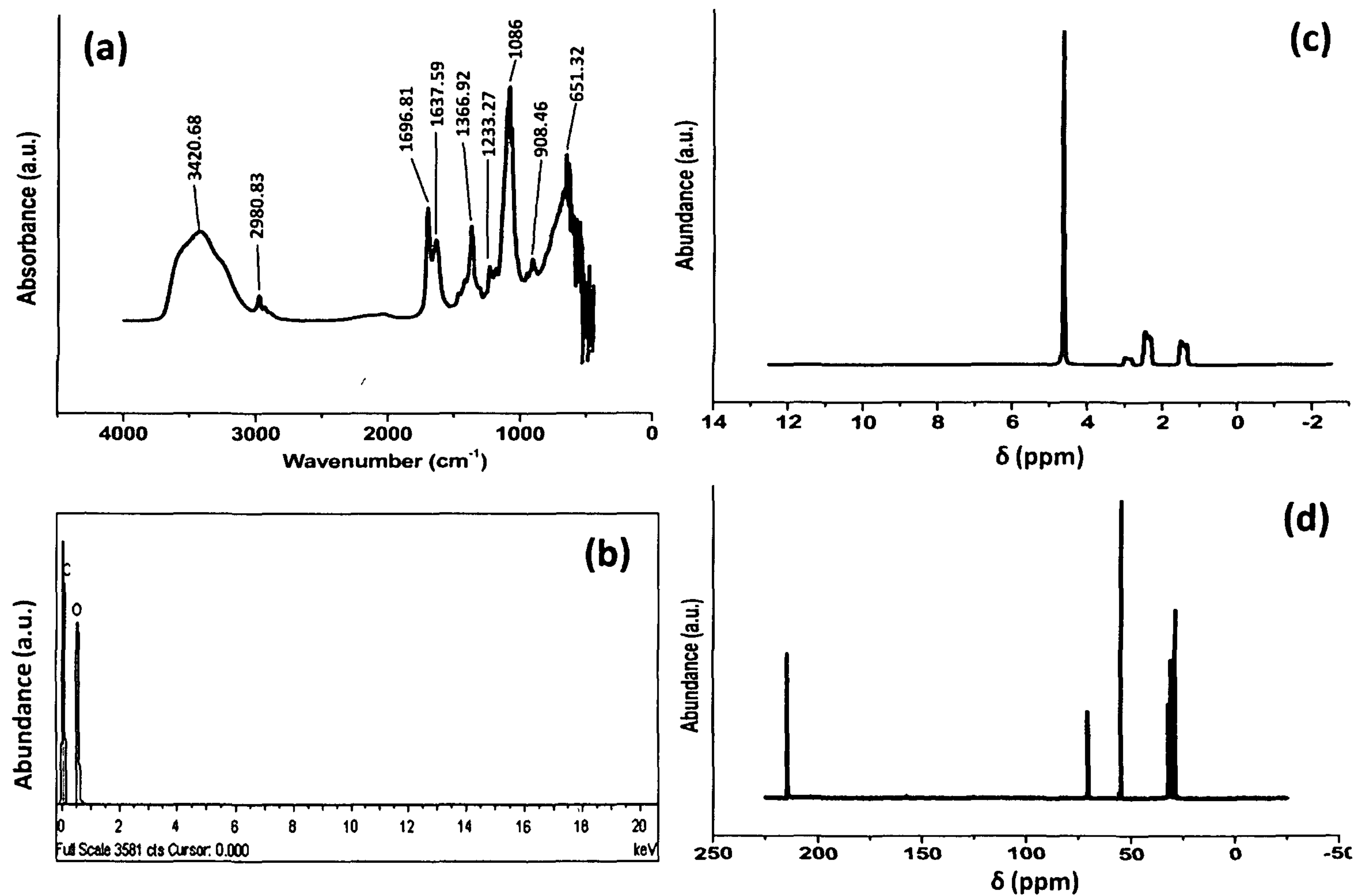


**Fig. 6.4** (a) FTIR spectrum; (b) EDX data; (c) $^{1}$H-NMR; and (d) $^{13}$C-NMR of FCDs.

The thermal behavior of FCDs was investigated by TGA. The thermogram (**Fig. 6.3f**) exhibited a two-step degradation pattern with the initial degradation (<100 °C) ascribed to the loss of moisture and volatile products, while the final degradation (286.24–358.21 °C) was associated with the decomposition of oxygen-containing groups.[41]

The FTIR spectrum was acquired to gain further structural insights and surface state of the synthesized FCDs. In this spectrum (**Fig. 6.4a**), the stretching frequencies observed at 651, 908, 1086, 1233, 1367, 1638, 1697, 2981 and 3421 $cm^{-1}$ indicate the presence of olefinic C-H (out-of-plane), epoxy ring, C-O or C-O-C, carboxylate group, C-N, C=O, C-H, hydroxyl (-OH) stretch, respectively.[9] The different oxygenous functional groups as evident from FTIR, proves that the surface of FCD is partially oxidized. Origin of these functional groups can be ascribed to the degradation of the carbohydrates, crude fibres and residual protein present in the spent-meal through hydrothermal treatment.[10,14] Additionally, the presence of these hydrophilic functional groups imparts excellent water solubility to the C-dots without further chemical modification, which in-turn promotes the environmental friendliness of the product.[9]

EDX data of C-dots (**Fig. 6.4b**) further confirms the fact that the as-prepared NPs mainly consist of carbon (47.81 wt%) and oxygen (52.18 wt%). EDX spectrum of 'oil-and-protein' spent meal, showing its various elemental compositions, is given in **Fig. 6.5** for comparison.

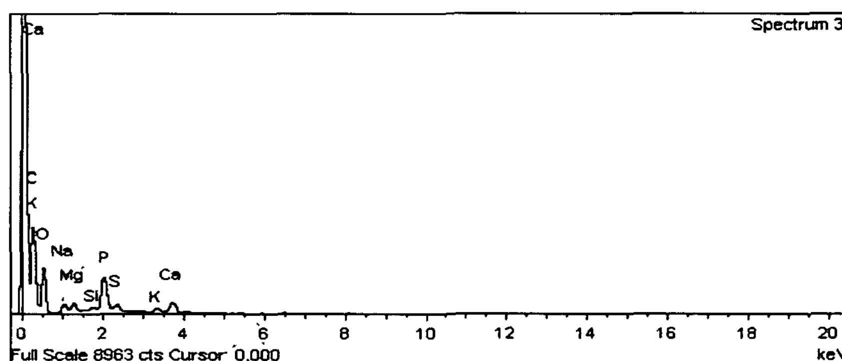


**Fig. 6.5** EDX spectrum of 'oil-and-protein' spent meal.

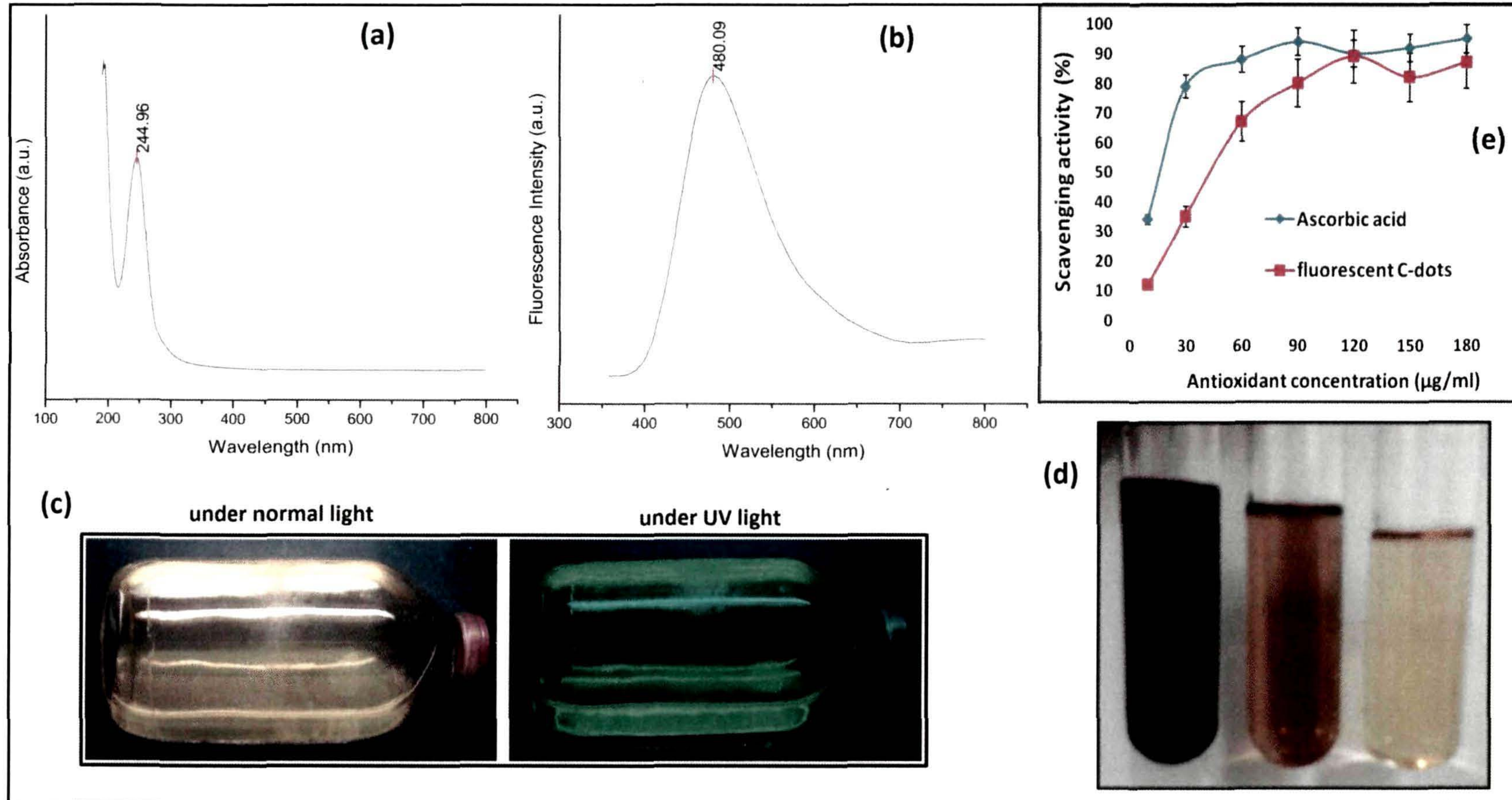


**Fig. 6.6** (a) UV-vis spectrum; (b) PL spectrum of aqueous dispersion of FCDs (0.1 mg/ml) (Excitation at 350 nm); (c) Photographs of aqueous FCD dispersion (0.25 mg/ml) observed under normal light and the same under UV light; (d) gradual bleaching of DPPH solution by FCDs; (e) DPPH scavenging activity of FCDs in comparison to that of ascorbic acid.

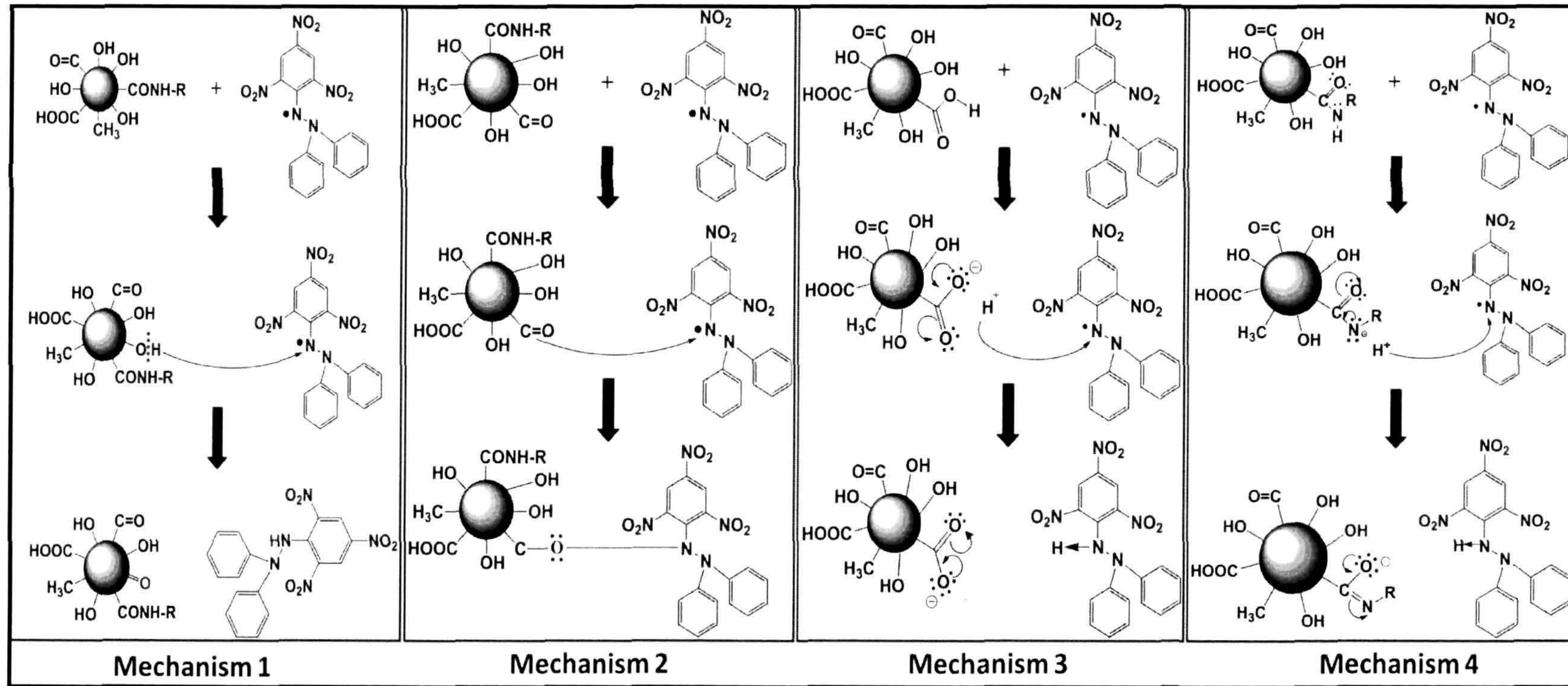


**Fig. 6.7** Plausible mechanism for the DPPH scavenging activity of FCDs.

NMR *spectra highlighted the presence of two different kinds of chemical* environments or regions, which are discussed as follows. In the $^{1}$H-NMR spectrum (**Fig. 6.4c**), the regions found are: δ=1-3 ppm (for $sp^3$ C-H protons) and δ=3-6 ppm (for protons attached with hydroxyl, ether and carbonyl groups).[9] Also, in the $^{13}$C-NMR spectrum (**Fig. 6.4d**), similar regions were found; namely, δ=20-100 ppm (for $sp^3$ carbons and for carbons attached with hydroxyl-group or ether linkage) and δ=214-215 ppm (for keto, –C=O carbon atoms).[9] Specifically, the peaks within δ=28-33 ppm depicts the presence of methyl groups in the surface functional moieties and the singlet peak at δ=70.67 ppm indicates the occurrence of carbohydrate units in FCDs.[6]

To explore the optical properties of FCDs, UV-vis absorption (**Fig. 6.6a**) and PL study were carried out. FCDs showed maximum UV absorption peak at 244.96 nm which likely to have originated from the formation of multiple polyaromatic groups where $\pi$-$\pi$* transitions of the aromatic C-C bond occurs.[8] Most C-dots are excited in the UV region (330-420 nm) and yield photoluminescence in the wavelength range of 400-600 nm depending on the size of C-dots.[16] **Fig. 6.6b** shows the PL emission peak of FCDs centered at 480.09 nm, with excitation at 350 nm. This is consistent with the literature. From the fundamental as-well-as application view-point, fluorescence is one of the most fascinating behaviors of the C-dots. The aqueous solution of FCDs is bright yellow and transparent in normal light but changes to an intense green colour under UV light (**Fig. 6.6c**), signifying the NPs to be fluorescent. It is worth noting that the origin of fluorescence from the obtained dispersion could be attributed only to the presence of C-dots because the preparatory ingredients (spent rapeseed meal, water and NaOH) are known to be non-fluorescent materials (i.e. non-emissive under UV light), further confirming the formation of luminescent NPs. QY measured using quinine sulfate as a reference was found to be 9.2%, which is comparable with those of the reported luminescent C-dots. The fundamental mechanism of fluorescence of C-dots is still an open question; however it is thought that the presence of quantum size and different surface trap sites could be the main factors. It is believed that radiative recombination

between electrons and holes (excitons) take place at the particle surface, under the illumination of excitation light, and thus fluorescence emission occurs.[42]

With the aim of detecting the radical scavenging activity of FCDs, the DPPH assay was performed. As known, a freshly prepared DPPH solution exhibits a deep purple colour with an absorption maximum at 517 nm. This purple colour generally fades/disappears as-soon-as an antioxidant is added into the medium, resulting in a decrease in absorbance at 517 nm. Interestingly, it was found that unlike other antioxidants, FCDs first turned the purple colour of fresh DPPH solution into a reddish-brown colour and then gradually into a colourless (bleached) product (**Fig. 6.6d**). Compared to ascorbic acid, the reaction of DPPH with FCDs proceeded at a slower rate. Nevertheless, the decrease in absorbance is detectable within minutes of reaction. This observation corroborates with the DPPH radical scavenging activity of carbonaceous fullerenes ($C_{60}$).[43] As shown in **Fig. 6.6e**, the scavenging activity of ascorbic acid and FCDs increased in dose-dependent manner upto 94% at the dose level of 90 μg/ml and to 89% at the dose level of 120 μg/ml, respectively. The $EC_{50}$ value was determined from the plotted graph of scavenging activity versus the concentration of sample/standard, which is defined as the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50%. $EC_{50}$ values of ascorbic acid and FCDs were found to be 35.83±4.17 and 56.16±2.97 μg/ml, respectively ($p<0.05$), confirming that the scavenging potential of FCDs was lower than that of ascorbic acid. Nonetheless, this noble facet of FCDs raises the prospects of its incorporation into oxidation-prone food models. Indeed, the nanochemistry involved in radical scavenging property of C-dots needs further investigation; however from the knowledge gained so far, it could be explained that the quenching behavior of FCDs may be attributed to its quantum size with increased surface-to-volume ratio, providing more available reaction sites, and also to the presence of different functional moieties on the surface (-OH, C=O, -CONR, -COOH, etc.), which tends to interact with and reduce DPPH species. It has been reported that among the water-soluble fullerenes, nanomaterials of $C_{60}(C(COOH)_2)_2$, $C_{60}(OH)_{22}$ and metallofullerenes possess high antioxidative function and can wipe out different types of

reactive oxygen species (ROS) depending on surface modification.[42] Amino acid-functionalized multi-walled carbon nanotubes were found to be more potent antioxidants than butylated hydroxyanisole and reduced glutathione.[44] A schematic illustrating the probable mechanism(s) is proposed in **Fig. 6.7**. Data from the literature suggests that similar to fullerenes, ROS or free radicals may be "grafted" or adsorbed at the surface of C-dots by radical addition to the carbon framework, which is due to their high electron affinity.[43,45] Thus, the mechanism of scavenging reaction is not straight forward in case of NPs and a generalized inference can be drawn that the organic functionalities of NPs can quench DPPH radicals (perhaps by providing hydrogen atom or by electron donation, conceivably via a free radical attack on the DPPH molecule, or by grafting/linking of radicals to C-dot's surface) and convert them to a colourless/bleached product (i.e., 2,2-diphenyl-1-picrylhydrazine, or a substituted analogous hydrazine).

### 6.3.3. Hemocompatibility

A hemolysis test was used to determine the toxicity of FCDs on mammalian RBCs. Since there are concerns about the health implication of NPs, it is important to understand how FCDs interact specifically with RBCs, as it would eventually come in direct/indirect contact with blood as soon they are exposed to the host's body. Hemolytic activity of NPs is usually determined by measuring the absorption peak of hemoglobin at 570 nm, which is released to the solution from hemolyzed cells.[46] Photographs showing precipitated RBCs at the end of the hemolysis experiment are given in **Fig. 6.8a**. The red colour of the released hemoglobin from damaged cells is clearly observable for positive control and 50 mg/ml concentration of FCDs. For rest of the samples (negative control and 0.1-10 mg/ml of FCDs), the supernatants are almost colourless, confirming the intactness of the RBC membrane in these cases. This result is in conformity with that of **Fig. 6.8b**. According to ASTM E2524-08 standard (Standard test method for analysis of Hemolytic products of NPs), hemolysis >5% indicates that the test material causes damage to RBCs; this criterion was exceeded at the FCD concentration of 50 mg/ml. Hence, FCDs were found to exhibit hemolytic activity only at higher concentration (≥50 mg/ml), which is

much lower than that reported for their other counterparts (fullerenes, carbon nanotubes, etc.) and several similar NPs.[47] This benevolent feature may be due to the presence of congenial surface functional groups, making FCDs tolerable at low concentration. Yildirim et al.[46] also found that surface functionalization of nano-structures can render them completely non-hemotoxic.

To get a closer insight into the FCD-induced biological effect on RBC, the uptake of FCDs by the RBCs was examined by fluorescence microscope (**Fig. 6.8c**). With the increasing dosages (20, 30 and 40 mg/ml), no marked changes in the morphological shape of RBCs was detected; however, higher concentration (50 mg/ml) caused dramatic hemolysis, showing ruptured cell membrane and patches of released cell content (**Fig. 6.8d**). It is possible that mechanical pressure exerted by excess permeation and accumulation of FCDs inside the cells caused lysis, especially at higher dosage. Thus, FCDs were found to be biocompatible in a dose-dependent manner, with no detectable hemotoxic effect up to 40 mg/ml. The C-dots were likely internalized into the RBCs through endocytosis,[16] and even after entering into the cells, the C-dots retained their fluorescence property and turned bright green under UV excitation (488 nm). In addition when the laser excitation was changed to 405 nm, a fluorescent blue color was observed. A similar observation was obtained by Sahu et al.[8] in case of orange juice-derived CNP. All these preludes demonstrate that the FCDs obtained from spent-rapeseed meal can serve as a potential substitute for organic dyes or semiconductor QDs in bio-imaging application.

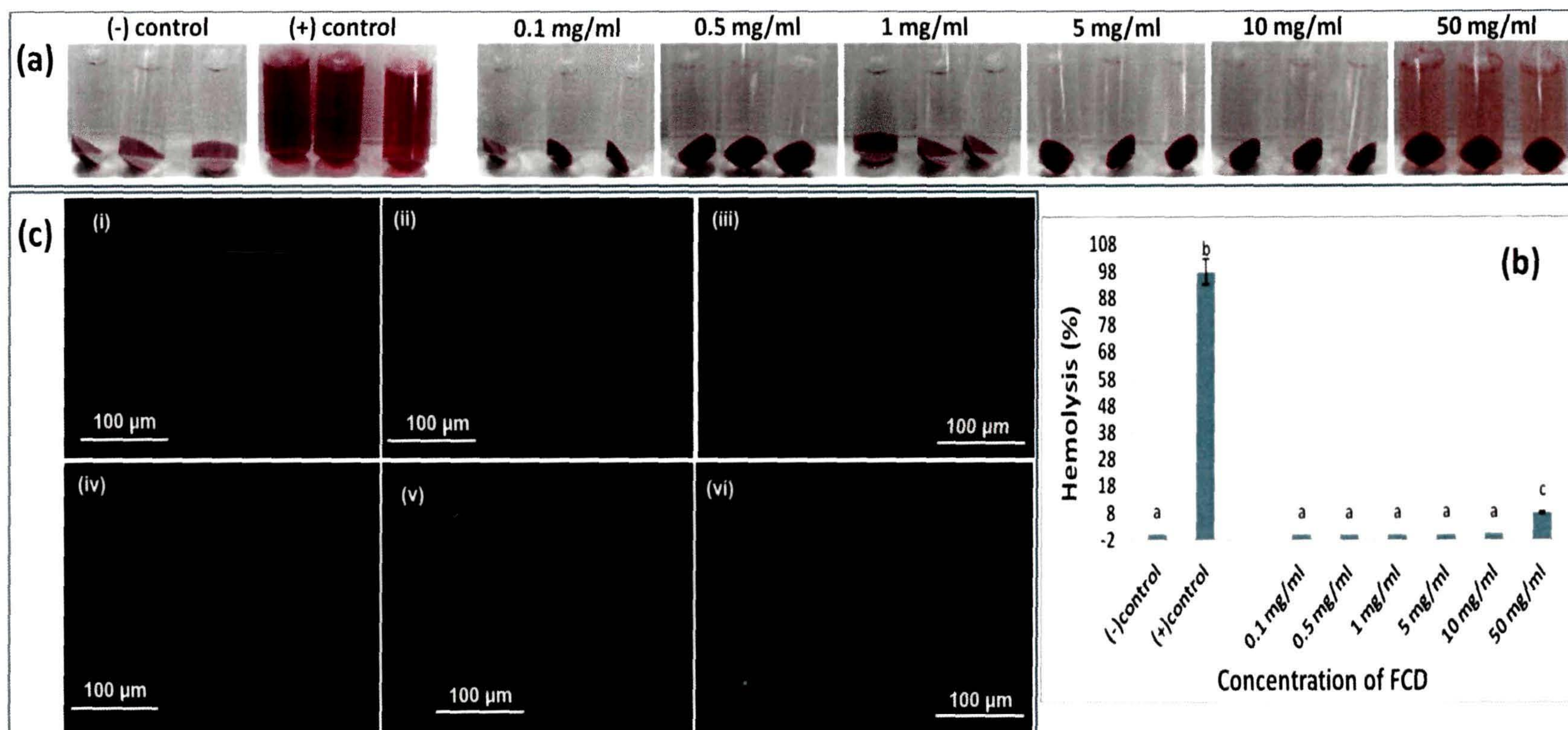


**Fig. 6.8** (a) Photographs of RBCs treated with FCDs at different concentrations. The released hemoglobin from the damaged cells in the supernatant can be seen from the photographs; (b) Hemolysis percentages of FCDs at different concentrations; (c) RBCs treated with various concentrations of FCDs (20, 30 and 40 mg/ml) observed under fluorescence microscope by excitation at 405 nm ((i)-(iii)) and 488 nm ((iv)-(vi)). Bars with different letters indicate significant difference ($p<0.05$).

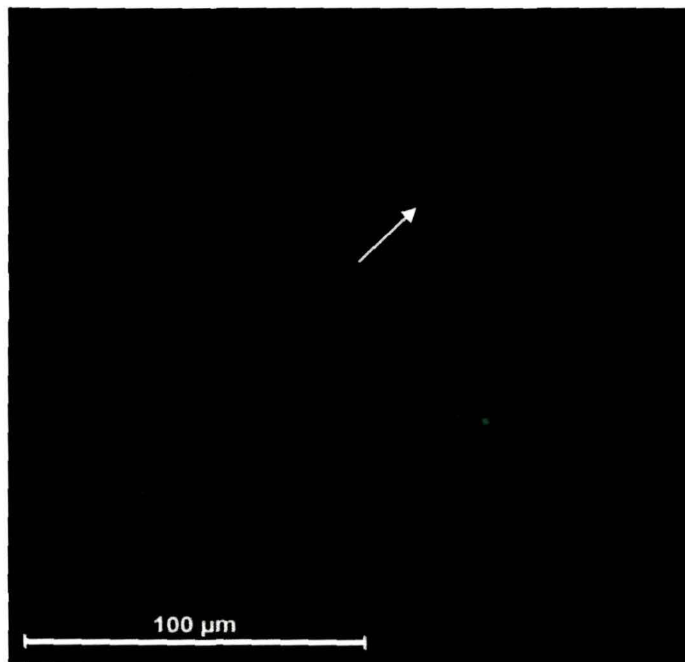


**Fig. 6.8d** Hemolytic activity of FCDs at 50 mg/ml concentration.

### 6.3.4. Characterization of rapeseed protein film, with (F1 and F2) or without (F0) added FCDs

Having established the biocompatibility, we introduced the FCDs into a biopolymer matrix (rapeseed protein) in a pursuit to make fluorescent bio-packaging material. One of the most important and difficult tasks in the incorporation of active compounds and new materials in a matrix is understanding if they will be miscible with the matrix and if they will not influence the processability and their structure.[48] In this study, the preliminary experiments allowed the determination of FCD concentrations that when added to the protein-based matrix do not affect significantly their appearance and processability. Accordingly, composite films having 20 or 30 mg FCDs per 100 ml of film-forming solution were prepared for further analyses.

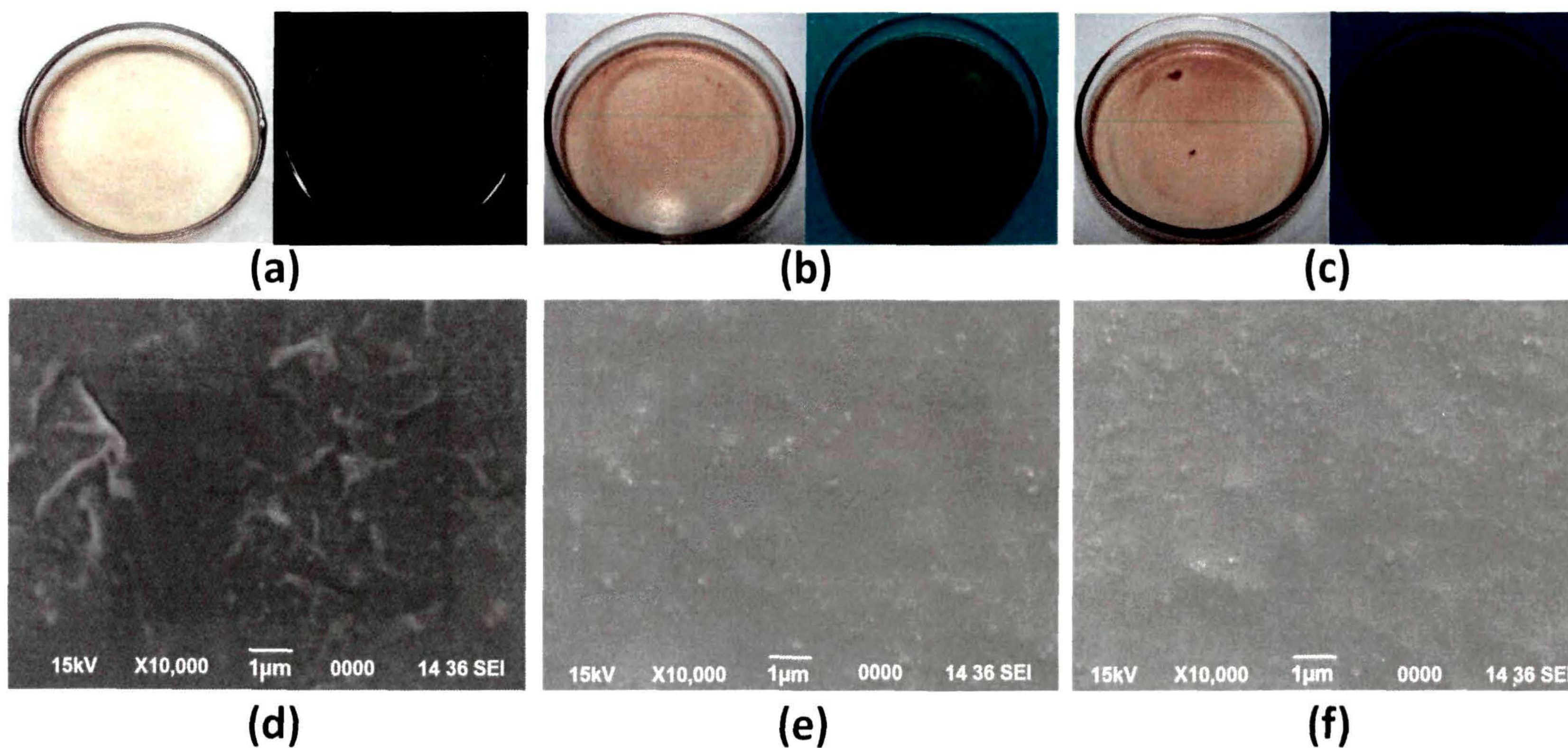


**Fig. 6.9** Photograph showing the pristine protein film and the nanocomposite films under normal light (on left) and UV light (on right) (F0 (a), F1 (b), F2 (c)); and their corresponding SEM images (F0 (d), F1 (e), F2 (f)).

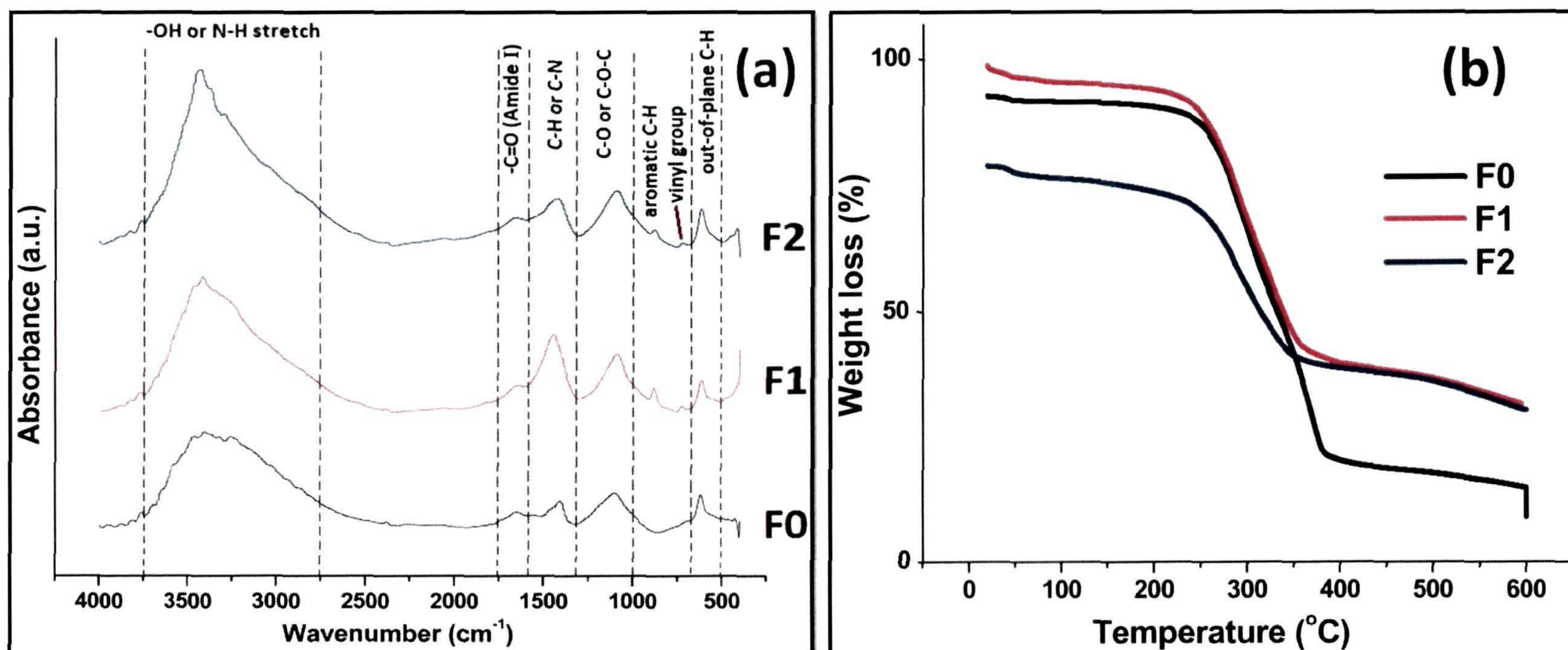


**Fig. 6.10** (a) FTIR spectra and (b) TGA curves of F0, F1 and F2.

On the basis of visual inspection, all the examined films (F0, F1 and F2) showed similar homogeneity, transparency and flexibility, with the exception of colour. The colour of the film changed from slightly yellow to dark brown-yellow, when FCDs were added (**Figs. 6.9a-c**). Generally, CNPs are blackish-brown, which might be the cause for dark colouration. As anticipated, F0 did not show any green glow under UV light; whereas under the same condition, F1 and F2 appeared as intense green– light emitting films. From this, it is clear that a homogeneous dispersion of the nano-component in the film-matrix is obtained, without the presence of any visible green-coloured fluorescent patches or clusters. Thus we were successful in fabricating fluorescent glowing edible films based on the luminescent C-dots, which is envisioned to be used in forgery-proof packaging. It is expected that such packaging would benefit consumers, industry stakeholders and food regulators. At this point, one of the objectives in this work was accomplished, and the comparative study of the physicochemical properties of these films was undertaken.

SEM was used to characterize the topography and morphology of the films (**Figs. 6.9d-f**). Microstructure of F0 presented rough ridge-like protrusions on the surface, typical of protein films, as has been also reported for films prepared with soy protein,[49] rapeseed protein[21] and pumpkin seed protein.[19] Surprisingly, F1 and F2 did not show any such ridge structures; rather, a good distribution of the additive (FCDs) in the matrix was found. FCDs appeared to be embedded on the polymeric matrix and exhibited a heterogeneous size distribution most likely due to some agglomeration. Such problems of agglomeration of nanomaterials in composite films have often been reported.[50] Agglomeration usually occurs when a relatively high loading of the nanomaterial is added to the matrix, as is the case here. Apparently such tiny clustering did not affect the green fluorescence of the films.

The effect of the addition of FCDs into protein film was initially evaluated by FTIR. When different compounds are mixed, physical bonds and chemical interactions are reflected by changes in characteristic spectral peaks.[48] FTIR spectra of F0, F1 and F2

are shown in **Fig. 6.10a**. The main broad peaks are maintained, the spectra of the films with FCDs being similar to that of the control. Special mention should be made of the peaks between 3500 and 3000 $cm^{-1}$, corresponding to the hydroxyl groups or hydrogen-bonds or to the N-H group, that are weaker in F0 when compared to F1 or F2. This stretch appeared to be more recognizable with increasing FCD concentration. This indicates higher amounts of O-H or N-H groups in F1 and F2 than in F0. Derived from this fact, it is feasible to believe that FCDs, with hydrophilic surface moieties, may form hydrogen-bonding between themselves or with amino groups of protein, especially when the protein is partially unfolded (denatured) during heating of the film-forming solution. This might be one of the possible reasons that F1 and F2 had more compact, uniform and homogeneous surface morphologies compared to F0, as viewed under SEM (**Figs. 6.9d-f**), mainly due to high compatibility of FCDs in protein matrix. Addition of FCDs led to the emergence of few new peaks in the region of 717-727 $cm^{-1}$ (ascribable to out-of-plane C-H bending in aromatic substitution) and 878-881 $cm^{-1}$ (correlating to the presence of vinyl groups). Moreover, in F1 and F2, the intensity of C-H or C-N stretch at 1400-1450 $cm^{-1}$ increased and seemed to be more distinguishable than that in F0. All these facets signify the possibility of new interactions and cross-links between FCDs and polymeric matrix.

The thermal stability of the composite films was tested by TGA. The thermograms in **Fig. 6.10b** show that all of the film samples follow a similar degradation pattern, indicating uniform dispersion and high interactions of FCDs with the film (polymer) matrix.[51] Surprisingly, in all the 3 samples, no significant mass loss was noted up to ≈260 °C, beyond which decomposition of glycerol and protein occured.[18] The char yield is the non-volatile carbonaceous material generated on pyrolysis, which is indicated by the residual weight after the decomposition step. It is worth mentioning that the increase of the residual yield (the char) with the increase of FCD concentration was detected, which points to the thermo-insulating nature of the C-dots as a nanofiller for the polymer matrix, a property similar to that of carbon nanotubes.[52] After degradation, the net weight loss of F0 was found to be ≈80.58%; whereas those of F1 and F2 were

≈59.73% and ≈45.65%, respectively. Thus, with the increase in FCD content, significant ($p<0.05$) reduction in weight loss was observed, thereby proving FCDs as potent thermo-insulators for proteinaceous films, a property desirous for high temperature processing.

Although the same amount of film-forming solution was used in all the three films, the slightly higher thickness of F0 (**Table 6.5**) may be due to its ridges-like rough outgrowths on its surface, as seen under SEM (**Figs. 6.9d-f**). Presence of FCDs led to an increase of water affinity of edible films, showing that moisture content increases in F1 and F2 significantly ($p<0.05$) as compared to F0 (**Table 6.5**). This may be due to the hydrophilic behavior of the –OH groups in FCDs, which probably influenced WVP increasing the adsorption of water molecules[48] in FCD-incorporated films when compared with F0. As a consequence, the water vapour barrier property of F1 and F2 is lesser than F0 (**Table 6.5**). The opposite behavior was observed for $O_2$ permeability, leading to a significant decrease ($p<0.05$) of the values for FCD-containing films when these were compared with F0. Usually it is perceived that higher WVP would render higher $O_2$ permeability;[48] however in this case, the radical scavenging functional groups of FCDs probably created additional sites in the film favouring the removal of oxygen, thereby minimizing the influence of adsorbed moisture in the film's transport properties. Thus the oxygen barrier property is improved due to the presence of FCDs.

TS, puncture strength and EB of a material are used to study their resistance to tensile stress, to determine the breaking force and to express the percentage of increase in length that occurs before the sample breaks (i.e. film's ability to stretch), respectively. The presence of FCDs in F1 and F2 influences their mechanical properties, decreasing the values of TS and puncture strength ($p<0.05$); on the other hand, an increase of EB values was observed (**Table 6.5**). The reason behind the decrease in TS and puncture strength by the addition of FCDs was the hydrophilic nature of C-dots which favours the interaction with water molecules,[48] as explained by the higher moisture content of the films. Due to their small sizes, FCDs fit easily into the polymer chains. Because of the hydrophilic groups of FCDs (especially –OH), it is likely to be strongly bonded by

hydrogen bridges with glycerol and protein molecules especially at amine, amide, carboxyl and hydroxyl sites.[53] As a result, protein-protein interaction decreases and polymer segmental mobility increases (flexibility or EB enhances).

**Table 6.5** Physico-chemical properties of pristine protein (F0) and nanocomposite film (F1 and F2)§

| **Property** | **F0** | **F1** | **F2** |
|---|---|---|---|
| Thickness (μm) | $54.1\pm0.2^a$ | $48.8\pm0.15^b$ | $49.7\pm0.09^b$ |
| Moisture content (% dry basis) | $28.3\pm1.6^a$ | $35.8\pm1.1^b$ | $37.9\pm1.4^c$ |
| WVP (ng m/m$^2$ s Pa) | $1.4\pm0.62^a$ | $2.5\pm1.6^b$ | $2.9\pm1.4^b$ |
| Oxygen permeability (ng $d^{-1}$ $Pa^{-1}$ $m^{-1}$) | $8.6\pm0.30^a$ | $4.7\pm0.2^b$ | $2.9\pm0.20^c$ |
| Tensile strength, TS (MPa) | $3.6\pm0.51^a$ | $2.1\pm1.8^b$ | $2.1\pm1.2^b$ |
| Puncture strength (N) | $1.2\pm0.03^a$ | $0.87\pm0.01^b$ | $0.53\pm0.09^c$ |
| Elongation at break, EB (%) | $9.6\pm0.02^a$ | $17.8\pm0.69^b$ | $16.1\pm1.03^b$ |
| Hunter-L | $81.0\pm0.13^a$ | $78.2\pm0.16^b$ | $77.2\pm0.09^b$ |
| Hunter-a | $0.34\pm0.01^a$ | $1.2\pm0.03^b$ | $1.6\pm0.05^c$ |
| Hunter-b | $11.9\pm0.12^a$ | $10.9\pm0.07^b$ | $8.6\pm0.04^c$ |
| Opacity | $2.4\pm0.17^a$ | $2.5\pm0.13^b$ | $2.6\pm0.02^b$ |
| DPPH scavenging activity (%) | $3.7\pm1.6^a$ | $64.9\pm2.8^b$ | $71.7\pm4.0^c$ |

§Results are mean±standard deviation of 3 replicates. Values followed by same superscript letter within a row are not significantly different ($p>0.05$)

Good optical properties of film are extremely important in food packaging for ensuring that the consumers can clearly see the food product. The darkness of the FCD-loaded films was significantly higher ($p<0.05$), as evidenced by the lower Hunter-L values compared to the control. Meanwhile, in comparison with F0, increase in FCD concentration increased Hunter-a (indicator of the tendency towards redness) and decreased Hunter-b (indicator of the reduction of yellowness) values. F0 was more transparent (lower opacity value) than F1 and F2 (**Table 6.5**). The opacity of the film samples significantly increased ($p<0.05$) with increasing FCD concentration. This

observation could be explained by the fact that a higher amount of added FCDs hinders light passage through the film. This result accords with several published studies in which addition of antioxidants, polyphenols, plant extracts or other additives to film matrices, has been found to affect the optical properties severely.

As the level of FCDs increased in the film formulation, so did the expected antioxidant character of the composite film (**Table 6.5**). In relation to F0, the DPPH scavenging activity increased by 17.5 and 19.3 fold in F1 and F2, respectively. F0 also showed some scavenging activity on DPPH. This is related to the fact that free radicals can react with the residual free amino (-$NH_2$) groups to form stable macromolecular radicals, and the -$NH_2$ groups can form ammonium ($NH_3^+$) groups by absorbing a hydrogen ion from the solution.[25] It should be noted that although a high amount of FCDs was incorporated into the film, the FCDs in the protein film showed much lower antioxidant capacity than in the free form (aqueous dispersion) at much lower concentration (**Fig. 6.6e**). This could be due to interaction between FCDs and the film matrix formed via hydrogen bonds and other cross-links, thereby reducing the availability of free structural factors such as hydroxyl, keto, carboxylic groups, etc.,[54] which imparts a scavenging feature to FCDs.

### 6.3.5. Oxidative stability analyses of rapeseed oil packed in nanocomposite films

On the basis of the above results, it was presumed that the use of FCD-incorporated composite packaging could be helpful in improving the oxidative shelf-life of lipid food items during storage. As such, rapeseed oil, packed in small pouches made of either nanocomposite films (F1 and F2) or pristine protein film (F0), was monitored over time to assess the antioxidative effect of these films on a real food system. The samples packaged in nanocomposite films (S1 and S2) can easily be segregated from the control (S0) under UV light, on the basis of the inherent green luminescence of C-dots (**Fig. 6.11a**). To consider the complexity of the lipid oxidation process, both the primary and secondary oxidation products have been assessed.

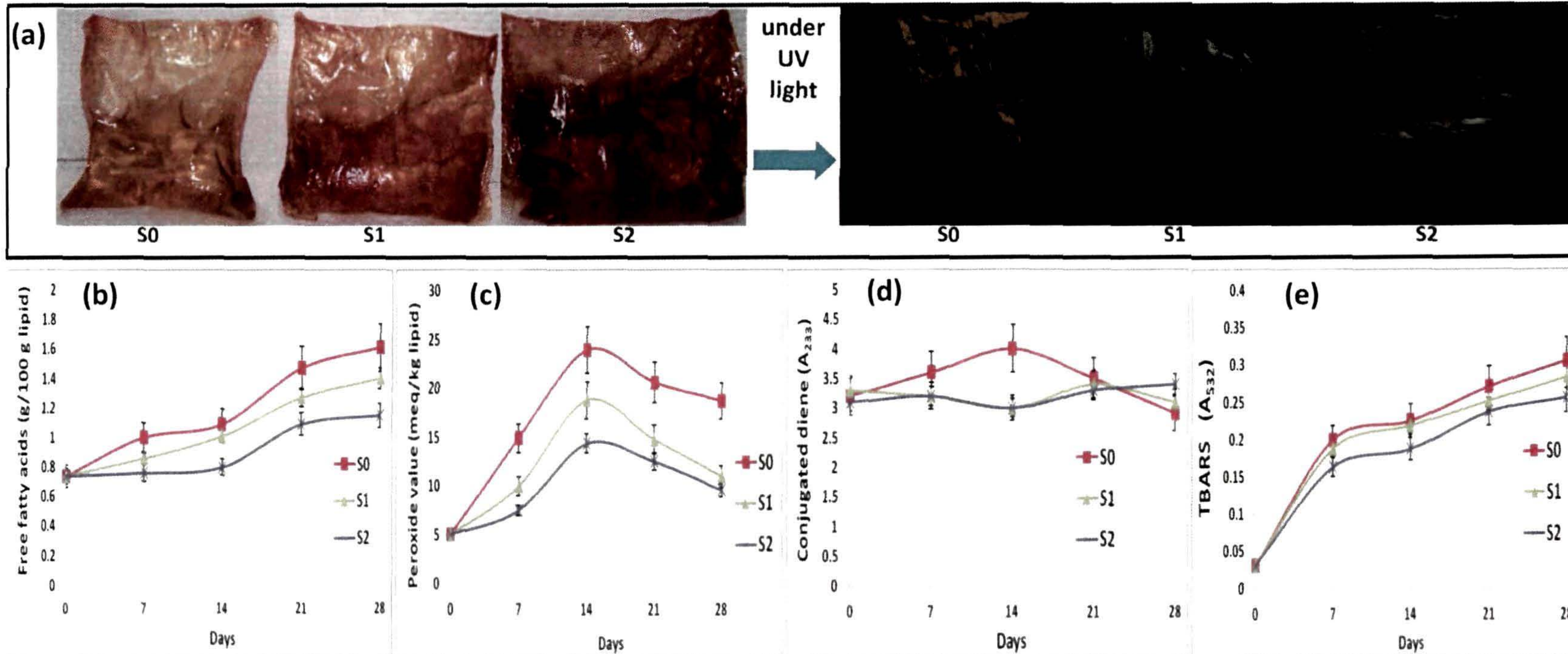


**Fig. 6.11** (a) Oil sample packed in F0, F1 and F2, as viewed under normal light and UV light; Changes in (b) Free fatty acids (FFA); (c) Peroxide value (PV); (d) Conjugated dienes (CD); and (e) TBARS values of S0, S1 and S2 during storage.

Within the first 14 days of storage, FFA content increased perceptibly in both S0 and S1 ($p<0.05$); however, S2 showed negligible changes as compared to day 0 ($p>0.05$) (**Fig. 6.11b**). Subsequently, a gradual increase was found up to 21 days, and the highest FFA value was obtained on day 28. Possibly, lipid hydrolysis, catalyzed by lipases, occurred to a greater extent at the end of the storage period. On day 28, the respective FFA values of S1 and S2 were ≈1.2 and ≈1.4 fold less than that of S0. A small amount of FFA is usually noticeable in cold-pressed oil due to the release of enzymes from crushed seeds along with the oil. Alternatively, the accumulation of FFA could be attributable to the lipases from micro-organisms, which probably enhanced with extended storage.[32]

Unsaturated fatty acids react with molecular oxygen, usually via a free radical mechanism, to form hydroperoxides, the primary oxidation products.[55] A marked increase in PV was observed in all the tested samples for up to 14 days ($p<0.05$) (**Fig. 6.11c**). Thereafter, a decrease in PV was noticeable with extended storage ($p<0.05$), which was presumed to be due to degradation of hydroperoxides, yielding a wide variety of secondary decomposition products including aldehydes. On day 28, the PV of S0 was almost 2 fold higher than those of S1 and S2, which indicates the higher deterioration rate of S0 compared to the other samples.

During oxidation of PUFAs containing methylene-substituted dienes and polyenes, there is a shift in the position of the double bond due to isomerization and conjugate bond formation (conjugated dienes).[33] This is accompanied by increased UV absorption at 233 nm. It is an indicator of auto-oxidation and is reported to increase with uptake of oxygen and formation of peroxides, during the early stages of oxidation. Chaijan et al.[32] reported that almost immediately after peroxides are formed, the non-conjugated double bonds (C=C-C-C=C) that are present in natural unsaturated lipids are converted to conjugated double bonds (C=C-C=C). Thus, conjugated dienes are the primary oxidation products formed. $A_{233}$ of S0 increased slightly up to 14 days of storage, after which its value showed a decreasing trend (**Fig. 6.11d**). S1 and S2 exhibited similar profiles up to 21 days. From day 21 onwards, $A_{233}$ of S1 decreased and that of S2

continued to increase, till the end of storage period ($p<0.05$). This decrease probably occurred because of their decomposition into secondary oxidation products.

Secondary lipid oxidation was studied by the TBARS value. Traditionally, the absorbance at 532 nm of the pink pigment formed in the reaction is measured. In all of the samples, TBARS value increased sharply throughout the storage ($p<0.05$) (**Fig. 6.11e**). The marked increase in TBARS during 14-28 days of storage was coincidental with the decrease in PV (**Fig. 6.11c**). This was probably due to the destruction of hydroperoxides into relatively polar secondary products, especially aldehydes in the later stages of lipid oxidation. It was most likely that a higher rate of lipid oxidation might be taking place at the end of storage period (days 14-28). Additionally, the loss in natural antioxidants (such as tocopherol, polyphenols, etc.) of rapeseed oil during extended storage might contribute to the increased lipid oxidation.

On the basis of the available data, it can be interpreted that the antioxidative FCDs in F1 and F2 retarded the lipid oxidation rate in S1 and S2 by quenching the free radicals. The formation of free radicals, which precedes lipid hydroperoxides, is the initial step in lipid oxidation.[35] Abstraction of hydrogen atoms from allylic carbon atoms in unsaturated lipids generates a lipid radical, which in-turn has the potential to initiate the chain reactions of the auto-oxidation cycle.[55] The cycle is terminated either through a recombination of two radicals or by a reaction of the radical with antioxidant, leading to the formation of more stable radicals. The chain-breaking antioxidant, therefore, interrupts the oxidation chain reaction to enhance stability. Thus, FCD-incorporated packaging materials showed a discernible impact on the oxidative shelf-life of lipid product. Further studies need to be undertaken to improve the antioxidative and antimicrobial properties of these composite films.

## 6.4. Conclusion

Arising from this work, it is concluded that high-value CNPs can be easily synthesized from spent rapeseed meal by a simple green hydrothermal route. The obtained NPs were found to be multi-functional such as water solubility, high reductive potential, photoluminescence and biocompatibility. This is due to the rich functionalities attached to the nano-carbons. Literature enumerates biopolymer-based films as good vehicles for the incorporation of active compounds; however, inclusion of fluorescent carbon nanostructures has rarely been evaluated. The present investigation shows the incorporation of FCDs into rapeseed protein film matrix, which adds new features to the film; for instance photoluminescence, enhancement in antioxidant potential and thermal stability. Moreover, oil samples packed in sachets made of FCD-protein composite film, are able to resist oxidation better than that stored in pristine protein-based sachet. The work opens up new possibilities for agricultural residues as a valuable precursor of useful nanomaterials, and can subsequently give rise to a new concept of bio-based edible fluorescent food packaging, suggesting new scalable and simple approaches to improve environmental sustainability in industrial processes. Further studies should be undertaken to analyze the release behavior of FCDs from the film into different food model systems, their effect on organoleptic properties, gastro-intestinal tract, and toxicological assessment using *in-vitro* and *in-vivo* models.

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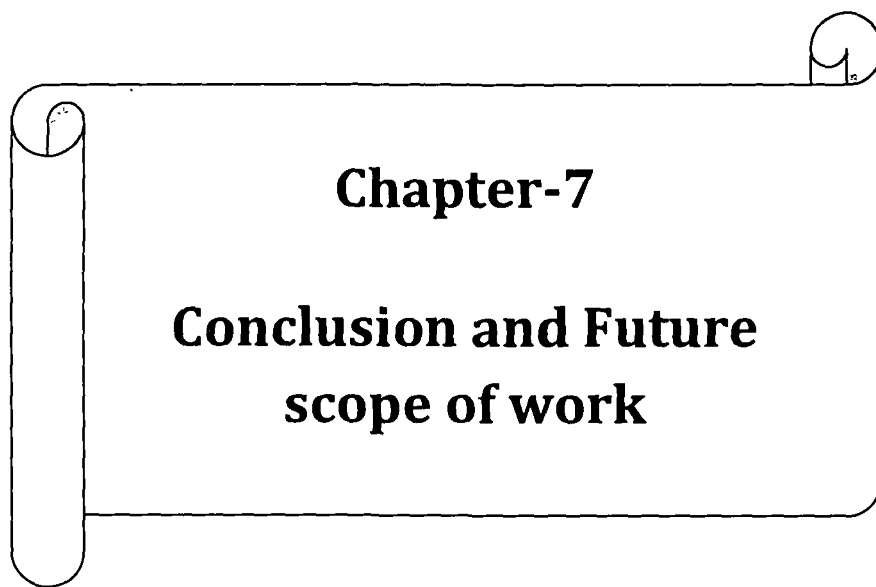
Chapter-7
Conclusion and Future scope of work

## 7.1. Salient findings

The present investigation was carried out to fulfill five clearly focused objectives. In the first objective, the effect of different solvent systems in removing anti-nutrients from rapeseed meal prior to protein extraction was tested, and the outcomes of such treatments on the physicochemical and functional properties of the meal protein were evaluated in the second objective. The third objective dealt with recuperation of protein from partially detoxified meal under statistically optimized conditions, and the protein extracted under the suggested optimal condition was chemically modified with varying concentration of maleic anhydride (maleylation) in the following (fourth) objective, with an intention to improve its functional properties. In the fifth objective, the residue left after oil and protein extraction (oil-and-protein spent meal) was utilized as a raw material for synthesizing multi-functional carbon nanoparticles by hydrothermal carbonization, which was then employed as an antioxidative and 'forgery-proof' ingredient in rapeseed protein-based bio-packaging material.

The salient findings of the thesis are summarized below:

1. **Removal of antinutrients from rapeseed press-cake with different solvent mixtures**
   - The most efficient extraction solvents for removal of the antinutrients were determined.
   - Acidified extraction mixtures (non-aqueous) were found to be superior to the non-acidified ones.
   - The characteristic differences in the efficacy of these wide varieties of solvents were studied by principal components analysis, based on which the mixture 0.2% perchloric acid in methanol-acetone (1:1 v/v) was deemed as 'the best' for detoxification of rapeseed meal.
   - Despite its high reductive potential, hemolytic activity of the extract from this solvent mixture clearly indicated the toxicity of the above-mentioned compounds on mammalian erythrocytes. The solvent treatment greatly reduced these compounds in the treated meal.

2. **Influence of antinutrients removal with acidified organic solvents from rapeseed meal on physicochemical and functional properties of meal protein**
   - Extraction resulted in substantial reduction of anti-nutrients from rapeseed protein isolate (RPI), especially polyphenols and phytates.
   - Efficiency of the removal of polyphenols and phytates from protein treated with perchloric acid containing solvent was much better than that with other acids.
   - Extent of allyl isothiocyanate removal was similar for all the acids tested. Isolates were essentially free of glucosinolates.
   - Treatment harbored significant improvement in their Degree of Whiteness, which was highest in the perchloric acid case.
   - The solvent treatment lowered protein yield and its solubility; conversely their interfacial properties were enhanced.
   - Treatment also resulted in distinct changes in proteins' secondary and tertiary conformations (Intrinsic emission fluorescence and FTIR spectra), especially higher level of acid led to detrimental effect of protein unfolding and aggregation.
   - Surface hydrophobicity and free sulfhydryl group of RPI changed considerably, with perchloric acid-treated samples showing higher values; while the disulfide content remarkably increased in trichloroacetic and phosphoric acid-treated samples, signifying aggregation.
   - Such aggregation-prone conformation tended to enhance thermal stability (higher denaturation temperature). Meals treated with lower level of acids seemed to consist of considerably structure-preserved proteins relative to that of control.
   - No appreciable alteration appeared among the electrophoretic profile of proteins from pristine meal and those treated with lower level of acids.

3. **Optimization of protein extraction from treated rapeseed meal**
   - The derived optimal conditions were extraction time of 2 h, solvent:meal ratio of 30:1 v/w, NaCl concentration of 0.0M and sodium sulfite level of 0.4%.

- The calculated regression model proved suitable for the evaluation of extraction process, whose adequacy was confirmed by Anderson-Darling Normality tests, Relative Standard Error of the Estimate (RSEE) and also by means of additional experiments performed at derived feasible experimental condition.
- Predicted values under the feasible optimum conditions were experimentally verified to be in general agreement (within 95% confidence interval).

4. **Effect of maleylation on physicochemical and functional properties of rapeseed protein**
   - Acylation increased whiteness value and dissociation of proteins, but reduced free sulfhydryl and disulfide content ($p<0.05$).
   - Intrinsic fluorescence emission and FTIR spectra revealed distinct perturbations in maleylated proteins' tertiary and secondary conformations.
   - Increase in surface hydrophobicity, foaming capacity, emulsion stability, protein surface load at oil-water interface and decrease in surface tension at air-water interface, occurred till moderate level of modification.
   - While maleylation impaired foam stability, protein solubility and emulsion capacity were markedly ameliorated ($p<0.05$), which were concomitant with decreased droplet size distribution ($d_{32}$).
   - *In-vitro* digestibility and cytotoxicity tests suggested no severe ill-effects of modified proteins, especially at low degrees of maleylation.
   - The study showed good potential of maleylated proteins as functional food ingredient.

5. **Synthesis of carbon nanoparticles (CNPs) from oil-and-protein spent meal and its application in fabricating rapeseed protein-based nanocomposite film**
   - Hydrothermal carbonization of oil-and-protein spent meal resulted in the formation of antioxidative, hemocompatible, fluorescent CNPs.

- Antioxidant potential of CNP-fabricated rapeseed protein-based fluorescent film improved by 17.5—19.3 fold, and has potential for use as forgery-proof packaging.
- Oil samples packed in nanocomposite film sachets showed significant delay in oxidative rancidity compared to that packed in pristine protein-film sachet (free fatty acids, peroxide value and thiobarbituric acid-reactive substances reduced upto 1.4, 2 and 1.2 fold, respectively).

## 7.2. Future scope

The results obtained from the current thesis can form the basis for further studies on rapeseed press-cake and its protein products.

A few of the future scope are outlined below:

- Further studies should be undertaken to analyze the release behavior of carbon nanoparticles from the film or other packaging materials into different food model systems, their effect on organoleptic properties, gastro-intestinal tract, and toxicological assessment using *in-vitro* and *in-vivo* models.
- Biological value and amino acids composition of the isolates extracted from different solvent treated meals and the maleylated proteins should be tested for assessing their qualities.
- High pressure hydrostatic treatment can be applied to the maleylated protein for increasing its gelation properties.
- Ultrasound-assisted protein extraction from rapeseed meal can also be tested to increase the yield.
- Combination of chemical and enzymatic modification can be applied for improving the techno-functional properties of protein.
- Supercritical fluid extraction can be tested for assessing its efficacy in removing antinutrients from the meal.
- Extrusion technology can be implemented to produce simulated meat analogs (chunks) from rapeseed meal protein.

# List of Publications

1. Das Purkayastha, M., et al. Physicochemical and functional properties of rapeseed protein isolate: Influence of antinutrient removal with acidified organic solvents from rapeseed meal, *J Agric Food Chem.* **62** (31), 7903-7914, 2014.
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5. Das Purkayastha, M., et al. Removing antinutrients from rapeseed press-cake and their benevolent role in waste cooking oil-derived biodiesel: Conjoining the valorization of two disparate industrial wastes, *J Agric Food Chem.* **61** (45), 10746-10756, 2013.
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8. Das Purkayastha, M., et al. Tackling correlated responses during process optimization of rapeseed meal protein extraction, *Food Chem.* **170**, 62-73, 2015.
9. Das Purkayastha, M., et al. Effect of maleylation on physicochemical and functional properties of rapeseed protein isolate. *Food Res Int.* (under review)

**Conference Presentation:**

1. Das Purkayastha, M., Barthakur, A., & Mahanta, C.L. Process for removal of tannins from rapeseed meal to obtain proteins for food utilization. *BIOFOODS 2011*, National seminar on "Role of Bioactive Compounds in Foods on Human health" organized by the Department of Food Engineering and Technology, Tezpur University, 14-16 Nov 2011.