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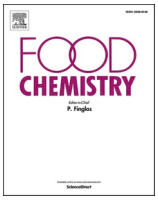

Combined effects of isolation temperature and pH on functionality and beany flavor of pea protein isolates for meat analogue applications

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ABSTRACT

The combined effects of isolation temperature (20, 30 and 40 °C) and pH (2.0–12.0) on yield, techno-functional properties, and beany flavor of pea protein isolates were investigated. Increasing pH from 2.0 to 9.5 and 11.0 increased yields from 37 % to 75 % and 79 %, respectively, at 20 °C. At a constant pH, increasing temperature from 20 to 40 °C did not increase protein recovery; rather, negatively affected the techno-functional properties such as protein solubility, foaming and gelation. Protein isolated at pH 11.0 (20 °C) provided a higher fat absorption, gelation capacity, gel hardness, cohesiveness, chewiness, and gumminess than at pH 9.5, due to higher protein denaturation as supported by their higher surface hydrophobicity. Volatile beany flavor marker hexanal was predominant in all isolates than the starting material, irrespective of isolation temperature, probably due to lipid oxidation. The results provide a basis for tuning the isolation process for producing pea protein isolates with desired techno-functional properties for meat analogue applications.

1. Introduction

The global demand for plant-based meat analogues has increased at a compound annual growth rate of 15 % from 2019 (MarketsandMarkets, 2019) due to ongoing protein shift trend towards a healthy and more environmentally sustainable choices (Lam, Can Karaca, Tyler, & Nickerson, 2018). It is expected that its market value will increase from USD 12.1 billion in 2019 to USD 27.9 billion by 2025 (MarketsandMarkets, 2019). This in turn has increased the search for plant-based protein ingredients to replace animal-based proteins. Pea (*Pisum sativum* L.) is one of those ingredients which has the potential to be used for plant-based meat analogues production because of its low cost, availability, low allergenicity, high nutritional value, and being non-genetically modified (Lam et al., 2018). Further, pea cultivation has lower greenhouse gas intensity per unit of nutritional density compared to animal foods (Saget, Costa, Santos, Vasconcelos, Styles, & Williams, 2021); its cultivation requires negligible amount of nitrogen fertilizer, and has less negative impact on biodiversity due to low pesticide use (Tidåker, Potter, Carlsson, & Röös, 2021).

The main classes of proteins found in pea are e.g. globulin, albumin and glutelin, with their amount being varied in the range of 55 %-65 %, 18 %-25 %, and 3 %-4%, respectively (Lu, He, Zhang, & Bing, 2020). The major class globulin is a storage protein and further classified into legumin, vicilin and convicilin, based on their sedimentation coefficient (Tanger, Engel, & Kulozik, 2020). Legumin (11S) has a molecular weight of 360 kDa, is hexameric with subunits being 60 kDa each. Each subunit is composed of an α-chain (40 kDa) containing chiefly of acidic amino acids and a β-chain (20 kDa) containing chiefly of basic amino acids, and are connected by a disulfide bond. (Tanger et al., 2020) Vicilin (7S) on the other hand has a molecular weight of 150 kDa, is trimeric with subunits being 50 kDa each, and does not contain any disulfide bonds. Pea globulin usually follows a 2:1 ratio of 11S legumin and 7S vicilin, but may vary depending on genotype. The other protein convicilin has a molecular weight of 280 kDa, is tetrameric with subunits being 70 kDa each, and has no disulfide bonds. (Kyriakopoulou, Dekkers, & van der Goot, 2019; Tanger et al., 2020) The sulfur-containing amino acids of legumin e.g., cysteine creates disulfide bridges during e.g., extrusion and provides texturization. (Lu et al., 2020; Sha & Xiong, 2020) Besides this, other protein functionalities such as protein solubility, gelation properties, emulsification, foaming, water and oil holding capacities play important roles in meat analogue structure formation (Kyriakopoulou et al., 2019). For example, proteins in general provide water holding and network formation properties (Kyriakopoulou et al., 2019), which depend largely on their structural properties (Abdollahi, Rezaei,

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Jafarpour, & Undeland, 2018). Thus, it is important to find the right functional properties of proteins giving a suitable meat analogue structure formation (Kyriakopoulou et al., 2019).

The process conditions used for protein isolation from the legumes can largely influence protein isolation yield, structure, and functionality, defining product forming capacity and thus the final application of protein isolates. For example, it has been reported that the isolation pH can affect protein solubility (Day, 2013), surface hydrophobicity (Timilsena, Adhikari, Barrow, & Adhikari, 2016), and water holding capacity of protein isolated from legumes (Peng, Kersten, Kyriakopoulou, & van der Goot, 2020). Further, a positive correlation between pH and the reactivity of disulfide bond formation has been reported in gelation studies with oats and whey (Monahan, German, & Kinsella, 1995; Nieto-Nieto, Wang, Ozimek, & Chen, 2015). One of the structural properties of proteins i.e., surface hydrophobicity, which represents the degree of protein unfolding and denaturation, changes with pH and affects e.g., intermolecular protein–protein and protein-lipid interactions, thus affecting proteins' water solubility, emulsification, foaming, and gelation properties (Timilsena et al., 2016). Protein isolation temperature is also an important factor which can affect e.g. denaturation, aggregation, and structure formation of proteins (Zhang, Liu, Liu, Yoon, Rizvi, & Wang, 2019). Besides these, both temperature and pH affect volatile compound formation (Gao et al., 2020; Sajib & Undeland, 2020), often reported as beany flavor compounds for plant-based ingredients, which is an outstanding challenge for meat alternative applications as it is not desired. The most reported beany flavor markers found in pea proteins are e.g. hexanal, 1-hexanol, 2-pentylfuran, 3-methyl-1-butanol, 1-octen-3-ol, (*E,E*)-2,4-decadienal, and (*E,E*)-2,4-nonadienal (Ferawati, Witthöft, & Bergström, 2020; Xu, Jin, Lan, Rao, & Chen, 2019).

To date however very little is known how a combination of pea protein isolation temperature and pH affect the functional properties and beany flavor of pea proteins. For example, several authors reported only the effect of alkaline isolation pH, typically in the pH range of 8.0–10.5 (Ferawati et al., 2021; Gao et al., 2020), on pea protein and the effect of temperature and their combined effect has been neglected. Therefore, the aim of this study was to investigate the combined effect of protein isolation temperature and pH on functional, rheological, textural, structural, and volatile beany flavor properties of protein isolates from yellow pea. The results of this study will guide us towards a better understanding of key drivers of pea protein functionality and flavor to find optimum protein isolation process condition suitable for meat analogue applications.

2. Material and methods

2.1. Materials

Dry fractionated pea protein concentrate (Vestkorn A/S, Denmark), referred to as pea flour hereafter, was used as the starting raw material to further improve its protein purity and techno-functional properties suitable for meat analogue applications. The pea flour contained around 90 %-93 % dry matter, 54 %-56 % protein, 15 %-20 % carbohydrates, 15 %-17 % dietary fiber, 3 %-5% total fat, and 5.5 %-6.5 % ash. All chemicals used were of reagent grade.

2.2. Protein solubility analysis at different temperature and pH

Solubility of proteins at different pH was measured according to Abdollahi and Undeland (2018). Briefly, 50 g pea protein powder was dispersed in 750 ml of distilled water, and the pH was adjusted to 2.0–12.0 using 2 M NaOH or 2 M HCl, and incubated either at 20 or 30 or 40 °C for 60 min. Two different sets of experiments were performed, one to adjust the pH in the alkaline direction and the other in the acidic direction. Then, the solutions were centrifuged at 3,000 × g for 30 min at 4 °C, and the soluble protein content in the supernatants was measured using a modified version of the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). The relative solubility of proteins was measured based on the solubility at pH giving the maximum solubility.

2.3. Protein isolation process

Based on the results from the step 2.2, pea proteins were extracted at pH 2.0, 9.5, and 11.0 at 20, 30, and 40 °C. Briefly, 60 g pea flour was homogenized with 840 ml deionized water in 1-liter glass beaker at either 20, 30, or 40 °C; the pH was adjusted to either 2.0, 9.5, or 11.0 using 2 M NaOH and/or 2 M HCl and incubated for 60 min with continuous stirring using an overhead stirrer (R 3003 Spiral stirrer, IKA). Upon completion, the mixture was centrifuged at 3000 × g for 20 min (4 °C); the pellet was discarded, the pH of the supernatant was adjusted to 4.0 using 2 M HCl, and held for 20 min. Afterwards, the mixture was centrifuged as above, the supernatant was discarded, the pH of the pellet was adjusted to 7.0, stored at −80 °C, and thereafter freeze-dried. This freeze-dried protein isolate was used for analyses. The soluble protein content of the supernatant was measured using a modified version of Lowry method (Lowry et al., 1951). Protein solubilization and precipitation yields were calculated using equations below, where H, S1, and S2 represent soluble protein content of the homogenate at selected solubilization pH, at supernatant after the first centrifugation, and second centrifugation, respectively.

$$\text{Protein solubilization yield } (\%) = \frac{\text{Soluble protein content of S1 } \left(\frac{\text{mg}}{\text{ml}}\right) \times \text{volume S1}}{\text{Soluble protein content of H } \left(\frac{\text{mg}}{\text{ml}}\right) \times \text{volume H}} \times 100.$$

$$\text{Protein precipitation yield } (\%) = \frac{\left(\text{Soluble protein content of S1 } \left(\frac{\text{mg}}{\text{ml}}\right) \times \text{volume S1}\right) - \left(\text{Soluble protein content of S2 } \left(\frac{\text{mg}}{\text{ml}}\right) \times \text{volume S2}\right)}{\text{Soluble protein content of S1 } \left(\frac{\text{mg}}{\text{ml}}\right) \times \text{volume S1}} \times 100.$$

Mass yield and protein recovery of protein isolation process were calculated using equations below:

$$Massyield(\%) = \left(\frac{\text{amount of dry protein isolate}}{\text{amount of dry starting material}}\right) \times 100$$

$$Proteinrecovery(\%) = \left(\frac{\text{amount of final product} \times \text{total nitrogenous protein content}}{\text{amount of starting material} \times \text{total nitrogenous protein content}}\right) \times 100$$

2.4. Analyses of protein isolates

2.4.1. Total nitrogenous protein content

Nitrogen content in the sample was first analyzed using Dumas method with a nitrogen analyzer (LECO, St. Joseph, MI, USA), and then the nitrogen content was converted to total nitrogenous protein content, representing both soluble and insoluble proteins, using a nitrogen to protein conversion factor of 6.25 (Mariotti, Tomé, & Mirand, 2008). For the analysis, approximately 0.1 g sample was loaded on the sample tray, which was then combusted in the nitrogen analyzer at approximately 1000 °C. The resulting nitrogen, after reduction of nitrogen oxides to nitrogen and separation of water and carbon dioxide, was then determined by a thermal conductivity detector.

2.4.2. Proteins solubility in water

To measure protein solubility at different pH, 0.5 g of each protein isolate was dispersed in 20 ml distilled water, and then the pH was adjusted to 3.0–11.0 using 1 M NaOH or 1 M HCl. Two different sets of experiments were performed, one to adjust the pH in the alkaline direction and the other in the acidic direction. The protein dispersion was stirred using magnetic stirrer and held at each pH for 30 min at ambient temperature (i.e., 22 °C). The dispersion was then centrifuged at 15,000 × g for 30 min (4 °C). Thereafter, the soluble protein content in the supernatant was measured using a modified version of Lowry method (Lowry et al., 1951). The relative solubility of proteins was measured based on the solubility at pH giving the maximum solubility using the formula below.

$$\text{Protein solubility}\,(\%) = \left(\frac{\text{soluble protein concentration in the supernant}}{\text{Soluble protein concentration in pH with maximum solubility}}\right) \times 100$$

2.4.3. Fat absorption capacity

Fat absorption capacity of protein isolate was measure according to Sathivel, Yin, Bechtel, and King (2009) with some modification. Briefly, 250 mg protein isolate was weighed into 15-ml plastic tube, followed by addition of 5 g sunflower oil, and vortexed for 2 min. The mixture was incubated at ambient temperature (i.e. 22 °C) for 30 min with intermittent mixing every 10 min. Thereafter, the mixture was centrifuged at 2500 × g for 30 min, free oil was poured out, and the resulting mixture was weighted again. Fat absorption of the sample was calculated from the weight difference. The result was expressed in terms of milliliters of fat absorbed by 1 g of protein isolate.

2.4.4. Foaming capacity and foaming stability

Foaming capacity and foaming stability were measured according to Abdollahi and Undeland (2018). Briefly, 250 mg sample was mixed with 25 ml deionized water ($V_{initial}$) and homogenized at 11,000 rpm for 2 min using a Polytron Homogenizer (IKA T18 digital ULTRA TURRAX, IKA, Brazil). The volume of the mixture including foam was recorded immediately after homogenization (V_1) and after 60 min (V_{60}), and foaming capacity and foaming stability were calculated using equations below.

$$\text{Foaming capacity}\,(\%) = \left(\frac{\text{V1} - \text{Vinitial}}{\text{Vinitial}}\right) \times 100$$

$$\text{Foaming stability}\,(\%) = \left(\frac{\text{V60} - \text{Vinitial}}{\text{Vinitial}}\right) \times 100$$

2.4.5. Rheological (i.e. in-situ gelation) analysis

For both rheological (i.e. *in-situ* gelation) and textural analyses, 6 g protein isolate was mixed with 24 ml deionized water, and mixed for 30 *sec* using a chopper every 10th min over a period of 30 min. Then, rheological and textural analyses were performed as described in the following paragraphs.

A portion of the sample (i.e., around 1–2 g) from the chopper was loaded on the dynamic rheometer (Paar Physica Rheometer MCR 300, Anton Paar GmbH, Austria) for *in-situ* gelation analysis using a parallel-plate geometry (25 mm plate diameter and 1 mm plate gap) mounted on a dynamic rheometer operated in an oscillating mode. Sample edges were covered with inorganic oil to prevent evaporation during *in-situ* gelation. *In-situ* gelation was performed in three steps; i.e., ramping up of temperature from 20 °C to 90 °C at a constant heating rate of 5 °C/min, followed by maintaining temperature at 90 °C for 30 min, and, then the temperature was ramped down to 20 °C at a rate of 5 °C/min. The gelation test was done in a linear viscoelasticity region (i.e., 1 % strain and 0.1 Hz frequency) of the samples.

2.4.6. Textural analyses of gels

Sample for texture analysis was prepared as described in section 2.4.5. Then, a portion of the sample from the chopper was loaded in 10-ml plastic tubes, tightly sealed, and cooked in boiling water at 90 °C for 20 min. Upon completion, the syringe was cooled on ice-cool water for 1 h, and then stored overnight at 4 °C. Thereafter, the gels were cut in equal heights and diameter (i.e., 15 mm), and textural properties were analyzed using a texture analyzer (TVT 6700, Perten Instruments, Australia) equipped with a 5-kg load cell according to Abdollahi and Undeland (2019).

2.4.7. Water holding capacity of gels

Water holding capacity (WHC) of gels was measured according to Cardoso, Mendes, Vaz-Pires, and Nunes (2009). Briefly, 2 g properly chopped gel sample (X) was wrapped within two layers of pre-weighed (Y) filter paper, placed in 50-ml plastic tube, and centrifuged at 3,000 × g for 10 min at 20 °C. Then, the gels were removed from the filter paper, and the filter paper was weighed again (Z). WHC (%) was calculated using the equation below, where M is the initial moisture (%).

$$\text{WHC}\,(\%) = \left(\frac{\text{X} \times \left(\frac{\text{M}}{100}\right) - (\text{Z} - \text{Y})}{\text{X} - \left(\frac{\text{M}}{100}\right)}\right) \times 100$$

2.4.8. Molecular weight distribution of soluble proteins and peptides

Molecular weight distribution of soluble proteins and peptides was analyzed by high performance size exclusion chromatography (HP-*SEC*; Dionex HPLC, Dionex GmbH, Idstein, Germany) according to a method described by Sajib, Albers, Langeland, and Undeland (2020). The HPLC system was equipped with an Agilent Bio *sEC*-5 guard column (5 μm, 150 Å, 4.6 × 50 mm), and then chromatographic separation was performed by two serially connected *SEC* columns: Agilent Bio *sEC*-5 300 Å (5 μm, 300 Å pore size, 7.8 × 300 mm) and Agilent Bio *sEC*-5 100 Å (5 μm, 100 Å pore size, 4.6 × 300 mm). Sample was prepared by dissolving protein isolates in the mobile phase (i.e., 0.1 M sodium phosphate buffer at pH 7.0) to a protein concentration of 10 mg/ml. The mixture was then centrifuged at 10,000 × g for 10 min, the supernatant was filtered through 0.45 μm pore size cellulose acetate membrane (fisher scientific), and the resulting filtrate was used for HP-*SEC* analysis. Molecular weight of samples was calculated against AdvanceBio *SEC* 300 Å Protein Standard (Agilent Technologies).

2.4.9. Surface hydrophobicity

Surface hydrophobicity was analyzed according to Timilsena et al. (2016). Briefly, 40 mg pea protein isolate was mixed with 40 ml phosphate buffer (0.01 M, pH 7.0), centrifuged at 10,000 × g for 20 min, and the resulting supernatant was used for soluble protein content analysis by a modified version of Lowry method (Lowry et al., 1951) and for further analysis. The supernatant was further diluted to 0.01 – 0.1 mg/ml protein concentrations using phosphate buffer. Thereafter, 2 ml of diluted supernatant was mixed with 10 μL of 8 mM 8-anilinno-1-napthaleneslfonic acid solution (ANS; solubilized in the same buffer), vortexed for 30 *sec*, and incubated in dark for 15 min at ambient temperature (~22 °C). Upon completion, the fluorescence intensities of samples containing ANS solution, ANS blank (in phosphate buffer), and sample blank were measured at excitation and emission wavelengths of 374 and 485 nm, respectively, using a Tecan Safire 2 plate reader (Tecan, Switzerland). Fluorescence intensities of blanks were subtracted from samples, net fluorescence intensities were plotted against protein concentration, and surface hydrophobicity was calculated from the initial slope (of the linear regression).

2.4.10. Volatile beany flavor markers analysis

Selected volatile beany flavor markers were analyzed by headspace solid-phase microextraction (HS-SPME)-GC–MS according to a method described by Sajib and Undeland (2020) with slight modifications. Modifications were; a different GC–MS setup (TQ8030, Shimadzu) and the data acquisition scan was performed in the mass range of 30–500 amu. SPME fiber and GC column used were 75 μm Carboxen/ polydimethylsiloxane (CAR/PDMS) coated SPME fiber (Supelco, USA) and fused silica ZB-1701 capillary column (30 m × 0.32 mm, 1 μm, Phenomenex), respectively. Sample was prepared by dissolving 1 g sample in 9 ml MQ-water directly in 20-ml SPME vials. Then, SPME fiber was injected into vial for volatile compounds extraction at 60 °C for 20 min with stirring at 500 rpm. The fiber was then injected into GC–MS for the analysis in splitless mode for 5 min. The GC inlet temperature was maintained at 300 °C, and carrier gas helium was used at a constant flow rate of 1.5 ml/min. GC separation was performed at GC oven temperatures of 35 °C for 3 min, followed by an increase of 3 °C/ min to 70 °C, and then at 10 °C/min to 200 °C, and finally at 20 °C/min to 260 °C with a holding for 5 min. MS transfer line temperature was maintained at 265 °C, and ion source temperature was 200 °C. Volatile beany flavor compound markers were selected based on most commonly reported markers by other researchers e.g., Ferawati et al. (2020) and Xu et al. (2019), and are hexanal, 1-hexanol, 1-octen-3-ol, 1-octen-3-one, 2-pentylfuran, 1-pentanol, 1-Nonanal, 3-methyl-1-butanol, (*E,E*)-2,4-nonadienal, and (*E,E*)-2,4-decadienal and 2-isopropyl-3-methoxy pyrazine. Volatile compounds were identified by external standards, and relative quantification was performed against the peak area of the internal standard.

2.4.11. Sodium content analysis

To get an indication about the amount of NaCl salt residue in protein isolates, sodium (Na) content was analyzed according to a method by Gmoser, Fristedt, Larsson, Undeland, Taherzadeh, and Lennartsson (2020) with slight modifications. Briefly, around 150 mg samples were weighed into Teflon vials, and then 3 ml of milli-Q water, 750 μL of nitric acid and 150 μL of concentrated hydrochloric acid were added. The Teflon vials were then closed tightly and digested in microwave digestor (Milestone microwave laboratory system Ethos Plus Sorisole, Italy) at 180 °C for 35 min. Upon completion the samples were cooled to room temperature. Thereafter the samples were diluted using milli-Q water and analyzed in atomic absorption spectroscopy (240FS AA, Agilent Technologies, Australia). Quantification was made using standard curves of sodium standard (Fluka, Switzerland).

2.5. Extrusion processing

To investigate the applicability of selected pea protein isolates in meat analogue applications, both low-moisture extrusion (LME) and high-moisture extrusion (HME) trials were performed on a TwinLab-F 20/40 twin-screw extruder (Brabender, Germany). The extruder has 6 different temperature zones (including the die). In case of LME processing, a round head die with 2 mm diameter was used to produce low-moisture meat analogues (LMMA), which is also known as textured vegetable protein (TVP). And, in case of HME processing, a cooling die with 20 × 9 × 330 mm dimension was used to produce high moisture meat analogues (HMMA). Extruder screw configurations for both LME and HME processing can be found in supporting info. (see Figs. 4-5; supporting info.). The feeder was operated in the range of 2.25 – 3.43 kg/h capacity for both LME and HME trials. For LME trial, selected pea protein isolate was used together commercial oat protein (Lantmännen Oats AB; 1:1 ratio); and for HME trial only pea protein isolate was used as the raw material. The operational settings for LME were; H_2O: 35 %, temperature profile: 40–80-130–160-160 °C, screw velocity: 750 rpm, pressure: 10.3 bar, melt temperature: 174 °C, torque: 13, and throughput: 5.0 kg/h. The operational settings for HME were; H_2O: 62 %, temperature profile: 40–80-150–150-145–35 °C, screw velocity: 600 rpm, pressure: 8.3 bar, melt temperature: 149.2 °C, torque: 8.9, and throughput: 4.5 kg/h. Melt temperature refers to the temperature of the melt extrudate recorded by a thermocouple connected to the extruder barrel. Data on melt temperature, barrel temperature profile, screw speed, pressure, torque, and throughput were collected from the extruder's log records.

2.6. Water absorption index and internal sensory analyses of extrudate

Water absorption index (WAI) was measured gravimetrically according to a protocol by Munz (2021). Briefly, 2 g LME processed extrudate was weighed into 50-ml plastic tube, and then the tube was filled with cold water (10 °C) to soak the extrudates and kept for 10 min. Thereafter, the water was drained for 10 min using a strainer and the extrudate was weighed again (see Fig. 7; supporting info.). The WAI of extrudates was then calculated from weight difference according to the equation below.

$$\text{WAI (\%)} = \frac{(\text{hydrated extrudate weight (g)} - \text{initial extrudate weight (g)}) \times 100(\%)}{\text{initial extrudate weight (g)} \times (100\% - \text{extrudate moisture content (\%)}}$$

An internal sensory analysis (i.e., affective test) was performed by four panelists for LME processed extrudate to get a better understanding of consumer experience. The test was performed in three steps. In step 1, 25 g extrudate was mixed with 56.6 g water and 10 g rapeseed oil in a mixer (Snabbhack, Electrolux, Sweden) for approximately 15 *sec* to cut the extrudates into smaller pieces to increase the surface area and thus facilitate water and oil absorption. The mix was let to rest at room temperature for 30 min until further testing. In step 2, the soaked extrudates from step 1 were fried in oil without any seasoning. In step 3, the soaked extrudates from step 1 were mixed with 8 g of taco spice mix seasoning, fried in oil, and tested as part of taco lunch. The panelists were then evaluated extrudates from all three steps based on the likelihood of acceptance.

2.7. Statistical analysis

Results are expressed as mean (n = 2 or 3) ± standard error of the mean (SEM). The data were subjected to ANOVA analysis with Tukey's Honest Significant Differences (HSD) test on RStudio software (https://www.rstudio.com/); significant differences were accepted at $p < 0.05$.

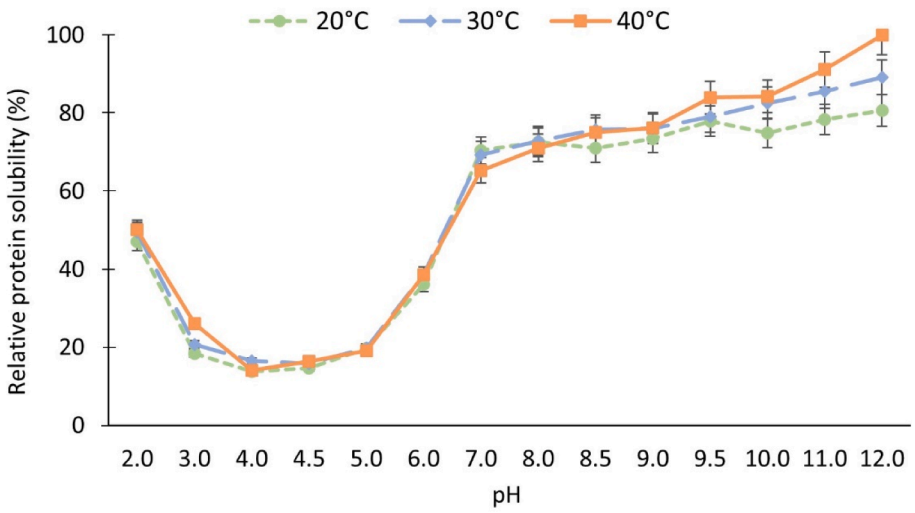

Fig. 1. Effect of solubilization temperature and pH on pea protein solubility. Relative protein solubility was measured based on the solubility at pH and temperature giving the maximum protein solubility. Protein contents refer to soluble proteins only as described in the method section.

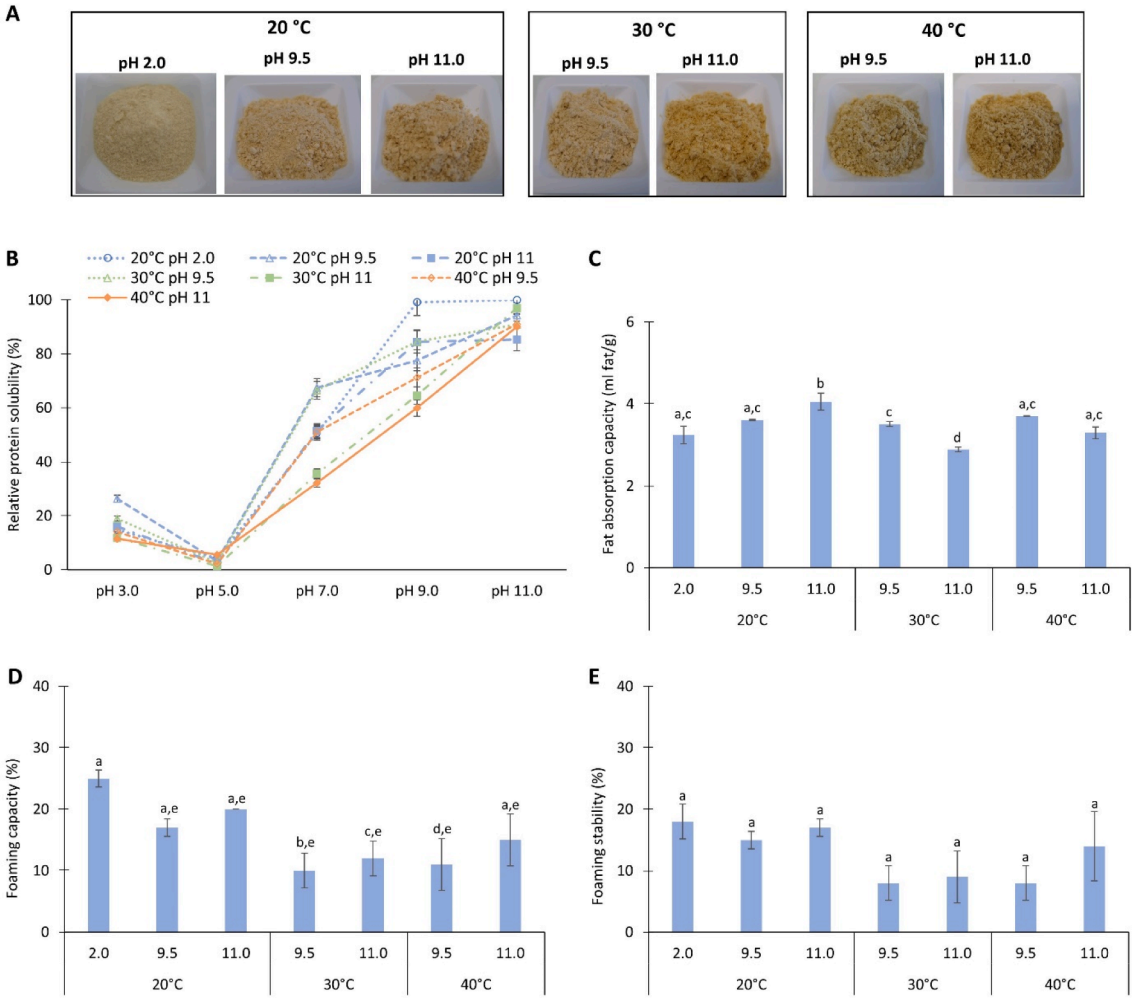

Fig. 2. Pictures of pea protein isolates (A), and results from water solubility (B), fat absorption capacity (C), foaming capacity (D) and foaming stability (E) analyses of protein isolated produced at different temperature and pH. Different lower-case letters represent significance ($p < 0.05$) differences.

3. Results

3.1. Effects of temperature and pH on pea protein solubility

Fig. 1 shows pea proteins' solubility at different temperature and pH values. In general, the solubility was higher at alkaline pH's than acidic pH's, and the highest solubility was noticed at pH 12.0. The lowest protein solubility was at pH 4.0, which is within the range of previously reported isoelectric point of 4.0–5.0 for pea proteins (de Oliveira et al., 2020). The solubility increased again by decreasing pH to 3.0, which was followed by a sharp increase in solubility at pH 2.0. In case of temperature, relatively higher protein solubility was noticed at higher temperature at alkaline pH's (e.g., pH > 9.0) than acidic pH's. Apart from that, there was no big difference in protein solubility when 20 or 30 or 40 °C temperature was used below pH 9.0. However, by increasing solubilization pH to 9.5 or higher the effect of temperature appeared. For example, the use of 40 °C gave significantly ($p < 0.05$) higher protein solubility at pH 9.5 and 12.0 than at 20 °C. Based on these results,

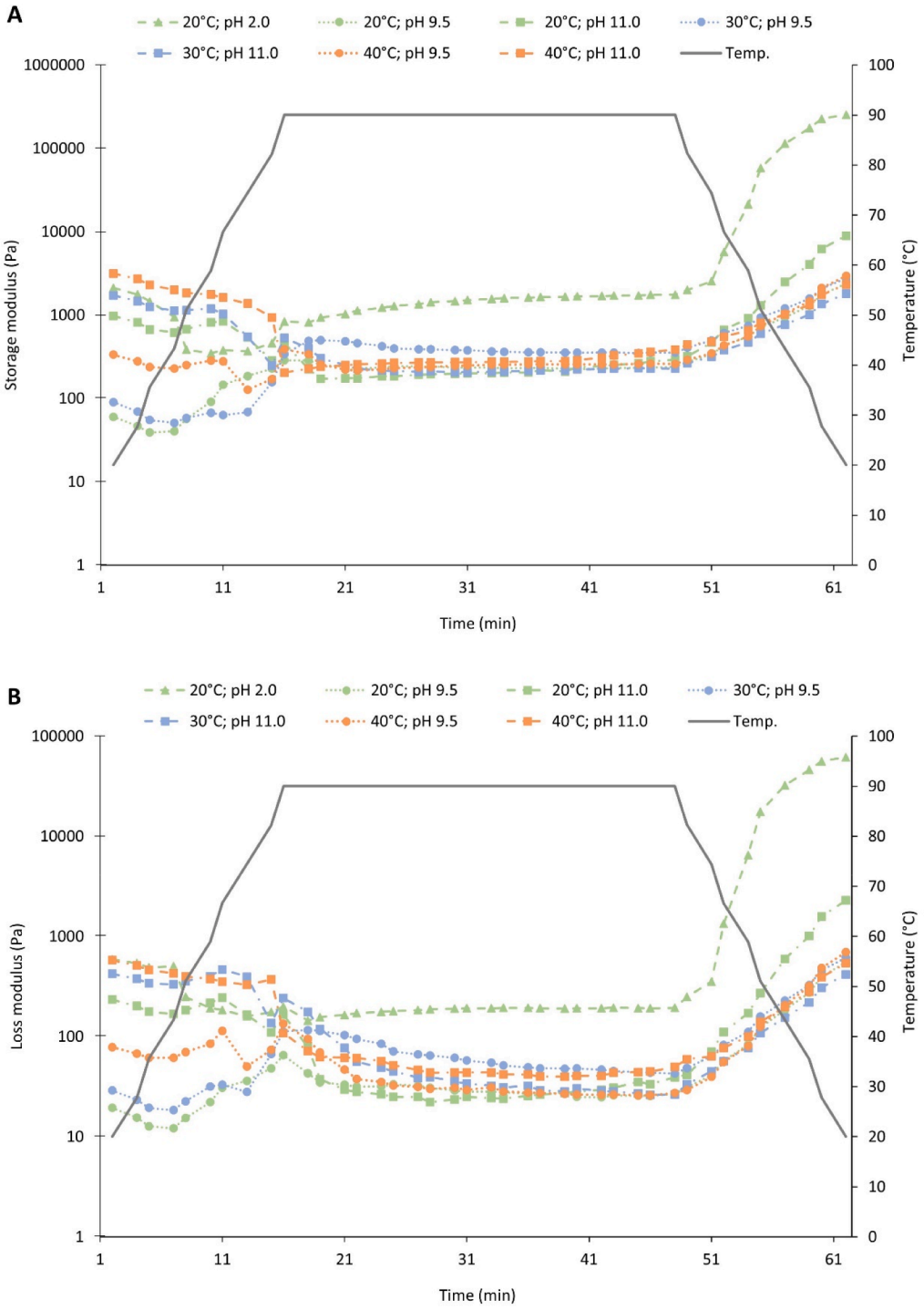

Fig. 3. Rheological behaviours (i.e., storage modulus G', Fig. A; and loss modulus G'', Fig. B) of protein isolates, produced at different temperature and pH, during *in situ* gelation via temperature ramp test including an initial heating step (5 °C/min, from 20 to 90 °C), followed by an isothermal step (90 °C, 30 min) and a final cooling step (5 °C/min, from 90 to 20 °C).

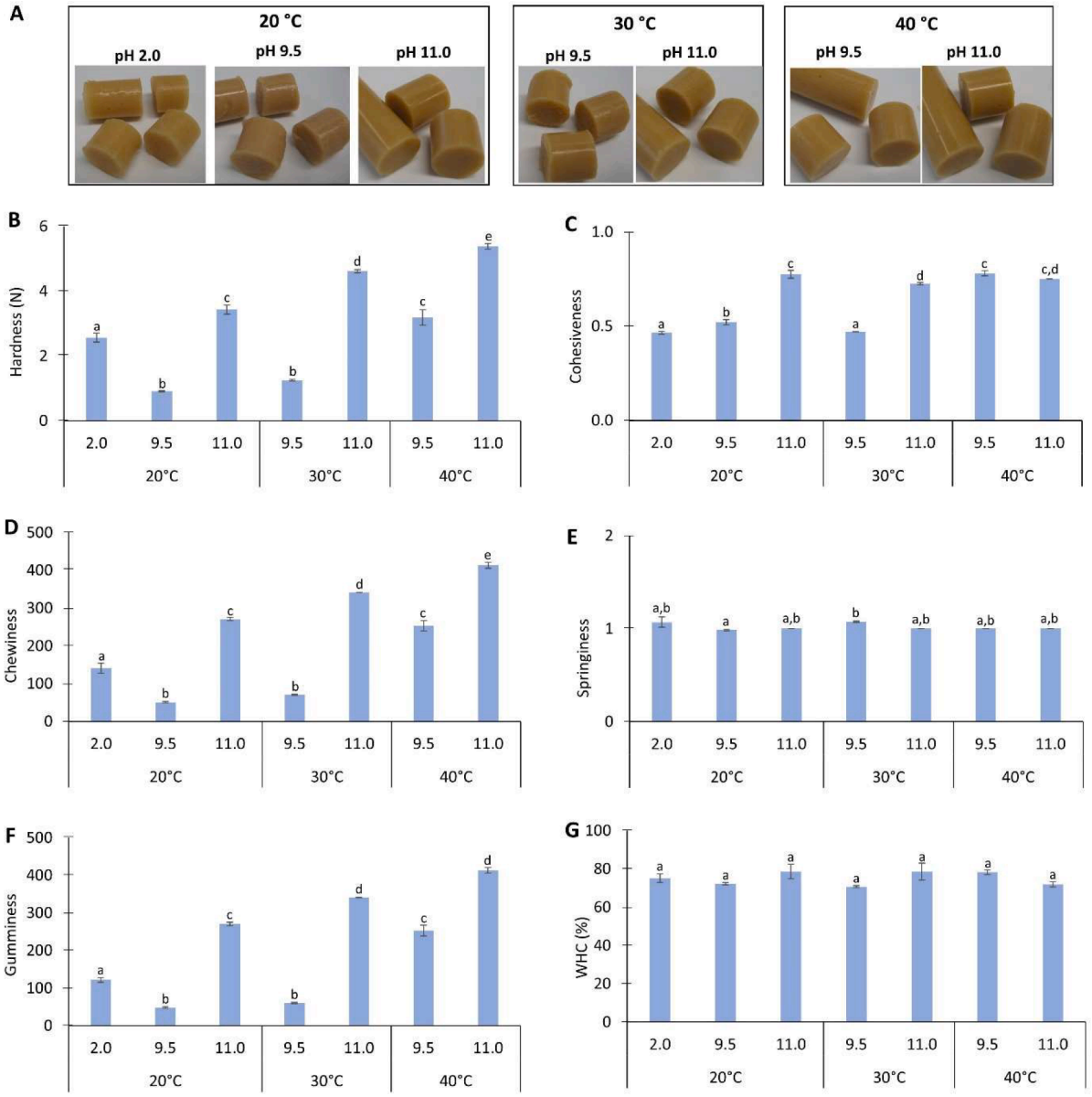

Fig. 4. Representative visual pictures of gels prepared from pea protein isolates (A) used for textural (B-F) and water holding capacity (G) analyses. Gel hardness (B), gel cohesiveness (C), gel chewiness (D), gel springiness (E), and gel gumminess (F) values of gels. Different lower-case letters represent significance ($p < 0.05$) differences.

pH 4.0 was selected as the protein precipitation pH; and pH 2.0, 9.5, and 11.0 were selected as protein solubilization pH for further experiments at 20 °C or 30 °C or 40 °C.

3.2. Effect of temperature and pH on protein isolation yields

Mass yield, which refers to "x" gram of protein isolate recovered from 100 g of starting material, increased with an increase in solubilization pH from 9.5 to 11.0 at both 20 and 30 °C (see supporting info. Fig. 1). The highest mass yield of 37.61 % was recorded at pH 9.5 (40 °C); however further increasing the pH to 11.0 at this temperature did not increase the yield. In case of protein purity, i.e., total nitrogenous protein content representing both soluble and insoluble proteins in the final isolate, all protein isolation conditions resulted in around 80 % protein purity, except that of pH 2.0 at 20 °C which gave the highest protein purity of 89.46 %, although the mass yield was the lowest (i.e., 12.16 %).

3.3. Characterization of protein isolates

3.3.1. Functional properties

Fig. 2 shows representative pictures of dried protein isolates, as well as their water solubility, fat absorption capacity, foaming capacity, and foaming stability. In general, the protein isolates were highly soluble at alkaline pH's (Fig. 2**B**). In case of solubility at pH 7.0, the most commonly found pH in food products, isolates produced at pH 9.5 had the highest solubility than isolates produced at pH 2.0 and 11.0 (see supporting info. Fig. 3). The lowest solubility was noticed at pH 5.0, which is close to the isoelectric point of the pea proteins (i.e., pH 4.0–5.0). In case of fat absorption capacity, which also resembles flavor retention capacity of protein isolates, isolates produced at pH 11.0 showed significantly ($p < 0.05$) higher values than the ones produced at pH 9.5 and 2.0 at 20 °C (Fig. 2**C**). However, this trend seems to reverse at 30 and 40 °C. Foaming capacity indicates protein isolate's ability to make foam, and in general isolates produced at pH 2.0 (20 °C) had better foaming capcity than the rest (Fig. 2**D**). In case of foaming stability,

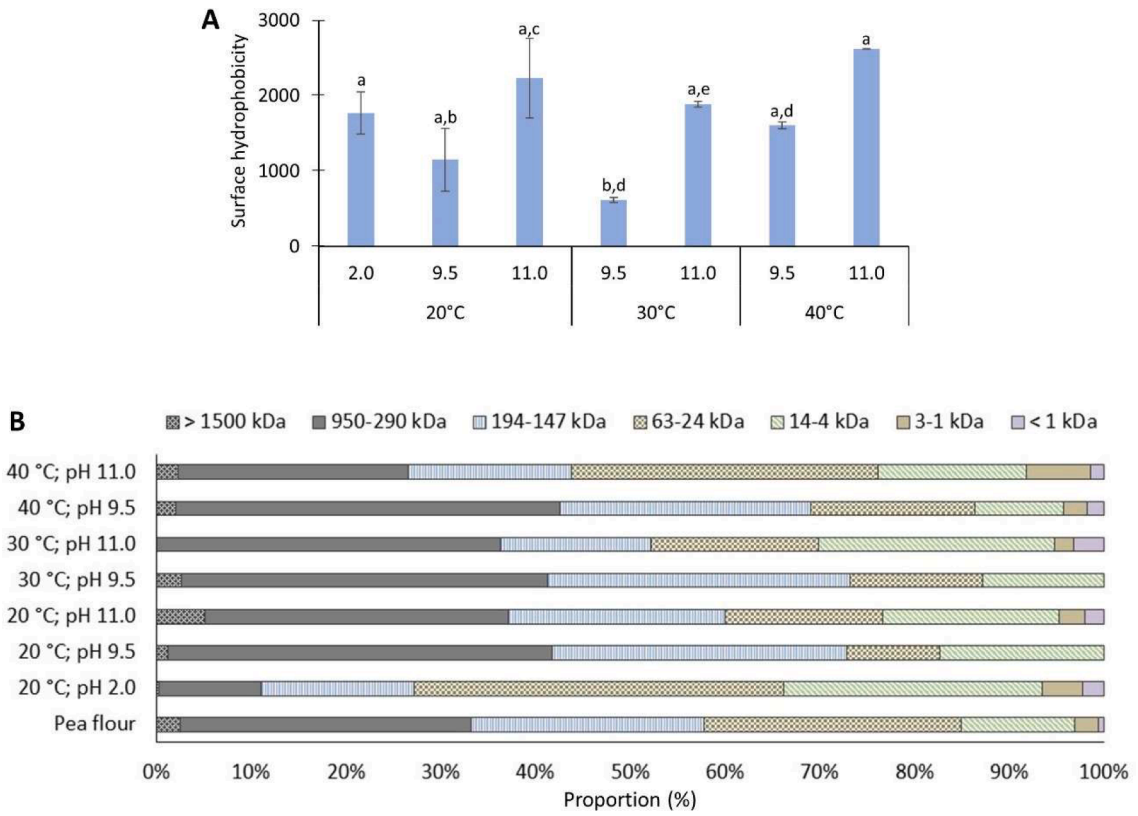

Fig. 5. Surface hydrophobicity of pea protein isolates (A), and molecular size distribution of protein isolates and the initial pea flour (B). Different lower-case letters represent significance ($p < 0.05$) differences.

foams from isolates produced at 20 °C were slightly more stable than others (Fig. 2**E**). In general, it was seen that the effect of isolation temperature surpasses the effect of solubilization pH on the functional properties of the pea protein isolates.

3.3.2. *Rheological (in situ gelation) properties of protein isolates*

The changes in rheological properties of protein isolates, i.e., storage modulus (G') and loss modulus (G''), at different temperatures over time is shown in Fig. 3. The storage modulus represents material's ability to store energy – the elastic portion, whereas the loss modulus represents

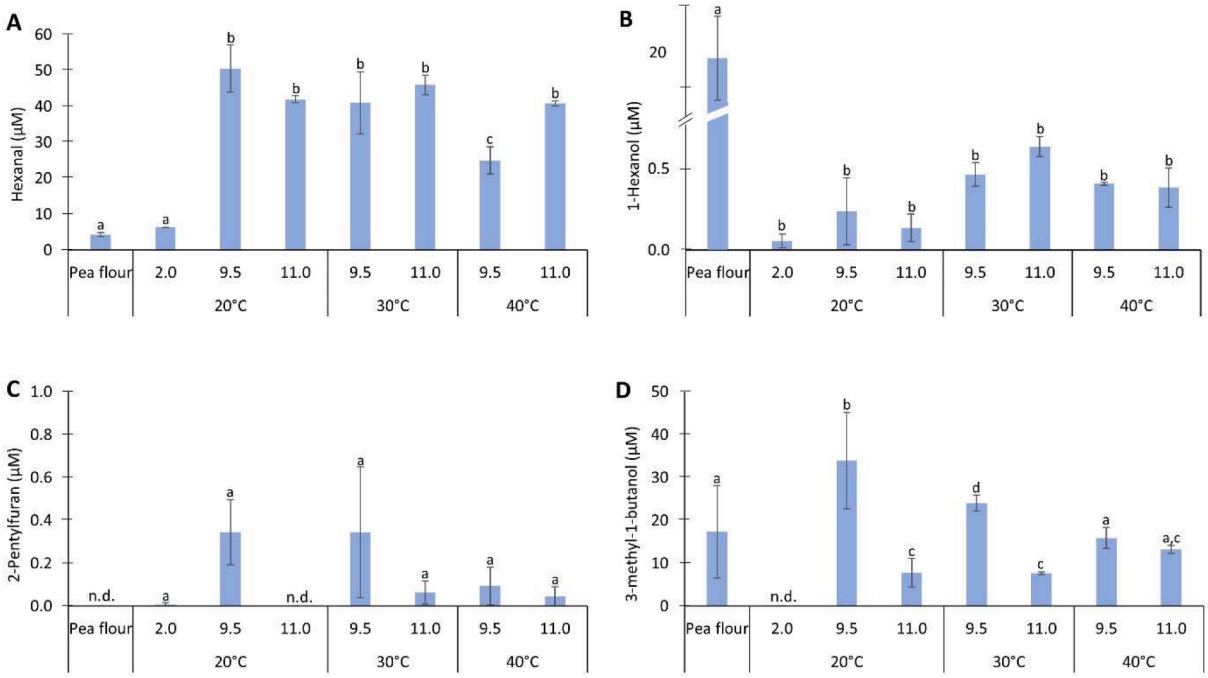

Fig. 6. Detected volatile compounds in pea protein isolates; hexanal (A), 1-hexanol (B), 2-pentylfuran (C), and 3-methyl-1-butanol (D). Different lower-case letters represent significance ($p < 0.05$) differences.

material's ability to dissipate energy as heat – the viscous portion. Overall, the gelation process started with an initial reduction in G' for all protein isolates (Fig. 3**A**), reflecting denaturation of proteins. Protein isolates produced at pH 9.5 had lower G' values than others in the beginning (i.e., 0–11 min). After an initial decrease in G', isolates produced at pH 9.5 at 20 and 30 °C showed an increase in G' from 5 to 11 min (i.e., 35–50 °C) reflecting structure formation by proteins. Further increasing the temperature to 90 °C and holding it for 30 min showed more or less similar G' values for all protein isolates, except for the isolate produced at pH 2.0 (20 °C) which had the highest G' values representing better structure formation than others. This isolate also had the highest G' value at the end of the *in situ* gelation process when the temperature was reduced from 90 to 20 °C during the cooling step. This trend was followed by the isolate produced at pH 11.0 (20 °C). Other isolates had more or less similar final G' values at the end of the cooling step. Similar trends were noticed for loss modulus G'' for all isolates (Fig. 3**B**).

3.3.3. Textural properties of gels prepared from protein isolates

Fig. 4**A** shows pictures of gels prepared from protein isolates produced at different temperature and pH. In general, gels prepared from isolates produced at pH 11.0 at all temperatures were more firm than others. This visual observation was also confirmed by textural analysis; i.e., gels prepared from isolates produced at pH 11.0 had significantly ($p < 0.05$) higher gel hardness, cohesiveness, chewiness, and gumminess than others (Fig. 4**B-F**). The recoreded higher gel hardness at pH 11.0 than at pH 2.0 at 20 °C (Fig. 4**B**) was in line with their loss modulus (G'') values (Fig. 3**B**); i.e., isolates produced at pH 2.0 had higher G'' values than at pH 11.0 showing slightly more viscous behavior than the other, which was also reflected on its slightly lower gel hardness value. There were no significant ($p > 0.05$) differences in springiness (Fig. 4**E**) and water holding capacity (Fig. 4**G**) among different gels.

3.3.4. Surface hydrophobicity and molecular size distribution

Surface hydrophobicity represents the extent to which hydrophobic amino acids, which are usually buried inside the protein molecule, are exposed to the surface of the protein by e.g., protein unfolding and denaturation (Timilsena et al., 2016). In our study we have noticed that both protein isolation temperature and pH affected surface hydrophobicity (Fig. 5**A**). Relatively higher surface hydrophobicity values were obtained at pH 11.0 than pH 9.5 at all isolation temperatures.

Molecular weight distribution of protein isolates (i.e., soluble proteins and peptides), analyzed based on major peaks identified by HP-*SEC* analysis, produced at different temperature and pH is shown in Fig. 5**B**. Compared to the starting raw material pea flour, slight changes in molecular weight distribution were noticed in the protein isolates. The largest change was noticed when pH 2.0 (20 °C) was used for protein isolation; that is, a sharp decrease in the relative amount of proteins with molecular weight of 290–950 kDa was noticed in this protein isolate compared to the pea flour. Also, protein isolates produced at pH 11.0 had a relatively lower amount of large proteins and peptides than isolates produced at pH 9.5.

3.3.5. Presence of volatile beany flavor marker compounds

Presence of four volatile beany flavor markers were noticed in the pea protein isolates (Fig. 6). Among these four markers, hexanal was predominant in all isolates, and significantly ($p < 0.05$) higher amounts of this compound were noticed in the isolates produced at pH 9.5 and 11.0 at all temperatures than pH 2.0 (20 °C) and pea flour (Fig. 6**A**). Contrary, significantly ($p < 0.05$) higher amount of 1-hexanol was noticed in the pea flour than the protein isolates (Fig. 6**B**). The marker 2-pentylfuran was not detected in pea flour and the amounts found in protein isolates were low. The other beany flavor marker 3-methyl-1-butanol was predominant in the isolates produced at pH 9.5 than pH 11.0 at all temperatures, although slightly lower amounts were noticed at elevated temperatures.

3.4. Produced meat analogues from LME and HME processing

Fig. 7 shows representative pictures of meat analogues produced from both LME and HME processing. As can be seen in Fig. 7**B**, LME processed extrudate showed fibrous muscle meat-like texture after rehydration. This extrudate had a water absorption index (WAI) of 3.5 %. HME processed extrudate also showed fibrous muscle meat-like

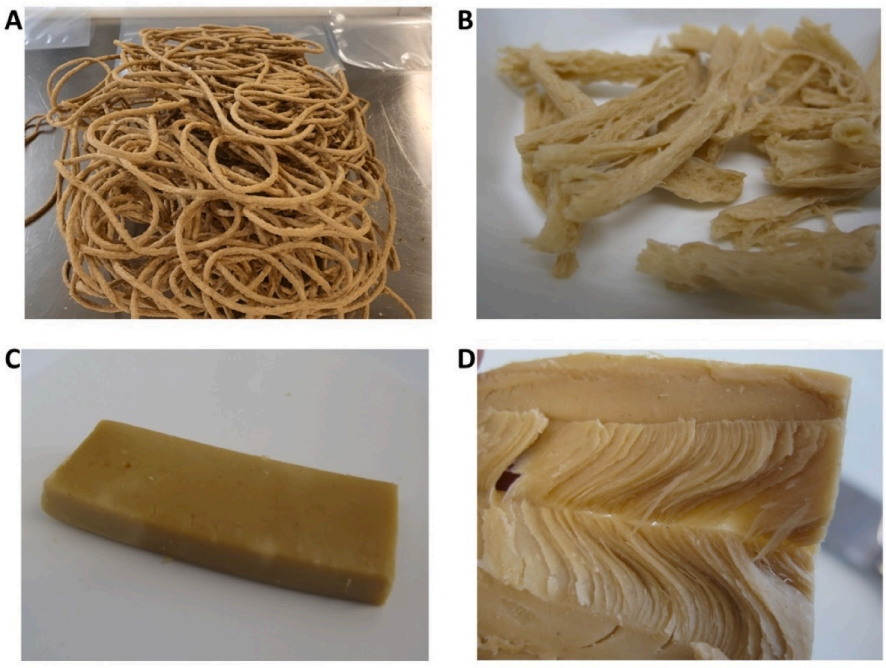

Fig. 7. Pictures of extrudates after (A) low-moisture extrusion and (C) high-moisture extrusion processing. Figure (B) show the fiber-like texture of low-moisture processed extrudate after soaking in cold water (10 °C) for 10 min, and (D) shows fiber-like anisotropic texture of high-moisture processed extrudate.

texture (Fig. 7**D**). An internal sensory analysis of LME processed extrudate showed likeliness of LME extrudate fried in oil with/without seasoning. Extrudate soaked in water and oil (i.e., step 1 as described in section 2.6) showed pleasant muscle meat-like fibrous texture and chewiness; however, the presence of off flavor and saltiness were perceived by the panelists. Extrudate fried in oil with/without seasoning (i.e., step 2 and 3) showed somehow a bit softer texture after cooking than step 1. The presence of off flavor disappeared after frying; however, the panelists still perceived the saltiness. Extrudate fried in oil with seasoning (i.e., step 3) increased the overall likelihood of acceptance of the extrudate in terms of flavor, texture, and juiciness.

4. Discussion

The main principle applied for protein recovery in this study was first to solubilize pea proteins at studied temperature and pH, separate the soluble protein fraction by centrifugation, and then precipitate proteins at their isoelectric precipitation pH (pI). So, the "solubilization" is a crucial step to ensure a satisfactory protein recovery with desired functionalities. Our results clearly showed that the isolation conditions greatly influenced protein solubility, which could possibly be due to protein denaturation and/or formation of aggregates affected by e.g., isolation pH, ionic strength, temperature (Tanger et al., 2020; Tanger, Müller, Andlinger, & Kulozik, 2022). In general, our observed highest protein solubility at alkaline pH's and the lowest solubility at pH around 4.0 was in line with previously reported study by Shand, Ya, Pietrasik, and Wanasundara (2007), where the authors used alkali isolation followed by isoelectric precipitation to investigate the physicochemical and textural properties of heat-induced pea proteins gels. Tanger et al. (2022) reported the highest solubility of pea protein at pH 9.0; however, the authors did not investigate protein solubility beyond pH 9.0. Our study shows that pea protein solubility increases even further beyond pH 9.0; the highest was at pH 12.0. The latter could possibly be due to a high ionic strength at this pH, and also due to the fact that the majority of pea proteins i.e., globulins is salt soluble (Tanger et al., 2022). And, probably for the same reason we have noticed a higher protein recovery at higher pH values e.g., at pH 11.0 than at pH 9.5 or pH 2.0 at both 20 and 30 °C.

Apart from the pH, we also noticed that the solubility was temperature dependent, indicating that proteins were denatured at varying extent at different temperatures (Tanger et al., 2020). Our observed highest protein solubility at 40 °C (pH 12.0) indicates that this isolation condition denatured proteins in a way that increased protein solubility than the other conditions used. However, the use of 40 °C temperature did not increase protein recovery that much compared to 20 and 30 °C, possibly due to changes in e.g., surface properties of proteins. It is worth mentioning that the isolation condition used may target different types of pea proteins, which will then influence protein solubility, isolation yield, and protein functionality. For example, globulin has a different isoelectric point (pI) pH than albumins (Tanger et al., 2020); since the majority of pea proteins is globulin (55 %-65 % globulin vs 18 %-25 % albumin) it is likely that the extracted proteins will mostly be globulins. And, even in case of globulins, the 11S legumin and 7S vicilin have different denaturation temperatures – i.e., 77 vs 68 °C (Tanger et al., 2022); so, they will denature at varying extent during isolation at different temperatures, which will also define their solubility. We have noticed that protein's water solubility was inversely correlated with surface hydrophobicity values; the latter indicating the extent of protein unfolding/denaturation. A decreasing water solubility trend was noticed with increased surface hydrophobicity values (see Fig. 2**B and 5A**). It is worth mentioning that protein solubility affects the gelatin behavior of protein (Tanger et al., 2022), which is an important protein functionality for use in meat analogue applications. Thus, based on our findings, the use of an isolation temperature > 30 °C is not recommended since it negatively affects both protein solubility and recovery yields. Similar to our observation, Timilsena et al. (2016) also reported an inverse relationship between surface hydrophobicity values and protein solubility of spray dried Australian chia seed protein isolate.

The functional properties of protein isolates dictate their final application in food industry. One of the most important protein functionalities is their water solubility, which largely depends on the state of protein i.e., native or denatured (Timilsena et al., 2016). The observed higher water solubility of protein isolates at alkaline pH values was expected, because the majority of pea protein is globulin which is soluble in alkaline solutions (Lu et al., 2020; McClements & Grossmann, 2021). And, all the protein isolates had their lowest water solubility at pH 5.0, which was close to their pI. This means that at pI protein molecules have the net zero charge, meaning that proteins have the lowest electrostatic repulsion enhancing protein aggregation, and thus the lowest water solubility around pI pH (Timilsena et al., 2016). As mentioned earlier that fat absorption capacity indicates proteins' ability to absorb fat, which, in case of plant protein-based meat analogue applications, suggests how much fat and/or fat-soluble flavors and other ingredients the isolates can hold. This fat absorption capacity depends on the extent of protein denaturation, i.e., the extent of hydrophobic groups exposure to the surface of the protein molecule (Timilsena et al., 2016), which was measured as surface hydrophobicity in this study. The observed higher fat absorption capacity by protein isolates produced at higher pH values, which was inversely related with water solubility, correlates well with higher surface hydrophobicity values at higher pH values, suggesting that protein denaturation was higher at pH 11.0 than at pH 9.0. Proteins with high surface hydrophobicity can easily orient themselves at the oil–water interface by placing their hydrophobic moieties towards the oil phase and the hydrophilic moieties towards the water phase (Damodaran, 2005), which is why higher fat absorption capacity was noticed from isolates with higher surface hydrophobicity values (i.e., isolates produced at higher pH values). Similar to this, an increased foaming capacity was noticed when isolates were produced at higher pH values, which is suggested to be positively correlated with surface hydrophobicity values (Kato & Nakai, 1980).

Rheological study provides a basis to understanding the heat induced gelation and how proteins will behave under varying temperatures, which is important in case of e.g., producing plant protein-based meat analogues by extrusion as it provides information about proteins' denaturation and structure formation behaviors. In such a test, the storage modules (G′) represents the elasticity of the gel network and the strength of the structure (Yang, Zamani, Liang, & Chen, 2021); i.e., the higher the G' the better the gel network and strength formation. The observed initial reduction in G' during *in-situ* gelation represents the reduction of electrostatic and hydrogen bond interactions due to increase in mobility of protein chains and thermal agitation induced by heat (Felix, Romero, Rustad, & Guerrero, 2017). The globular pea proteins denature at denaturation temperature resulting in exposure of its internal hydrophobic regions. The denaturation temperature depends on the composition of pea protein. For example, the two major protein fractions 7S and 11S of globulins have denaturation temperature of 68 and 77 °C, respectively. Thus, the ratio between 7S/11S determines the denaturation temperature, i.e., the higher the amount of 11S the higher the denaturation temperature. Upon denaturation, the unfolded protein interacts via covalent and/or non-covalent interactions, e.g., hydrogen bonds, disulfide bonds, van der Waal force, and hydrophobic interactions, to form protein aggregates giving a three-dimensional network. Here, it is important to note that a higher extent of protein aggregation may hinder protein unfolding as well as formation of intermolecular interactions. In this study, different protein isolates, isolated at different isolation conditions, behaved differently with an increase in temperature. Protein isolate produced at pH 9.5 showed a typical denaturation and structure formation behavior upon increasing the temperature from 20 to 90 °C, while proteins isolates produced at pH 11.0, especially those isolated at temperature > 20 °C, showed a reduction in G' during heating. This means that proteins isolated with a combination of high pH and high temperature were already denatured or not fully refolded due the condition used for their isolation. The

increased G' during the final cooling step is possibly due to physical interactions within the gel's primary network, e.g., hydrogen bonding, van der Waals, and hydrophobic bonds, which increased at low temperature strengthening the gel network. The hydrogen bonds play an important role here to immobilize water molecules in the protein network, which can affect the water holding capacity of gels. (Shand et al., 2007; Yang et al., 2021).

Following the rheological study, the textural analysis suggests gel formation capabilities by protein isolates, which can mimic the extrudate's properties after high moisture extrusion. A few authors previously reported that pea proteins form weak gels, which could probably be linked to their aggregate formation (Shand et al., 2007); however, our results suggest that pea protein isolates could form strong gels if isolated at pH 11.0 (20 °C). The observed higher gel hardness, cohesiveness, chewiness, and gumminess at this isolation condition were in line with rheological studies. Protein isolates produced at pH 2.0 provided slightly better texture formation than isolates produced at pH 9.5 (20 °C), probably because of its relatively higher protein content (89.46 % vs 82.10 %) and/or higher extent of protein denaturation (i.e., surface hydrophobicity values) than the one produced at pH 9.5. Similar to our observation, Ferawati et al. (2021) reported that the protein content of the raw material plays an important role in texture formation during HME processing of yellow pea and fava bean protein isolate/concentrate for meat analogue applications. In addition to that, we would like to stress that the structural properties of proteins e.g., surface hydrophobicity also play an important role in texture formation by contributing to formation of hydrophobic interactions during the cooling step.

The observed relatively lower amount of high molecular weight proteins and peptides in isolates produced at pH 11.0 than pH 9.5 corresponds well with proteins' water solubility at pH 7.0 (see supporting info. Fig. 3), where relatively higher water solubility was noticed in isolates produced at pH 9.5 than at pH 11.0. One plausible explanation for having relatively lower amount of soluble proteins and peptides in isolates produced at pH 11.0 than at pH 9.5 could be that the HP-*SEC* method used in this study analyzed proteins soluble in the mobile phase (i.e., 0.1 M sodium phosphate; pH 7.0); that is, isolates produced at pH 9.5 were more soluble in the HP-*SEC* mobile phase, than isolates produced at pH 11.0, giving a relatively higher proportions of proteins and peptides. Contrary, as discussed earlier, the observed relatively higher surface hydrophobicity values in isolates produced at pH 11.0 than at pH 9.5 suggests that proteins were slightly more denatured when pH 11.0 was used for isolation. This was in accordance with observed lower water solubility of proteins. However, as mentioned earlier, this slight protein denaturation probably helped in gaining a better texture formation, fat absorption, and foaming capacity than the rest, and can be considered as desirable. Nevertheless, the hexamer conformation of legumin (11S) has a molecular weight of 360 kDa, so the observed proteins in the molecular weight > 360 kDa could possibly indicate aggregation of proteins, which was noticed in all produced isolates at varying extent. Isolates produced at pH 2.0 (20 °C) had the lowest amount of proteins in the molecular weight range of 290–950 kDa, which could possibly be due to less aggregation and/or splitting of legumin into its subunits. The latter was evident by its relatively higher amount of proteins in the molecular weight range of 24–63 kDa, suggesting that legumin was split to its subunits of 60 kDa, as well as further split to its α- and β-chains of 40 and 20 kDa, respectively (Tanger et al., 2020). The trimeric vicilin (7S) has a molecular weight of 150 kDa, so the observed proteins in the range of 147–194 kDa could possibly represent vicilin. The splitting of vicilin into its subunits of 50 kDa each could also happen, which possibly elapsed together with legumin subunits in the molecular weight range of 24–63 kDa. There were no visible peaks in the HP-*SEC* chromatograph representing the 280 kDa-sized tetrameric convicilin; however, this could have been split to its subunits and coeluted with others. The observed proteins in the molecular weight range of 4–14 kDa could represent albumins (Tanger et al., 2020). Overall, our HP-*SEC* analysis suggests that all protein isolates were subjected to both aggregation and splitting to their subunits, but at varying extent depending on isolation condition used.

One of the most faced challenges with plant-based proteins for meat analogue applications is the beany flavor, coming from beany flavor compounds, which are either produced naturally in peas or are the products of secondary and/or tertiary lipid oxidation (Gao et al., 2020; Xu et al., 2019). Regarding the latter, secondary lipid oxidation product hexanal can be produced from the oxidation of n-6 polyunsaturated fatty acids like linoleic acid (18:2n-6) and/or from degradation of other preformed secondary lipid oxidation products like (*E,E*)-2,4-decadienal. Tertiary lipid oxidation product 2-pentylfuran can be generated from the interaction between preformed α,β-unsaturated secondary lipid oxidation products and amino acids/peptides/proteins. (Sajib & Undeland, 2020) Detailed discussion on lipid oxidation-derived volatile compounds can be found elsewhere (Sajib & Undeland, 2020), and will not be discussed further. We have observed the presence of four beany flavor marker compounds in protein isolates and in the pea flour, with hexanal, 1-hexanol, and 3-methyl-1-butanol being the predominant ones. Hexanal provides a grassy and green pea-like flavor, 1-hexanol provides grassy and greenish lemon-like flavor, and 3-methyl-1-butanol provides whiskey and fruity banana-like flavor (Xu et al., 2019). The other beany flavor maker 2-pentylfuran provides green bean-like flavor.

The observed significantly ($p < 0.05$) higher level of secondary lipid oxidation product 1-hexanol in pea flour, compared to protein isolates, could possibly be due to different oxidation kinetics of e.g., linoleic acid present in pea flour (Xu et al., 2019). During protein isolation process this 1-hexanol probably either degraded or reacted with pea proteins/peptides/amino acids to form e.g., non-enzymatic browning reaction products, and thus significantly ($p < 0.05$) lower levels were detected in pea protein isolates than the starting raw material pea flour. Contrary, the observed higher levels of hexanal in protein isolates than the pea flour could possibly be due to different oxidation kinetics involved behind the formation of hexanal. For example, hexanal can be formed due to lipoxygenase (LOX) activity, oxidation of n-6 polyunsaturated fatty acids like linoleic acid (18:2n-6) and arachidonic acid (20:4n6), and degradation of other preformed volatile compounds like 2-octenal and 2,4-decadienal (Sajib & Undeland, 2020). Gao et al. (2020) reported similar levels of hexanal both in pea flour and protein isolate produced at pH 9.5, which was not the case in our study. This observed difference could probably be due to varying extent of different reaction pathways taking place both in the starting pea flour and during protein isolation process, as well as during storage of protein isolates. The well-known prooxidant LOX could promote lipid oxidation at varying extent; for example, Gao et al. (2020) mentioned that protein isolation pH can affect LOX activity influencing lipid oxidation at varying extent and thus the formation of beany flavor compounds. Although Szymanowska, Jakubczyk, Baraniak, and Kur (2009) reported the highest activity of LOX enzymes isolated from pea seeds at pH 5.5, the optimal activity pH varies greatly depending on the source (Gao et al., 2020; Hsieh, German, & Kinsella, 1988; Szymanowska et al., 2009). Even though the direct link between LOX activity and volatile beany flavor compound formation was not investigated in our study, based on the findings from Gao et al. (2020), we can speculate that protein isolation at higher pH values e.g., at pH 11.0 (20 °C) was less affected by LOX activity and thus resulted in slightly lower levels of e.g., hexanal and 3-methyl-1-butanol formations. It has also been reported that LOX activity is inhibited at temperatures above 40 °C (Hsieh et al., 1988), which possibly partially supports a relatively lower levels of beany flavor marker formation when a temperature > 20 °C was used for protein isolation. Apart from the reduced LOX activity at temperatures > 20 °C, it is also possible that the preformed secondary lipid oxidation products can be degraded and/or interacted with proteins/peptides/amino acids at temperatures > 20 °C (Sajib & Undeland, 2020) giving slightly lower values, which requires further investigation. Here it is important to note that the formation of volatile compounds can occur via several reaction pathways as explained earlier. So, careful attention is required while explaining the

reason of volatile compound formation in a complex food matrix like pea protein isolate. Apart from the unwanted beany flavor, generation of such oxidation products may reduce nutritional value (Sajib & Undeland, 2020), and chronic uptake of such food ingredients/products may represent a health risk e.g., increased risk of tumor development (Esterbauer, 1993). Therefore, it is important to minimize such unwanted reactions; examples of such are solid dispersion-based spray-drying and/or addition of natural antioxidants, which could be the subject of a separate study.

Based on the results of this study, i.e., primarily protein recovery yield and texture formation, which are the main concerns for commercial application of pea protein isolates for meat analogue applications, pH 11.0 and 20 °C temperature were used to produce isolates for both LME and HME trials. The produced isolate performed well in both LME and HME applications and the extrudates showed fibrous muscle meat-like texture (Fig. 7). In general, the production of extrudates with desirable texture from both LME and HME trials suggest that the investigation of this current study helped in finding suitable protein isolation process settings and thus produced a protein isolate with desired properties for meat analogue applications. However, internal sensory analysis of LME processed extrudate was perceived somehow salty by the panelists. One possible reason for this perceived saltiness could be the use of a relatively high alkaline pH 11.0, which required more NaOH and HCl for pH adjustments and thus possibly formed more salt (e.g., NaCl) than pH 9.5 (see supporting info. Table 1). The presence of relatively higher sodium (Na) content was detected in protein isolates produced at pH 11.0 than at pH 9.5 (see supporting info. Fig. 6), which partially explains this perceived saltiness. However, NaCl is water soluble, and we expect it to remain soluble in the solution during both protein isolation and precipitation steps, and thus it is not expected to end up in the protein isolate. Contrary, the starting raw material pea flour and protein isolates produced at pH 2.0 had higher sodium contents than isolates produced at both pH 9.5 and 11.0. Possible reasons for having a relatively high sodium content in protein isolates produced at pH 2.0 could be that acidic condition resulted in the extraction of a larger portion of sodium already present in the starting raw material, or proteins' nucleophilic groups e.g., carboxylic acid groups reacted with sodium to form sodium carboxylate. Apart from saltiness, panelists also perceived some off flavor in produced meat analogue. In our HS-SPME-GC–MS analysis, we did detect the presence of a few volatile beany flavor markers like hexanal, 1-hexanol, 2-pentylfuran, and 3-methyl-1-butanol as discussed earlier, which probably explains this perceived off flavor by the panelists. However, the fact that this perceived off flavor disappeared after frying in oil with/without seasoning suggests that further processing and/or addition of off flavor masker is needed to increase the likeliness of pea protein-based meat analogues. Nevertheless, the perceived off flavor of plant protein-based isolates and meat analogues produced thereof is still an outstanding challenge, which the food industry usually solves by adding off flavor/taste masker, and thus requires thorough investigation to further increase the overall likeliness of plant protein-based meat analogues.

This study also opened up a few possible topics for future investigation, e.g., sodium content of produced pea protein isolates. The presence of sodium was related to the total volumes of NaOH and HCl used during protein isolation except for the one produced at pH 2.0 (see supporting info. Table 1 and Fig. 6), which may contribute to perceived saltiness when consumed and thus require further investigation on e.g., understanding the mechanism of sodium formation and/or reduction of sodium content during processing. Also, the isolates produced at alkaline pH values (i.e., pH 9.5 and 11.0) had slightly dark brownish color than the one produced at acidic pH 2.0 (see Fig. 2**A**), and the reasons of such including/excluding lipid oxidation and/or pyrrole formation (Sajib & Undeland, 2020) can be investigated further as the color of ingredients partly dictates the quality and consumer acceptance. Nevertheless, the results of this study provide an understanding of the effects of pea protein isolation process conditions on functional properties of the isolates, and can be used in producing a range of pea protein isolates with different functionalities for different types of applications by tuning the process conditions. It is foreseen that pea protein isolates can not only be used as meat analogues, but its usage can also be explored in other areas e.g., egg analogues, cheese analogues, and fish analogues (McClements et al., 2021).

5. Conclusion

Combined effects of isolation temperature and pH on functional, rheological, textural, structural, and beany flavor properties of pea protein isolates have here been reported for the first time. Both pH and temperature affected the above-mentioned properties at varying extent. For example, the use of pH 11.0 gave better protein recovery than pH 9.5, probably because the majority of pea proteins i.e., globulins were more soluble at this pH facilitating a higher protein recovery yield. Also, at this pH proteins were probably more unfolded and denatured than pH 9.5, providing a better structure formation as shown with gel formation capacity, which could be suitable for meat analogue applications too. Protein isolated at pH 11.0 provided better fat absorption capacity, gel hardness, cohesiveness, chewiness, and gumminess than isolation at pH 9.5. Protein isolation under acidic conditions (at pH 2.0) did not provide a good protein recovery, probably because pea proteins were not soluble that much at this pH than alkaline conditions. However, the gels prepared from this provided slightly better textural properties than isolation at pH 9.5, as well as produced less volatile beany flavor compounds than the rest. Therefore, it can be investigated further for possible improvements in recovery yields and/or other application areas. All isolation conditions, except pH 2.0, generated significantly higher amount of volatile beany flavor marker hexanal than the starting material pea flour, and probably will require addition of masking agents during further processing like extrusion to cover this undesired beany flavor. The use of a higher isolation temperature e.g., 30 and 40 °C did not provide better techno-functional properties than 20 °C and are not recommended. It is also possible to use pH 9.5, which will require slightly less NaOH and HCl than pH 11.0, for protein isolation if the final application requires slightly less firm textural properties than the one we reported here for isolation at pH 11.0. Overall, the results of this study provide a basis for tuning of the isolation process for producing pea protein isolations with desired functionalities for a wide range of applications.

CRediT authorship contribution statement

Mursalin Sajib: Data curation, Investigation, Methodology, Visualization, Writing – original draft, Conceptualization, Funding acquisition, Project administration. **Bita Forghani:** Data curation, Methodology, Writing – original draft. **Naveen Kumar Vate:** Data curation, Writing – original draft. **Mehdi Abdollahi:** Supervision, Conceptualization, Writing – review & editing, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.135585.

References

Abdollahi, M., Rezaei, M., Jafarpour, A., & Undeland, I. (2018). Sequential extraction of gel-forming proteins, collagen and collagen hydrolysate from gutted silver carp (Hypophthalmichthys molitrix), a biorefinery approach. *Food Chemistry, 242*, 568–578.

Abdollahi, M., & Undeland, I. (2018). Structural, functional, and sensorial properties of protein isolate produced from salmon, cod, and herring by-products. *Food and Bioprocess Technology, 11*(9), 1733–1749.

Abdollahi, M., & Undeland, I. (2019). Physicochemical and gel-forming properties of protein isolated from salmon, cod and herring by-products using the pH-shift method. *LWT, 101*, 678–684.

Cardoso, C., Mendes, R., Vaz-Pires, P., & Nunes, M. L. (2009). Effect of dietary fibre and MTGase on the quality of mackerel surimi gels. *Journal of the Science of Food and Agriculture, 89*(10), 1648–1658.

Damodaran, S. (2005). Protein stabilization of emulsions and foams. *Journal of Food Science, 70*(3), R54–R66.

Day, L. (2013). Proteins from land plants–potential resources for human nutrition and food security. *Trends in Food Science & Technology, 32*(1), 25–42.

de Oliveira, A. P. H., Omura, M. H., Barbosa, É. d. A. A., Bressan, G. C., Vieira, É. N. R., Coimbra, J. S. d. R., & de Oliveira, E. B. (2020). Combined adjustment of pH and ultrasound treatments modify techno-functionalities of pea protein concentrates. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 603*, 125156. 10.1016/j.colsurfa.2020.125156.

Esterbauer, H. (1993). Cytotoxicity and genotoxicity of lipid-oxidation products. *The American Journal of Clinical Nutrition, 57*(5), 779S-786S.

Felix, M., Romero, A., Rustad, T., & Guerrero, A. (2017). Physicochemical, microstructure and bioactive characterization of gels made from crayfish protein. *Food Hydrocolloids, 63*, 429–436.

Ferawati, F., Witthöft, C., & Bergström, M. (2020). Characterization of volatile compounds in Swedish yellow and gray peas: Implications for new legume-based ingredients. *Legume Science, 2*(4), e55.

Ferawati, F., Zahari, I., Barman, M., Hefni, M., Ahlström, C., Witthöft, C., & Östbring, K. (2021). High-moisture meat analogues produced from yellow pea and faba bean protein isolates/concentrate: Effect of raw material composition and extrusion parameters on texture properties. *Foods, 10*(4), 843.

Gao, Z., Shen, P., Lan, Y., Cui, L., Ohm, J.-B., Chen, B., & Rao, J. (2020). Effect of alkaline extraction pH on structure properties, solubility, and beany flavor of yellow pea protein isolate. *Food Research International, 131*, Article 109045.

Gmoser, R., Fristedt, R., Larsson, K., Undeland, I., Taherzadeh, M. J., & Lennartsson, P. R. (2020). From stale bread and brewers spent grain to a new food source using edible filamentous fungi. *Bioengineered, 11*(1), 582–598.

Hsieh, R. J., German, J. B., & Kinsella, J. E. (1988). Lipoxygenase in fish tissue: Some properties of the 12-lipoxygenase from trout gill. *Journal of Agricultural and Food Chemistry, 36*(4), 680–685.

Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimica et biophysica acta (BBA)-Protein structure, 624*(1), 13–20.

Kyriakopoulou, K., Dekkers, B., & van der Goot, A. J. (2019). Chapter 6 - Plant-Based Meat Analogues. In C. M. Galanakis (Ed.), *Sustainable Meat Production and Processing* (pp. 103–126). Academic Press.

Lam, A., Can Karaca, A., Tyler, R., & Nickerson, M. (2018). Pea protein isolates: Structure, extraction, and functionality. *Food Reviews International, 34*(2), 126–147.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*(1), 265–275.

Lu, Z., He, J., Zhang, Y., & Bing, D. (2020). Composition, physicochemical properties of pea protein and its application in functional foods. *Critical Reviews in Food Science and Nutrition, 60*(15), 2593–2605.

Mariotti, F., Tomé, D., & Mirand, P. P. (2008). Converting nitrogen into protein—beyond 6.25 and Jones' factors. *Critical Reviews in Food Science and Nutrition, 48*(2), 177–184.

MarketsandMarkets. (2019). Plant-based Meat Market by Source (Soy, Wheat, Pea, Quinoa, Oats, Beans, Nuts), Product (Burger Patties, Sausages, Strips & Nuggets, Meatballs), Type (Pork, Beef, Chicken, Fish), Process, and Region - Global Forecast to 2025. https://www.marketsandmarkets.com/Market-Reports/plant-based-meat-market-44922705.html.

McClements, D. J., & Grossmann, L. (2021). The science of plant-based foods: Constructing next-generation meat, fish, milk, and egg analogs. *Comprehensive Reviews in Food Science and Food Safety*.

Monahan, F. J., German, J. B., & Kinsella, J. E. (1995). Effect of pH and temperature on protein unfolding and thiol/disulfide interchange reactions during heat-induced gelation of whey proteins. *Journal of Agricultural and Food Chemistry, 43*(1), 46–52.

Munz, K. (2021). *Protein Workshop - Water Absorption Index*. Bühler AG.

Nieto-Nieto, T. V., Wang, Y. X., Ozimek, L., & Chen, L. (2015). Inulin at low concentrations significantly improves the gelling properties of oat protein–A molecular mechanism study. *Food Hydrocolloids, 50*, 116–127.

Peng, Y., Kersten, N., Kyriakopoulou, K., & van der Goot, A. J. (2020). Functional properties of mildly fractionated soy protein as influenced by the processing pH. *Journal of Food Engineering, 275*, Article 109875.

Saget, S., Costa, M. P., Santos, C. S., Vasconcelos, M., Styles, D., & Williams, M. (2021). Comparative life cycle assessment of plant and beef-based patties, including carbon opportunity costs. *Sustainable Production and Consumption, 28*, 936–952.

Sajib, M., Albers, E., Langeland, M., & Undeland, I. (2020). Understanding the effect of temperature and time on protein degree of hydrolysis and lipid oxidation during ensilaging of herring (Clupea harengus) filleting co-products. *Scientific Reports, 10*(1), 9590. https://doi.org/10.1038/s41598-020-66152-0

Sajib, M., & Undeland, I. (2020). Towards valorization of herring filleting by-products to silage 2.0: Effect of temperature and time on lipid oxidation and non-enzymatic browning reactions. *LWT - Food Science and Technology, 109441*. http://www.sciencedirect.com/science/article/pii/S0023643820304308.

Sathivel, S., Yin, H., Bechtel, P. J., & King, J. M. (2009). Physical and nutritional properties of catfish roe spray dried protein powder and its application in an emulsion system. *Journal of Food Engineering, 95*(1), 76–81.

Sha, L., & Xiong, Y. L. (2020). Plant protein-based alternatives of reconstructed meat: Science, technology, and challenges. *Trends in Food Science & Technology, 102*, 51–61.

Shand, P. J., Ya, H., Pietrasik, Z., & Wanasundara, P. K. J. P. D. (2007). Physicochemical and textural properties of heat-induced pea protein isolate gels. *Food Chemistry, 102*(4), 1119–1130. https://doi.org/10.1016/j.foodchem.2006.06.060

Szymanowska, U., Jakubczyk, A., Baraniak, B., & Kur, A. (2009). Characterisation of lipoxygenase from pea seeds (Pisum sativum var. Telephone L.). *Food Chemistry, 116*(4), 906–910.

Tanger, C., Engel, J., & Kulozik, U. (2020). Influence of extraction conditions on the conformational alteration of pea protein extracted from pea flour. *Food Hydrocolloids, 107*, Article 105949. https://doi.org/10.1016/j.foodhyd.2020.105949

Tanger, C., Müller, M., Andlinger, D., & Kulozik, U. (2022). Influence of pH and ionic strength on the thermal gelation behaviour of pea protein. *Food Hydrocolloids, 123*, Article 106903. https://doi.org/10.1016/j.foodhyd.2021.106903

Tidåker, P., Potter, H. K., Carlsson, G., & Röös, E. (2021). Towards sustainable consumption of legumes: How origin, processing and transport affect the environmental impact of pulses. *Sustainable Production and Consumption, 27*, 496–508.

Timilsena, Y. P., Adhikari, R., Barrow, C. J., & Adhikari, B. (2016). Physicochemical and functional properties of protein isolate produced from Australian chia seeds. *Food Chemistry, 212*, 648–656.

Xu, M., Jin, Z., Lan, Y., Rao, J., & Chen, B. (2019). HS-SPME-GC-MS/olfactometry combined with chemometrics to assess the impact of germination on flavor attributes of chickpea, lentil, and yellow pea flours. *Food Chemistry, 280*, 83–95.

Yang, J., Zamani, S., Liang, L., & Chen, L. (2021). Extraction methods significantly impact pea protein composition, structure and gelling properties. *Food Hydrocolloids, 117*, Article 106678. https://doi.org/10.1016/j.foodhyd.2021.106678

Zhang, J., Liu, L., Liu, H., Yoon, A., Rizvi, S. S., & Wang, Q. (2019). Changes in conformation and quality of vegetable protein during texturization process by extrusion. *Critical Reviews in Food Science and Nutrition, 59*(20), 3267–3280.