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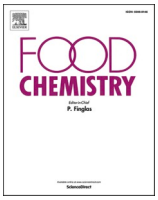
Food Chemistry 506 (2026) 148188

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Radio frequency-assisted mitigation of off-flavor formation in pea protein isolate

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ARTICLE INFO

Keywords:
Plant-based protein
Radio frequency
Lipid oxidation
Lipoxygenase enzyme
Off-flavor

ABSTRACT

The efficiency of radio frequency (RF) seed pretreatment to mitigate lipid oxidation-related beany volatile compounds during pea protein isolation via wet fractionation and its underlying mechanism were evaluated. RF treatments above 110 °C for >20 min fully inactivated lipoxygenase (LOX) but caused up to 50% loss in protein yield. By optimizing sample thickness, electrode distance, and heating duration, RF heating achieved >90% LOX inactivation below 100 °C while preserving protein yield, outperforming conventional heating. Moderate RF treatment (~75 °C) reduced LOX activity by 80% while retaining intrinsic phenolic compounds, which stabilized EPR-detectable free radicals and promoted the accumulation of hydroxyoctadecadienoic acids (HODE-13) rather than volatile off-flavor compounds. In contrast, more intense RF treatments (~85–95 °C) degraded intrinsic antioxidants and accelerated lipid breakdown to hydroperoxides and volatile off-flavor compounds. Overall, optimized RF pretreatment can effectively limit lipid oxidation and off-flavor formation during pea protein isolation by combining LOX inactivation with phenolic-mediated radical stabilization, without compromising protein yield.

1. Introduction

There is a growing interest in plant-based proteins as sustainable alternatives. Among these, peas stand out as a leading commercial protein source with their high yield, protein quality, wide availability, and low production cost (Schutyser et al., 2015). Despite these advantages, the widespread adoption of pea protein in food products is hindered by persistent off-flavors, often described as “beany,” “green,” “grassy,” “earthy,” and “mushroom-like” (Utz et al., 2022). These undesirable flavors arise from a complex mixture of volatile compounds, including aldehydes (notably hexanal), alcohols, ketones, furans, and alkyl methoxypyrazines. Some of these compounds are naturally present in peas, while most of them are produced by the oxidation of unsaturated fatty acids (USFAs), such as linoleic and linolenic acids, initiated or intensified during harvesting, storage and processing. Recent studies have shown that formation of lipid oxidation-related off-flavor compounds during the protein extraction via wet fractionation can result in their accumulation increasing by 4- to 100-fold in the pea protein isolate (Gultekin Subasi et al., 2024; Manouel et al., 2024).

Enzymatically initiated lipid oxidation by lipoxygenase (LOX), which catalyzes the degradation of USFAs into lipid hydroperoxides that subsequently break down into a wide range of volatile compounds, such as hexanal, is widely recognized as the major driver of off-flavor formation in plant proteins (Trikusuma et al., 2020; Utz et al., 2022). LOX is a non-heme iron-containing oxygenase that specifically catalyzes the formation of hydroperoxides with conjugated double bonds from USFAs containing cis, cis-1,4-pentadiene moiety and their corresponding lipids (Borowski & Brocławik, 2003).

Considering the heat sensitivity of LOX as a protein, thermal treatments, both wet and dry, such as steam blanching with or hot air roasting, are traditionally employed to mitigate its contribution to off-flavor formation in legumes (Chong et al., 2019). In solution or extracted form, most plant-derived LOX enzymes (e.g., from soy or pea) undergo irreversible denaturation at temperatures above ~60–70 °C, with complete inactivation typically achieved at ~70–90 °C within 10–15 min, depending on legume variety (Liburdi et al., 2021). However, when

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https://doi.org/10.1016/j.foodchem.2026.148188
Received 15 October 2025; Received in revised form 1 January 2026; Accepted 25 January 2026
Available online 27 January 2026

LOX is embedded within intact seed matrices, the structural integrity and protective effects of the matrix often require more intense heat treatments, typically above 100 °C for very long time, to achieve effective inactivation, especially when using conventional heating methods (B. Wang et al., 2021) Despite the positive effect reported for such treatments in reducing beany flavor formation and perception in seeds, their high temperatures and prolonged exposure can compromise product quality by causing uneven heating, extensive protein denaturation, reducing protein solubility and loss of nutritional value (B. Wang et al., 2021). This situation presents a particular challenge when the seeds are intended for subsequent protein extraction via wet fractionation, where maintaining high protein solubility is essential for the technical and economic viability of the process. Therefore, novel heating methods based on irradiation with higher efficiency have attracted increasing attention. Microwave and radio frequency (RF) heating have been adopted for industrial processing, and among these RF has the higher penetration depth with more uniform heating and easier process control advantage due to its longer wavelength and lower frequency.

RF processing quickly generates heat within the samples with dipolar heating and ionic conduction while the latter one is more pronounced compared to the microwave heating. This allows RF to efficiently deliver thermal energy through bulk food products, helping to inactivate natural enzymes (Hou et al., 2016; Manzocco et al., 2008).

RF heating has demonstrated its effectiveness in inactivating LOX in various food matrices, including soybeans (Jiang et al., 2018) and green peas (Zhang et al., 2021), while reducing heating time and preserving product quality (Tang et al., 2004). For example, Jiang et al. (2018a) reported a 94% LOX inactivation rate in a small batch (~30 g) of soybeans after RF treatment for 210 s, which effectively reduced hexanal content in the seeds. A major challenge in applying the RF still lies in achieving a uniform temperature distribution and avoiding localized overheating, especially at the edges and corners of the products in larger (e.g., industrial scale) scale processing., (Ling et al., 2020). Overheating and temperature non-uniformity can not only reduce protein extraction efficiency from the seeds but also impose the risk of autoxidation of USFAs through free radical chain reactions in the presence of oxygen or light due to the presence of lower temperature regions and lack of inactivation efficiency. This provides a non-enzymatic pathway for off-flavor formation during protein extraction, even when LOX has been inactivated (Trindler et al., 2022). To the best of our knowledge, no studies have evaluated the effectiveness of RF treatment as a pretreatment for LOX inactivation in yellow peas, nor its impact on protein extraction efficiency during wet fractionation, or its potential to reduce lipid oxidation and off-flavor formation during protein extraction. Successful implementation of this approach after harvesting pea seed could potentially enhance their stability during storage but also mitigate off-flavor development during protein extraction and subsequent steps of the value chain, without compromising protein yield or quality.

The present study aimed to investigate: (1) the effect of RF treatment of pea seeds, for LOX inactivation and following protein extraction efficiency using the alkaline solubilization and isoelectric precipitation; (2) whether temperature distribution (uniformity) during RF treatment can be optimized to effectively inactivate LOX without compromising protein extraction efficiency; and (3) whether LOX inactivation in pea seeds can mitigate subsequent lipid oxidation and off-flavor formation in the resulting protein isolate. To address these objectives, the study was designed as a sequential, integrated workflow. First, RF pretreatment was evaluated as a strategy to reduce lipoxygenase activity in pea flour while maintaining protein recovery during wet extraction. Based on this initial assessment, RF processing conditions were optimized to achieve effective enzyme inactivation without compromising extractability. Subsequently, the impact of RF pretreatment on lipid oxidation and off-flavor formation during alkaline solubilization and isoelectric precipitation was investigated and benchmarked against conventional thermal treatment. Finally, complementary analyses were conducted to examine potential side effects of RF pretreatment on oxidative pathways in the resulting protein isolates, providing mechanistic insight into the interplay between enzymatic and non-enzymatic oxidation processes throughout extraction.

2. Materials and methods

2.1. Materials

Dry field pea (*Pisum sativum* L.) seeds from the Ingrid variety were harvested and further dried to 14% relative humidity using hot air at 30 °C, filled into paper bags, and then stored under farmhouse storage (4 °C) following the harvest by Lantmannen Lantbruk, Sweden, before transferring to the facilities of Chalmers University of Technology in 2023. Immediately after receiving each sample (around 1 kg, without vacuum) in sealed plastic bags at the division laboratory, they were placed at −80 °C in dark conditions until further use.

2.2. Radio frequency (RF) treatment of pea seeds

2.2.1. Evaluating the effect of RF treatment on LOX activity and protein yield

2.2.1.1. Pretrials to find suitable RF heating conditions for pea seeds. A preliminary trial was conducted to determine the suitable RF processing conditions, in terms of power level, electrode distance, and treatment time, capable of heating pea seeds to the temperature range of 70–120 °C, which is the reported range for irreversible LOX inactivation. Results from this preliminary trial are not presented here. Next, the most effective identified treatments were repeated, and the seeds were used for measuring protein extraction yield using alkaline solubilization and isoelectric precipitation as explained in Section 2.4.

RF treatment of pea seeds was conducted using a pilot scale free-oscillator 10 kW 27.12 MHz staggered through-field electrode system (Sonar Machinery, Izmir, Turkey) equipped with a conveyor belt (25 mm above the bottom potential applied electrode). The electrode gap of the RF system could be adjusted from 8 to 16 cm using the movable top ground electrode (Altin et al., 2023). The RF system had 4 adjustable power levels, 1 at the lowest and 4 at the highest. While power level 1 leads to longer processing due to the lower potentials along the charged electrode, power level 4 resulted in rather higher potentials with significant temperature non-uniformities for the processing of pea seeds. Pretrials showed that applying power level 2 resulted in a potential of 4000 V at the charged bottom electrode at 10 cm electrode gap, 5000 V at 15 cm electrode gap while at applied power level 3, the potential of the charged bottom electrode was 5000 and 5800 V at 8 and 10 cm electrode gaps. Based on the pre-trials, power levels of 2 and 3 were determined to be the optimum process conditions to apply in the RF heating experiments. The experiments were then performed at the electrode gap of 8 cm at power level 3 and electrode gaps of 10 cm and 15 cm at power level 2.

2.2.1.2. RF treatment process. To apply RF heating, each batch of 1 kg yellow pea seeds at 6.5 cm thickness was placed in a rectangular polypropylene (PP), transparent to the RF energy, container (20 cm × 13.8 cm × 6.5 cm). The sample thickness was equalized with the height of the container for better reproducibility and homogeneity. During the process, the PP container was placed on top of the bottom charged electrode over the conveyor belt within the cavity in the middle of two parallel electrodes. Based on the pretrials, treatments were conducted under three conditions: (i) 10 cm electrode gap and heating for 20 min or (ii) 10 cm electrode gap and heating for 30 min and (iii) 15 cm electrode gap and heating for 30 min. The temperature distribution of the yellow pea seeds was monitored using a handheld infrared thermal imaging camera (Fluke VT04 Visual IR Thermometer, Fluke Corp., USA), which captured full thermal images. Thermal images were taken from the top and

bottom layers of the pea seed bed after RF heating. The bottom layer of the sample mass is located close to the geometric mid-layer relative to the electrode configuration and therefore serves as a representative internal region where temperature non-uniformity is most likely to occur. Immediately after each treatment, the samples were removed from the RF cavity, spread in a thin layer and cooled to room temperature. Untreated samples were considered as the control. Treated and control seeds were then sealed in zip-lock bags, frozen and stored at −80 °C for LOX activity measurement and protein extraction. Each treatment was performed in duplicate. The results from this trial defined the treatments for the subsequent further optimization in Section 2.2.2.

2.2.2. Optimizing RF conditions for maximum yield and mitigating off-flavor formation

To mitigate the negative effect of RF heating on protein yield loss and to evaluate its impact on off-flavor formation in pea protein isolate, RF processing parameters, including sample thickness, electrode gap, and heating time, were further optimized to achieve milder, more homogeneous, and faster heating. At this step, two sample thicknesses were tested:

Thick layer setup: as in the previous step, 1 kg of yellow peas was placed in the same rectangular polypropylene container (20 cm × 13.8 cm × 6.5 cm) with a layer thickness of approximately 6.5 cm. Considering the results from the previous trial, for this setup, the treatment at an electrode gap of 10 cm was reduced to 10 min and the treatment with 15 cm gap for 30 min was repeated.

Thin layer setup: 750 g of yellow peas were spread to a thickness of approximately 1.2 cm in a rectangular polypropylene tray (27.045 cm × 20.036 cm × 1.2 cm). The tray was positioned between the parallel electrodes set to an 8 cm gap in the RF equipment, and samples were heated for 15, 30, or 45 min.

After all RF treatments, seed temperature distribution was measured at both top and bottom surfaces of the sample mass using a visual infrared thermometer (Fluke Electronic Instrumentation Co., Ltd., USA) to assess the heating and temperature uniformity.

For conventional heating control (hot-air oven heating), 1 kg of yellow pea seeds were placed in the same PP container as the one used for RF treatment and heated using hot air in a convection oven at 120 °C for 30 min. The condition for oven heating was selected based on the literature to ensure effective LOX inactivation under this treatment (Jiang et al., 2018, 2021). Following all treatments, including the untreated control, the samples were cooled to room temperature, sealed in zip-lock bags, and stored at −80 °C until further evaluation. Each treatment was performed in duplicate.

2.3. LOX activity measurement in pea seeds

The LOX activity in dehulled pea seed flours was determined as explained before (Gao et al., 2020; Gökmen et al., 2002). Each sample of pea flour (0.1 g) was mixed with 10.0 mL of a phosphate buffer (10 mM) and stirred for 5 h at an ambient temperature. Subsequently, the mixture was centrifuged at 9100 ×*g* for 10 min, and the resultant supernatant was utilized as an enzyme extract. To prepare the model substrate solution, linoleic acid (140 μL) and Tween 20 (140 μL) were mixed and emulsified into 8 mL of phosphate buffer. Following this, 1.1 mL of 0.5 M NaOH was added to enhance the clarity of the solution, and the total volume was adjusted to 50 mL using phosphate buffer. The stock substrate solution was subsequently diluted (1:40, *v*/v) with 0.2 M sodium borate buffer (pH 9.0) before its combination with the enzymatic aliquot (1.25 mL: 50 μL). The absorbance increase attributed to the presence of a conjugated hydroperoxide moiety within the mixture at 234 nm was meticulously recorded for 3 min using a UV–visible spectrophotometer (60UV-vis, Agilent technologies, Santa Clara, USA). The unit of measurement for LOX activity was expressed as U/g, wherein U is defined as the quantitative increase in absorbance per minute, calculated using the following equation.

LOX activity quantification: A singular unit of LOX activity was delineated in terms of a variation in absorbance of 1 U at a wavelength of 234 nm, as represented in the following equation [Eq. 1].

$$LOX\left(\frac{U}{g}\right)=\frac{\Delta OD_{234\ nm}\times V_2}{\Delta t\times V_1}\times\frac{V_0}{m} \tag{1}$$

where $\Delta OD_{234\ nm}$ denotes the change in optical density (OD) over the time interval Δt at 234 nm; Δt signifies the cumulative duration of the reaction (min); V_0 represents the total volume of the crude enzyme extract (mL); V_1 indicates the specific volume of the crude enzyme extract added (mL); V_2 denotes the overall volume of the reaction mixture (mL); and m signifies the mass of pea flour (g)(Bi et al., 2022).

2.4. Pre-processing of pea samples and protein isolation

Whole dry pea samples were subjected to dehulling using a Satake TM05 abrasive mill (Satake, Japan) (Möller et al., 2021), with hull fractions being effectively eliminated. The resultant split peas were subsequently ground employing a Retsch ZM 200 ultra-centrifugal mill (Retsch, Haan, Germany) fitted with a 500 μm screen at a rotational speed of 12,000 rpm. Subasi et al. (2024).

The alkaline solubilization coupled with the isoelectric precipitation technique was used for the isolation of pea protein. Each pea flour was mixed with distilled water at a 1:10 ratio (*w*/*v*), its pH was adjusted to 8.5 using 2 M NaOH and maintained under stirring for 1 h (Karaca et al., 2011). The slurry was then centrifuged (4000 ×*g*, 20 min, 20 °C) and the supernatant was separated and its protein coagulated by pH readjustment to 4.5 using 2 M HCl and a 10 min incubation period. After a second centrifugation (4000 ×*g*, 20 min, 20 °C), the precipitated protein was collected and redissolved in distilled water (1:1) for pH neutralization. The protein was subsequently frozen at −80 °C, subjected to freeze-drying, and preserved in zipped plastic bags in the dark at −80 °C for subsequent analysis.

2.5. Protein recovery and mass yield measurement

The protein content of both the pea flours and pea isolates was quantified using an elemental analyzer by the Dumas method. A nitrogen-to-protein conversion factor of 5.4 was employed. The mass yield and protein recovery of the protein isolation process were calculated using using Eq. 2 and Eq. 3, respectively:

$$\text{Mass yield}\ (\%)=\frac{\text{Amount of dry protein isolate}\ (g)}{\text{Amount of dry starting material}\ (g)}\times 100 \tag{2}$$

$$\text{Protein recovery}\ (\%)=\frac{\text{Amount of final protein isolate}\times\text{protein content}}{\text{Amount of starting material}\times\text{protein content}}\times 100 \tag{3}$$

2.6. Determination of total phenolic content in pea protein isolate

The extraction of total phenolic compounds from the samples and their quantification were conducted according to the method described by Capanoglu et al. (2008). For this purpose, 0.5 g of each sample was mixed with 3 mL of 75% methanol, sonicated at room temperature for 15 min, centrifuged (2500 ×*g* for 10 min at 20 °C) and the supernatant was collected. The same application was repeated with the same pellet again using 2 mL of 75% methanol this time. The total phenolic content of the methanolic extracts was determined by the method of Singleton and Rossi (1965) using Folin-Ciocalteu reagent. A calibration curve was prepared with gallic acid and results were expressed as mg gallic acid equivalent (GAE) in g of protein isolate.

2.7. Quantification of lipid hydroperoxides

Lipid hydroperoxide (LOOH) is the main primary product of lipid oxidation and was quantified using the International Dairy Federation (IDF) method, with some modifications (Shantha & Decker, 1994). Prior to analysis, lipids were extracted by adding 8 mL of chloroform: methanol (1:2 *v*/v) to a test tube containing 2 g of each pea protein isolate and vortexing for 5 s. Then, 5 mL of chloroform and 2 mL of 0.5% KCl were added to the mixture and vortexed for another 5 s. These solutions were centrifuged at 10,000 ×*g* for 10 min. The bottom layer containing the hydroperoxides was collected. Next, 2 mL of collected layer was mixed with 2 mL of a chloroform: methanol (7:3 v/v) and mixed with 25 μL of ammonium thiocyanate and iron (II) solutions. After 5 min, their absorbance was recorded at 500 nm using a UV–vis spectrophotometer (Genesys 10S, Thermo Fisher Scientific, Waltham, MA, USA). The peroxide value (PV) was calculated using a standard curve of iron (III) chloride solution (Soendjaja & Girard, 2024).

2.8. Quantification of free radicals by Electron paramagnetic resonance (EPR)

Free radicals (FRs) were extracted and identified following the method outlined by Amft et al. (2020), which was modified and adapted specifically for pea protein isolate. Each pea protein was suspended in a mixture of ethyl acetate and methanol (EtAc:MeOH, 3:2, v:v-1) at a ratio of 1:6 (w: v-1), incorporating 100 μL of 200 mM N-tert-butyl-α-phenylnitrone (PBN) solubilized in ethanol. Following a 10 s vortexing and incubation at 50 °C for 30 min, the mixture was centrifuged (4000 ×*g*, 10 min, 4 °C), and the resulting supernatant was collected. Microcapillaries (6.5 cm in length) were filled with 50 μL of the sample and sealed at both terminals with wax. For EPR measurement, the microcapillary was placed inside an NMR tube. EPR measurements were conducted using a Spinscan X-band EPR spectrometer (LINEV Systems, Germany) operating at a frequency of 9.40 GHz, with a power setting of 10 mW, an attenuation of 10 dB, a modulation amplitude of 200 μT, and a modulation frequency of 109 kHz, at 20 °C. The data were analyzed using e-Spinoza (v1.1.02) and Origin 8.5, employing both single and double integration techniques on the spectral data to elucidate radical line shapes and to quantify spin concentration, respectively. A calibration curve using DPPH dissolved in ethanol (10 μM to 1 mM) was employed to determine spin concentration, with Avogadro's number (6.02214076 × 10^23 mol − 1) being utilized in the subsequent calculations. The results were articulated as the number of spins per gram of isolated protein dry weight (DW).

2.9. Analysis of 13-hydroxyoctadeca-9,11-dienoic acid (13-HODE)

The lipid oxidation intermediate derivative, specifically 13-hydroxyoctadeca-9,11-dienoic acid (13-HODE) was analyzed by developing a method following the principles described by Tullberg et al. (2016) with a modified derivatization procedure, employing 3-nitrophenylhydrazine (3-NPH), a carbodiimide coupling agent, to enhance detection sensitivity for carboxylic acid-containing oxidation products like HODE. First, 3-NPH (MW = 189.6 g.mol^{-1}) was diluted in LC-MS-grade methanol to a final concentration of 2 mg.mL^{-1}. This reagent was prepared fresh daily, protected from light, and stored at 4 °C until use to prevent degradation.

Similarly, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC-6; MW = 191.7 g mol^{-1}) was dissolved in LC-MS-grade methanol to a final concentration of 6 mg.mL^{-1}, with 6% (*v.v*$^{-1}$) pyridine, prepared fresh before use, as EDC is moisture-sensitive and can hydrolyze over time. Each pea protein was diluted in water (DF = 5) and vortexed for 10 s to ensure complete homogenization and dissolution of any aggregates. An aliquot of 50 μL of sample was transferred into a 1.5 mL Eppendorf tube, mixed with 50 μL 3-NPH and 50 μL EDC-6 + 6% pyridine. To facilitate the carbodiimide-mediated coupling reaction between the carboxylic acid group of HODE-13 and 3-NPH, forming a stable nitrophenylhydrazone derivative. The tubes were sealed and shaken for 60 min at 2000 rpm at room temperature and darkness in a Multi-Tube vortexer (DVX-2500, VWR, USA). After shaking, the tubes were centrifuged (15,000 ×*g*, 10 min, room temperature), and 200 μL were collected in a separate 1.5 mL Eppendorf tube. For quantification, an external standard curve of 13-HODE was prepared in concentrations ranging from 0.005 to 1 μM. The standards were also subjected to derivatization as described above. Targeted LC-MS analysis was performed using a Kinetex C18 column. The mobile phase included 20 mM acetic acid in water (A) and methanol (B). The gradient program was run for 40 min. It started with 70% eluent A and 30% eluent B for 2 min, followed by a linear increase of A to 95% over 8 min. This composition (95% A, 5% B) was maintained for 10 min. From 20 to 25 min, A was further increased linearly to 98% (2% B) and held for 2 min. Eluent A was then reduced linearly to 70% within 1 min, and this ratio (70% A, 30% B) was maintained until the end of the program. Detection used a QTRAP 6500+ mass spectrometer (Sciex, Singapore) in both positive and negative ESI modes. The results were expressed as μg HODE.g^{-1} of pea protein in DW.

Targeted liquid chromatography-mass spectrometry (LC-MS) analysis was performed using a Kinetics C18 chromatographic column. The mobile phase consisted of 20 mM acetic acid in water (solvent A) and methanol (solvent B). Detection was carried out with a QTRAP 6500+ mass spectrometer (AB Sciex, USA), operating in both positive and negative electrospray ionization (ESI) modes. Results were expressed as micrograms of 13-HODE per gram of pea protein isolate.

2.10. Analysis of volatile off-flavor compounds headspace solid-phase

Microextraction coupled to gas chromatography–mass spectrometry (HS-SPME-GC–MS) was used for the identification of volatile off-flavor contributors, employing a method described by Sajib et al. (2023) with some modifications. Around 0.1 g of protein isolates were dissolved in 5 mL of Milli-Q water in 20 mL SPME vials. The volatiles from the vial headspace were collected by an SPME fiber (75 μm Carboxen/polydimethylsiloxane (CAR/PDMS), Supelco, Bellefonte, PA, USA) during a 40 min extraction at 60 °C under continuous stirring (500 rpm). Subsequently, the adsorbed volatile compounds were thermally desorbed and injected into the GC–MS instrument for 5 min using the split less injection mode. A Shimadzu TQ8030 GC–MS setup equipped with a ZB-1701 capillary column (30 m × 0.32 mm, 1 μm, Phenomenex, Shinagawa, Japan) was used for analysis, with data acquisition scans ranging from 30 to 500 amu. Helium was employed as the carrier gas at a flow rate of 1.5 mL/min. The GC inlet temperature was held at 300 °C, and the GC separation occurred within an oven with temperatures ranging from 35 to 260 °C. The MS transfer line temperature was set to 265 °C, while the ion source temperature was maintained at 200 °C. During this analysis, selected number of volatile off-flavor compounds in peas were

targeted based on the most reported volatile beany associated flavor markers, including isovaleraldehyd (3-methylbutanal), valeraldehyde (pentanal), hexanal, 1-hexanol, benzaldehyde, 2-pentylfuran, 1-pentanol, nonanal, and 3-methyl-1-butanol.

Compound identification and quantification: Volatile compounds were identified using a combination of three complementary approaches: (1) mass spectral matching with the NIST library (match ≥85%), (2) retention index (RI) confirmation, calculated using a series of C8–C20 n-alkanes under the same chromatographic conditions and compared with published RI values for the ZB-1701 column (or equivalent 1701-type stationary phases), and (3) verification with authentic standards analyzed under identical GC–MS conditions for all targeted beany-associated volatiles. Quantification was performed by calculating the peak area of each compound relative to the internal standard. Where applicable, external standard curves were used to determine relative concentrations.

2.11. Statistical analysis

The RF pretreatments of the seeds and protein extraction trials were conducted at a minimum of two replicates. The determination of significant differences was achieved through the application of one-way analysis of variance (one-way ANOVA) to the data. Utilization of Duncan's multiple range test enabled the comparison of mean values, with significance attributed to data when $p < 0.05$. Statistical analysis was carried out using the Statistical Package for Social Science (IBM SPSS 28.0 for Windows, SPSS Inc., Chicago, IL, USA). Also, principal component analysis (PCA) was performed using OriginPro 2023 (OriginLab Corporation, Northampton, MA, USA) to visualize the relationships between LOX activity, TFC, PV, FRs, volatile compounds and 13-HODE.

3. Results and discussion

3.1. Screening RF treatments for LOX activity inhibition and its side effect on yield

Fig. 2 shows the change in relative LOX activity in pea seeds after RF treatment at different durations (20 and 30 min) and different electrode gaps (10 and 15 cm). The untreated control samples exhibited a very high LOX activity of 65.39 ± 0.9 U/g which is in line with the previous studies on pea seeds (Subasi et al., 2024). The LOX activity in the seeds decreased significantly ($p < 0.05$) with increasing RF heating temperature. The treatment with a 10 cm electrode gap for 20 min (bottom layer (BL): 130 °C and top layer (TL): 101 °C), the average LOX activity was reduced to 4.61 ± 0.1 U/g. Extending the treatment to 30 min (BL: 141 °C and TL: 113 °C) at the same gap eliminated LOX activity entirely (0.0 U/g). When the gap was increased to 15 cm and applied for 30 min (BL: 110 °C and TL: 94 °C), LOX activity was nearly undetectable, measuring 0.1 ± 0.02 U/g. These findings are consistent with those reported by Zhang et al. (2021), where LOX activity in fresh green peas significantly declined with increasing temperature, regardless of electrode gap. They showed that at 85 °C, residual LOX activity was reduced to less than 2% across different electrode gaps (105, 110, and 115 mm), indicating that temperature played a dominant role in enzyme inactivation, while electrode gaps had minimal influence. As demonstrated by the potential values, the electrode distance only affected the potential of the charged electrode, and this directly affected the electromagnetic field strength and resulting temperature change of the samples. In staggered field electrode system, the effect of electrode distance and orientation of the charged electrode on the electromagnetic field strength and distribution are analyzed and discussed in detail by Altin et al. (2025).

Sun et al. (2022) have also reported similar trends in sweet corn, where RF heating reduced LOX activity from 27.2 to just 4.6 as the final temperature increased from 50 °C to 80 °C. The rapid reduction in enzyme activity during RF treatment is likely due to structural changes in the LOX protein caused by high temperature. It should also be noted that the electric field in RF systems can accelerate enzyme inactivation by promoting protein unfolding, especially in high-moisture foods like sweet corn, which increases the mobility of protein molecules and enhances the unfolding rate of proteins, resulting in lower enzyme thermostability (Sun et al., 2022). Although LOX inactivation during RF treatment is mainly driven by temperature, the electric field may also contribute by promoting structural destabilization. LOX is a non-heme iron dioxygenase whose catalytic activity depends on a mononuclear Fe^{3+} center coordinated by conserved histidine residues and surrounded by a tightly folded active-site pocket (Skrzypczak-Jankun et al., 1996). Small perturbations around this site, such as partial unfolding or disruption of ligand geometry, are known to reduce catalytic efficiency even before global denaturation occurs (Wu & Wang, 1998). Studies on LOX inactivation by non-thermal physical treatments, such as pulsed light, demonstrate that the enzyme is highly sensitive to secondary-structure disruptions, showing losses in α-helix content and changes in intrinsic fluorescence tightly correlated with activity loss (Pellicer et al., 2019). These findings suggest that the oscillating RF electric field, combined with rapid dielectric heating, may accelerate conformational changes in the active-site environment and thereby promote faster LOX inactivation compared with slow, conductive heating (conventional heating). While LOX-specific electric-field effects have not yet been fully characterized, the structural sensitivity of the catalytic iron center and surrounding residues provides a plausible mechanistic explanation for the enhanced inactivation observed under RF processing.

LOX extracted from dried pea seeds and subjected to heating showed a decrease in their activity by exceeding 60 °C, which substantially decreased by increasing the temperature to 90 °C (Liburdi et al., 2021). However, a complete LOX inactivation was not observed at 90 °C and still more than 30% of enzyme activity was retained in all three pea varieties that they studied. These results could motivate the necessity of applying temperatures above 100 °C to achieve complete LOX inactivation in the pea seed matrix, as observed here.

The mass yield and protein recovery of yellow peas decreased significantly ($p < 0.05$) with increasing RF heating temperature (see Fig. 2b). The untreated control samples had a mass yield of 14.8% and protein recovery of 61% which is in line with our previous results (Subasi et al., 2024 and Manuel et al., 2025). In contrast, RF treatment using a 10 cm electrode gap for 20 min reduced the mass yield to 8.9% and the protein recovery to 39%. Extending the treatment to 30 min at the same electrode gap further decreased the mass yield to 3.6% and the protein recovery to 18%. However, when a 15 cm gap was used for 30 min, the mass yield was less compromised and reached 11.5% which can be related to the lower average amount of heat exposure at this treatment.

These findings agree with previous studies showing that high temperatures during RF or other thermal treatments can negatively affect protein extraction efficiency and yield. For example, Ling et al. (2019) found that RF heating above 100 °C negatively affected the yield, purity, solubility, and functional properties of rice bran protein isolate, primarily due to heat-induced protein denaturation. Similarly, Xu et al. (2017) observed the same trend in roasted chickpea seeds, linking the lower protein extraction to heat-induced changes that reduce solubility and extractability. The decrease in yield is mainly caused by structural changes in proteins when exposed to heat. High temperatures can disrupt hydrogen and disulfide bonds, causing proteins to unfold and form aggregates, which makes it harder to extract using alkaline solubilization (Akharume et al., 2021).

As can be seen in Fig. 2c, treatment with a 10 cm electrode gap for 20 min resulted in discoloration of some pea seeds. Increasing the treatment duration to 30 min at the same gap resulted in almost complete discoloration and burning of the seeds. While RF heating offers better heat penetration and more even temperature distribution than conventional or microwave methods, especially in low-moisture, granular materials,

non-uniform heating is still a challenge. Overheating at the edges or corners can occur due to uneven electromagnetic fields, which are influenced by the dielectric properties of both the sample and its surroundings (Ling et al., 2020). However, treatment with a 15 cm electrode gap for 30 min, resulting in an average temperature of around 100 °C, produced far fewer peas with partial discoloration zones, which may also explain the reduced adverse effects of RF treatment on protein mass yield. This indicated that tuning the RF condition can be a promising approach to achieve effective heating sufficient for LOX inactivation but mitigating its side effect on protein yield induced by protein denaturation.

3.2. Optimizing RF condition for maximum yield and mitigating lipid oxidation and off-flavor formation

3.2.1. Temperature distribution and heating rate of yellow peas during RF treatments

Visualizing and monitoring the temperature distribution within pea seeds under different RF conditions provides direct insight into heat uniformity, enabling the identification of settings that achieve target temperatures while avoiding localized overheating and associated quality losses. This is also essential for accurately interpreting the observed results and optimizing conditions to balance effective LOX inactivation and lipid oxidation mitigation with minimal negative impact on protein yield. Fig. 3. shows the effect of electrode gaps (8, 10, and 15 cm at different power levels), and different potentials of the charged bottom electrode (4000 V at the electrode gap of 10 cm, and 5000 V at the electrode gap of 15 cm at power level 2. In addition, 5000 V at the electrode gap of 8 cm at power level 3) treatment duration (10–45 min), and the sample thickness (2 and 6.5 cm) on the RF heating rate and temperature distribution of yellow pea sample.

In the same electrode gaps, the RF heating rate increased as the treatment duration increased. The maximum heating rate was obtained when the electrode gap was 15 cm and the RF was applied for 30 min on the 10 cm sample thickness, resulting in a maximum temperature of 120 °C at the BL of the sample. Sun et al. (2022) reported that the heating rate slowed when the electrode gap increased from 16 cm to 18 cm. This is because a smaller electrode gap results in a higher electric field intensity and higher energy absorbed by the treated samples through RF heating (K. Wang et al., 2022). Applying a short treatment of 10 min at a 10 cm gap and the same sample thickness could only result in an average temperature of 66 °C at the BL of the sample with a maximum of 90 °C, while increasing the time to 20 and 30 min caused overheating and sample discoloration, as previously shown in Fig. 2c.

The sample thickness of 6.5 cm and an electrode gap of 15 cm also exhibited uneven heating with localized hot spots, as shown in Fig. 3. In contrast, the treatment with a reduced sample thickness to 2 cm and a smaller electrode gap of 8 cm resulted in more homogeneous heat distribution with fewer intense hot spots at the same treatment duration of 30 min. Similarly, Ling et al. (2020) suggested that to achieve higher heating efficiency in RF treatments, containers should be designed with larger inner corners and lower heights, as these features help concentrate electromagnetic energy and enhance overall heating performance. However, increasing the time to 45 min under this condition also caused reaching the zone with a temperature of 136 °C.

3.2.2. LOX activity under optimized/milder RF conditions

Fig. 4 illustrates the changes in relative activity of LOX in yellow peas over varying treatment times (10 to 45 min) and different electrode gaps (8, 10, and 15 cm) during RF heating. The results show that residual LOX activity decreased significantly ($p < 0.05$) as the RF heating temperature increased, highlighting the effectiveness of RF heating in reducing LOX activity in yellow peas. The untreated control samples exhibited a much higher LOX activity of 45.95 ± 1.16 U/g. When the electrode gap was set to 10 cm and the peas were treated for 10 min (BL: 66 °C, TL: 53 °C), LOX activity measured 12.43 ± 0.22 U/g. Increasing the gap to 15 cm and applying the treatment for 30 min (BL: 96 °C, TL: 92 °C) resulted in a further decrease, with LOX activity reaching just 3.5 ± 0.4 U/g. Using thinner samples and an electrode gap of 8 cm and treating the samples for 15 min at 52.52 °C, the average LOX activity dropped to 13.63 ± 0.57 U/g. Extending the treatment to 30 min (72.46 °C) and 45 min (84.62 °C) at the same gap further reduced the LOX activity to 9.34 ± 0.4 U/g and 5.32 ± 0.4 U/g, respectively.

These results demonstrate that RF treatment at average temperatures below 100 °C can still achieve over 90% reduction in LOX activity. Even mild conditions, such as 75 °C, resulted in approximately an 80% reduction in its activity in pea seeds. Unlike traditional heating technologies, RF simultaneously heats the sample both internally and externally. Since an alternating electric field induces heat generation through molecular friction and space charge displacement, RF heating can effectively inactivate the LOX enzyme (Jiang et al., 2018a). Our findings are in line with those reported by Ling et al. (2018) where after treating rice bran at 80 °C for 45 min, 90 °C for 30 min, and 100 °C for 15 min, LOX activity decreased to 4.1%, 4.4%, and 5.5%, respectively.

A significant ($p < 0.05$) difference in LOX activity was observed between the seed subjected to conventional heating (120 °C for 30 min) and RF treatment (72 °C, 8 cm, 30 min). The LOX activity in the RF-treated samples was 9.34 ± 0.4 U/g, which was notably lower than that of the conventionally heated samples (13.5 ± 0.45 U/g), despite the conventional heating temperature being nearly twice as high as that used in the RF treatment. These results align well with a previous study where Jiang et al. (2021) showed that RF treatment resulted in significantly higher inactivation rates of LOX (95.2% in 270 s) compared to conventional heating (91.6% LOX in 600 s). This again confirms that RF heating requires lower temperatures (average 113 °C) and shorter times to inactivate LOX due to its higher heating rate and more uniform energy distribution. However, the conventional oven heating requires longer durations to expose the entire seed to high enough temperatures and energy to achieve similar enzyme inactivation as RF. Together, these findings support the effectiveness of RF heating as a fast and effective method for LOX inactivation in pea seeds under mild conditions, i.e., $T = 75$ °C, without inducing seed discoloration.

3.2.3. Mass yield and protein recovery under optimized/milder RF conditions

As can be seen in Fig. 4b, applying the milder RF treatment conditions mitigated the loss in mass yield and protein recovery from the treated seeds using the alkaline solubilization and isoelectric precipitation, which was seen in the previous trial. There were no significant ($p > 0.05$) differences in mass yield among the untreated control, oven-treated samples, and those treated with RF at 8 cm for 15 min, 8 cm for 30 min, and 10 cm for 10 min, with a mass yield of 15.95–16.1%. In contrast, more intensive treatments, 8 cm for 45 min and 15 cm for 30 min, led to a significant ($p < 0.05$) reduction of mass yield down to 13.96 ± 0.45% and 11.14 ± 0.55%, respectively. The absence of yield loss in treatments with average temperatures below ~80 °C could be attributed to avoiding the thermal denaturation threshold of pea storage proteins, especially globulins. Vicilin (7S), the major protein in peas, typically denatures around 72–78 °C, while legumin (11S) denatures at higher temperatures, approximately 85–92 °C, depending on moisture content, pH, and heating rate(Jean-Luc et al., 2013; Kuang et al., 2023). Under milder RF conditions, where the average temperature remained below this critical range, proteins likely retained their native structure and solubility, enabling efficient extraction during alkaline solubilization and isoelectric precipitation. In contrast, more intensive treatments that exceeded these thresholds most likely promoted protein unfolding, aggregation, and reduced solubility, leading to the significant decreases in recoverable protein mass yield observed. While thermal denaturation is a likely primary cause, other heat-induced processes such as protein–lipid interactions, Maillard reactions, or cross-linking could also contribute to the reduced protein extractability (Q. Wang, 2013).

Protein recovery of the seed showed a similar trend to mass yield,

with significant declines observed as RF heating temperatures increased (Fig. 4b). No significant differences in protein recovery were noted between the untreated control, oven-treated samples, and those subjected to milder RF treatments; specifically, 8 cm for 15 min and 10 cm for 10 min. These approaches maintained a relatively high protein recovery of 60–62%. However, when the intensity of the RF treatment was increased, such as 8 cm for 45 min or 15 cm for 30 min; protein recovery dropped substantially, reaching 56% and 45%, respectively. Previously, Ling et al. (2019) reported that applying RF treatment at 80 °C did not negatively affect protein recovery or purity from wheat bran. However, both parameters declined markedly at temperatures above this threshold, particularly when exceeding 90 °C. These results underscore the importance and possibility of tuning RF heating profiles to remain below key protein denaturation temperatures, thereby preserving protein extractability from pea seeds while still enabling effective reduction of LOX activity.

As can be seen in Fig. 4c, applying RF treatments at milder conditions (8 cm for 15 min, 8 cm for 30 min, and 10 cm for 10 min) where the maximum temperature did not exceed 100 °C, regardless of the thickness of the samples during the treatment. However, in samples treated more intensively, especially at 8 cm for 45 min, signs of discoloration in some seeds appeared. This supports the above observations that remaining at a mild heating condition is necessary to avoid the physical side effects of heating on the seed quality.

3.2.4. *Effect of RF treatment on total phenolic content (TPC) of protein isolate*

Determining the total phenolic content is important after heat treatment, as these compounds act as major natural antioxidants in pea seeds, and any degradation induced by RF treatment could substantially influence the lipid oxidation dynamics in the samples. Fig. 5a illustrates the TPC of protein isolated from yellow peas subjected to the various RF treatments and conventional heating. The control group and samples subjected to oven processing exhibited similar TPC values (approximately 9.5 and 9.3 mg/g, respectively), with no statistically significant difference observed between these groups and the milder RF treatments (8 cm, 15 min: 10 mg/g; 8 cm, 30 min: 9.7 mg/g; 10 cm, 10 min: 10.1 mg/g). The results indicate that TPC in yellow peas varies depending on the intensity of the RF treatment. Under milder conditions, such as 8 cm for 15 min or 10 cm for 10 min, TPC was either maintained or slightly increased compared to the control and oven-treated samples.

This is likely because moderate heat helps break down cell structures, making it easier to release and detect bound phenolic compounds. Similar effects were reported by Jogihalli et al. (2017), who observed

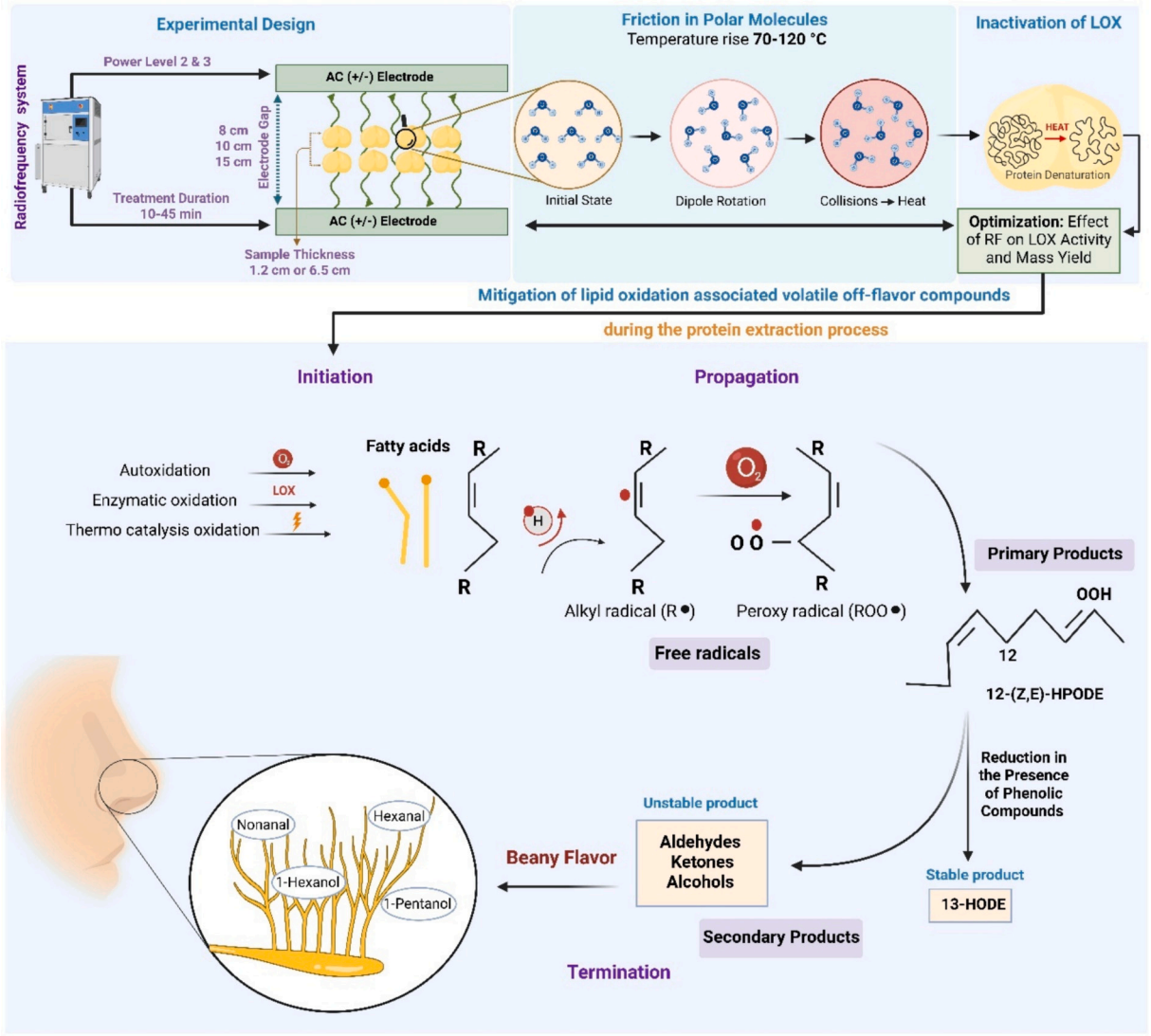

Fig. 1. Schematic representation of the experimental setup used in this study, alongside a simplified illustration of the lipid oxidation pathway induced during the protein isolation using the wet fractionation process and specific lipid oxidation markers targeted in this study to track it.

that roasting at 180 °C led to a 10.7% increase in phenolic content.

Conversely, more intense RF treatments (8 cm, 45 min: 7.1 mg/g; 15 cm, 30 min: 6.5 mg/g) led to a significant ($p < 0.05$) decline in TPC, with the resultant values being up to 30% lower than those of both the control and all other treatment conditions. This trend is consistent with findings from high-temperature roasting (200–220 °C), where phenolic content decreased by up to 26.5 (Jogihalli et al., 2017). At these higher temperatures and longer exposure times, phenolic compounds are more susceptible to oxidation and thermal degradation (Mir et al., 2016). Other studies also support the idea that intense heat treatments can lead to major losses of these compounds. For example, Singh et al. (2015) found that boiling and autoclaving chickpeas reduced their phenolic content by about 80%. While RF heating doesn't involve water like boiling does, exposing the peas to it for too long can still cause similar damage to the phenolic compounds.

In summary, the results support earlier findings showing that applying heat carefully can help release phenolic compounds and boost their measurable levels. However, if the heat is too intense or lasts too long, it can start to break these compounds down. This highlights the importance of fine-tuning RF treatment settings to get the most LOX inactivation benefits while protecting these valuable antioxidants.

3.2.5. *Effects of RF treatment on EPR-detected free radicals (FRs) in pea protein isolates*

Lipid oxidation happens through a well-known free radical chain reaction that unfolds in three main stages: initiation, propagation, and termination, as shown in Fig. 1. It all starts in the initiation phase, where highly reactive molecules like hydroxyl radicals (•OH) remove a hydrogen atom from an unsaturated fatty acid. This creates a lipid radical (L•), which quickly reacts with oxygen to form a peroxyl radical (LOO•). These peroxyl radicals continue the chain by pulling hydrogen atoms from nearby lipids, leading to the buildup of lipid hydroperoxides (LOOH). Eventually, in the termination phase, the reaction winds down as radicals either combine with each other or are neutralized by antioxidants, resulting in stable, non-reactive end products (Yildiz et al., 2025).

Fig. 5b shows the calculated relative radical abundance in the pea protein isolates (PPIs) obtained from the seeds subjected to various RF treatments and Fig. 6 provides a schematic illustration of RF induced variation in free radicals and their relation with RF treatment intensity, LOX inactivation and lipid oxidation pathways. The untreated control exhibited a free radical concentration of approximately 5.0×10^{14} spins g^{-1}, while oven-treated and milder RF treatments (e.g., 8 cm, 15 min; 10 cm, 10 min) displayed significantly ($p < 0.05$) lower levels (3.2–3.9 $\times 10^{14}$ spins g^{-1}). The reduced EPR signal in short-duration RF treatments, despite RF being applied to whole seeds rather than protein isolates, likely reflects biochemical changes induced before and during the extraction. Short RF exposures reduced LOX activity as shown before (see Fig. 4a), while also partially preserving endogenous antioxidants (see Fig. 5a), which perhaps reduced radical formation and facilitated radical recombination and thereby reduced the pool of pre-formed radicals in the seed matrix (Mildaziene et al., 2022).

The RF treatment at 8 cm, 30 min produced the highest EPR signal (≈7.5 × 10^14 spins g^{-1}) and was significantly higher than the untreated

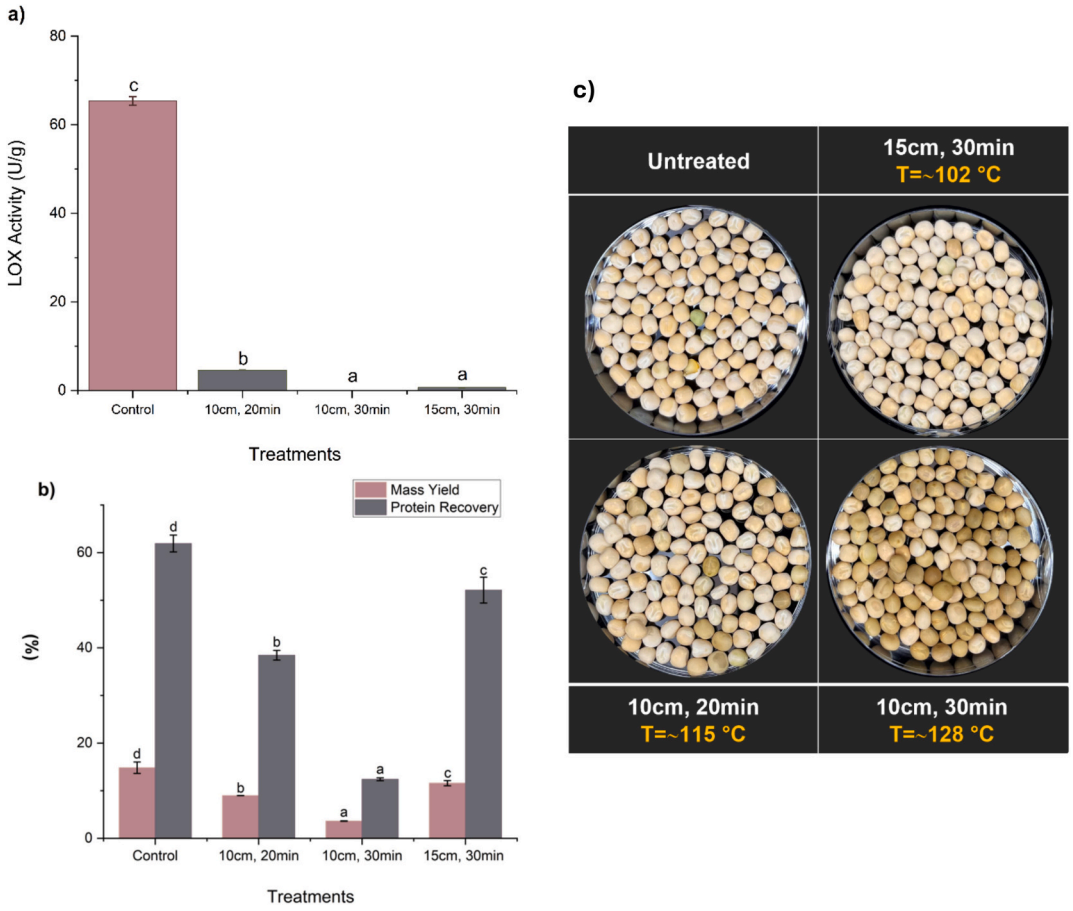

Fig. 2. Effect of radio frequency (RF) treatment on (a) LOX activity in yellow pea seeds, (b) their mass yield and protein recovery (c) representative pictures after treatment under different electrode gaps (10 and 15 cm) and treatment times (20 and 30 min). Different letters indicate statistically significant differences between treatments ($p < 0.05$). Untreated samples served as the control. T shows the average temperature obtained in each sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

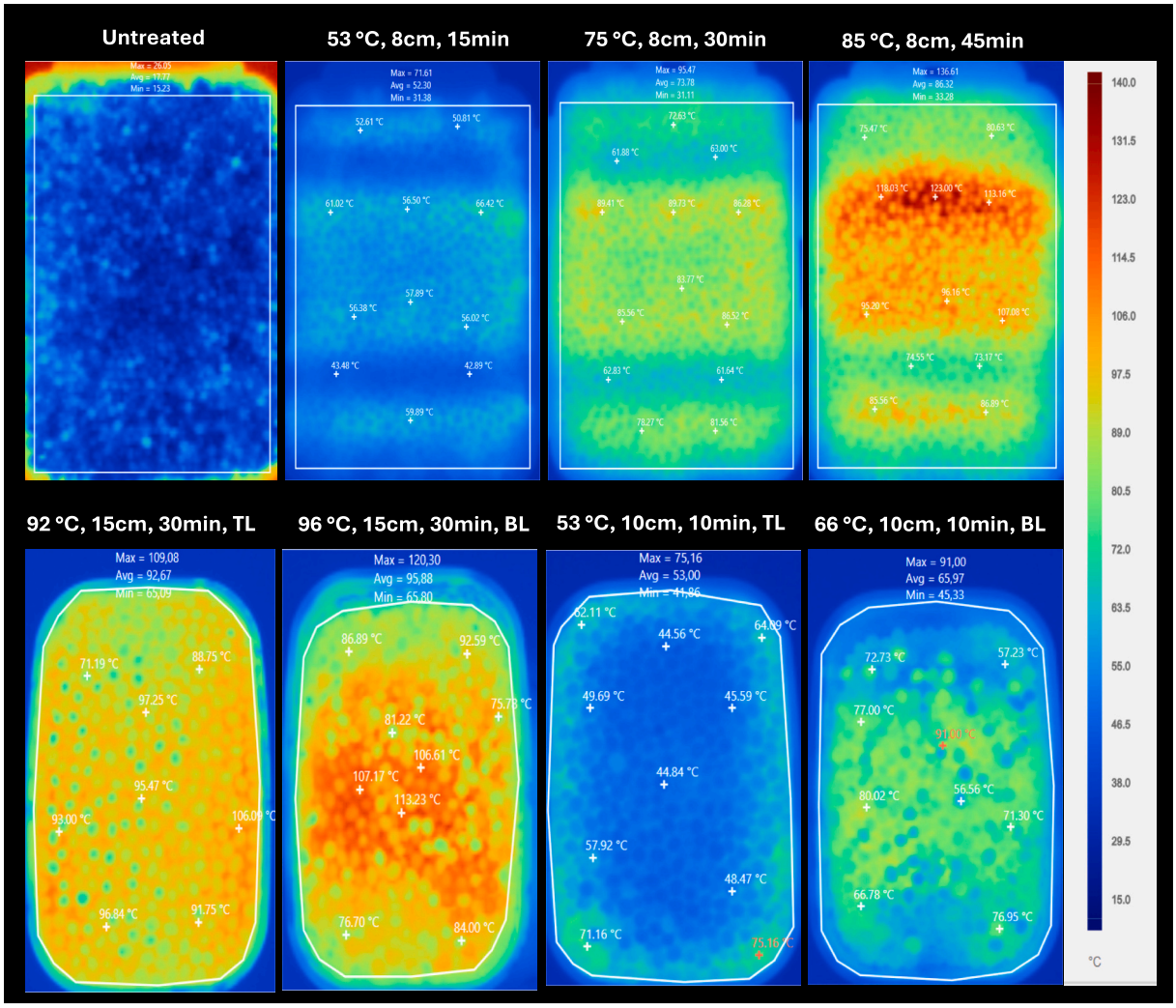

Fig. 3. Temperature distribution in yellow peas during radio frequency (RF) treatments (8 cm, 15 min; 8 cm, 30 min; 8 cm, 45 min; 15 cm, 30 min; 10 cm, 10 min) and untreated sample. Samples treated with the 8 cm gap had a thickness of 1.2 cm and the rest had a 6.5 cm thickness. The reported temperatures show the average temperature measured for each sample. BL and TL mean bottom layer and top layer, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

control and all other treatments ($p < 0.05$). This suggests that prolonged dielectric heating in seeds accelerated oxidation beyond the initiation stage, generating both lipid hydroperoxides and protein-bound radicals that persisted through extraction (Wang, Li, Shi, Huang and Chen, 2022), considering the preserved TPC in this sample (see Fig. 6). This is in line with prior findings that moderate thermal conditions can promote radical stability before decomposition begins (Jacobsen, 1999; Kamal-Eldin, 2003). It has been previously reported that both microwave treatment of rice starch (Fan et al., 2016) and RF treatment of barley (X. Wang et al., 2022) can result in the formation of free radicals. Wang et al. (2022) also showed that the free radical intensity in barley increased with increasing RF heating temperature and holding time, proving a relationship between the intensity of the heat generated by the treatments and the final quantity of long-term radicals. The isolation process itself, conducted under alkaline conditions, may have amplified these pre-existing differences by promoting secondary reactions between proteins and lipid oxidation products. Thus, the observed radical levels in the isolates could still reflect the oxidative state of the proteins and associated lipids at the moment of seed processing, and artefacts of the isolation procedure.

By contrast, the more intense RF treatments of 8 cm for 45 min and 15 cm for 30 min led to a sharp drop in EPR-detected FRs levels (0.7 and 0.3×10^{14} spins/g, respectively). These low EPR signals likely result from advanced oxidation stages, where radicals are consumed or transformed into EPR-silent products such as hydroperoxides, carbonyls, protein cross-links (e.g., dityrosine), and Maillard Adducts. (X. Wang et al., 2022) reported that increasing RF heating intensity and time, especially at 115 °C and 125 °C, increased FRs in barley due to the decreased RF heating uniformity. This could mean that the very low FRs quantity in our protein isolates produced from peas subjected to the intense treatments should most probably be due to radical consumption and transformation during the protein extraction process.

Overall, the data indicate a non-monotonic relationship between RF severity and measurable radicals in the proteins: (i) short time RF heating reduces radical generation and accumulation detectable by EPR but their prolongation enhances it, whereas (ii) severe RF heating accelerates downstream reactions that deplete radicals and yield stable oxidation products, contributing to the observed reductions in extractable protein mass yield under the harshest conditions.

3.2.6. Effects of RF treatment on primary and secondary lipid oxidation in pea protein isolate

Lipid oxidation is a complex, multi-phase process involving the formation and breakdown of unstable molecules such as FRs and hydroperoxides. These changes are heavily influenced by enzymatic activity (LOX), antioxidant levels (TPC), and the conditions of heat treatment

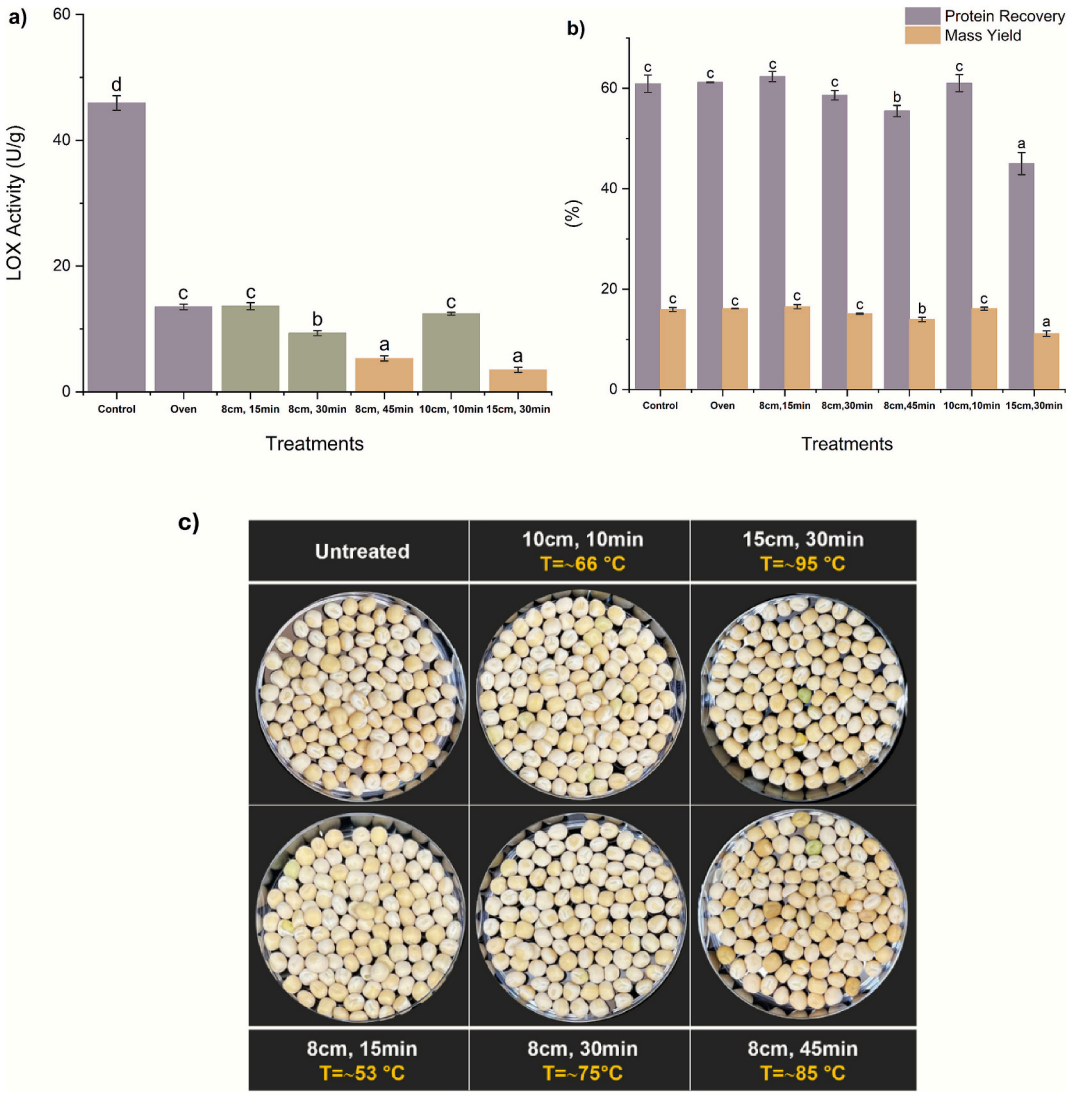

Fig. 4. Effect of optimized radio frequency (RF) conditions on (a) LOX activity in yellow peas, (b) their protein recovery and mass yield and (c) representative seed image after different treatments (8 cm, 15 min; 8 cm, 30 min; 8 cm, 45 min; 15 cm, 30 min; 10 cm, 10 min), conventional heating (oven) and untreated sample (control). Bars labeled with different letters indicate statistically significant differences between treatments ($p < 0.05$). T: average temperature of the samples during the RF treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Choe & Min, 2006; Frankel, 2005; Zhang et al., 2022). When lipids are heated, they oxidize and break down to create peroxide, which is quickly degraded into oxidized secondary products as the temperature rises. The peroxide value tends to rise when the rate of peroxide generation exceeds the rate of peroxide decomposition; otherwise, it tends to fall (Deng et al., 2022). The control sample, which underwent no heat treatment, showed the lowest PV (1.03 μg g^{-1}) (see Fig. 7a). This suggests that some lipid oxidation occurred naturally during the protein extraction, likely initiated by LOX activity, which was the highest in this sample, but it remained controlled due to the presence of phenolic compounds (natural antioxidants), which were also the highest in the control (9.5 mg/g) or mostly degraded into oxidized secondary products. The oxidation in the untreated sample most probably followed a typical enzyme-driven pathway, with limited radical propagation but more active peroxide breakdown during the protein extraction process to the secondary oxidation products or volatile compounds.

All the PPIs from heat-treated seeds showed a significant rise in their PV. Among the RF-treated samples, as the intensity of RF heating increased, the PV in the PPI also went up. Samples exposed to higher temperatures, like 85 °C and 96 °C, had noticeably higher PV levels compared to those treated at lower temperatures, such as 53 °C, 66 °C, and 75 °C. Among the RF treatments, the sample treated at 8 cm for 30 min was the most noteworthy. It showed a moderate PV (2.7 μg g^{-1}) but the highest level of FRs. In general, higher temperatures or longer heat exposures tend to increase PV and related oxidation indices (Abbas Ali et al., 2017). This suggests that oxidation had entered its propagation stage, where free radicals were actively being formed and stabilized, supported by a still strong antioxidant system (TPC: 9.7 mg/g). At this point, hydroperoxides had begun to accumulate but had not yet broken down into secondary volatiles. This condition appears to represent an optimal balance, where radicals are still measurable, oxidation is underway, and antioxidant defenses are partially intact.

By contrast, the more intense RF treatments, 8 cm for 45 min and 15 cm for 30 min, led to much higher PV (6.8 and 7.8 μg g^{-1}, respectively) despite the reduction of LOX activity, which showed a sharp drop in FRs levels and TPC. A similar trend was reported by de Freitas Floriano et al. (2020), who found that peanut oils roasted in the microwave exhibited the highest levels of primary and secondary oxidation products when roasted at the highest temperature (200 °C). These results indicate that oxidation has progressed to a more advanced stage. Under these harsh conditions, hydroperoxides were forming quickly but were also rapidly breaking down into secondary oxidation products such as aldehydes.

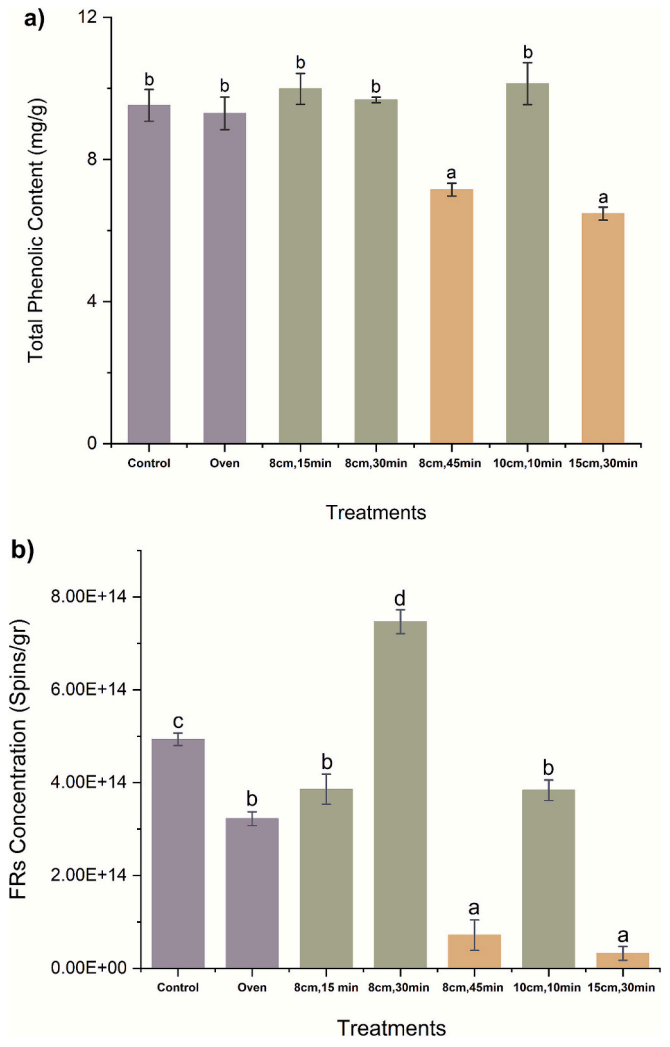

Fig. 5. Total phenolic content (TPC) (a) in yellow pea seeds and EPR detected free radicals (FRs) (b) in their protein isolated after treatment with various radio frequency (RF) conditions (8 cm, 15 min; 8 cm, 30 min; 8 cm, 45 min; 15 cm, 30 min; 10 cm, 10 min), conventional heating (oven) and untreated sample (control). Bars labeled with different letters indicate statistically significant differences between treatments ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The extreme heat likely caused free radicals to either decompose, recombine, or convert into more stable non-radical compounds as seen in the low EPR signals. Simultaneously, the total phenolic content declined significantly, weakening the antioxidant barrier and accelerating oxidation. This shift from controlled radical formation to extensive peroxide formation and decomposition reflects the transition from propagation to termination stages of lipid oxidation (Frankel, 2005; Loganathan et al., 2022).

Overall, these results reveal a non-linear relationship between treatment intensity and hydroperoxides. Moderate RF heating creates conditions that favor radical formation and peroxide build-up without major degradation. In contrast, more extreme treatments push oxidation into its final stages, characterized by high peroxide levels and low radical presence. The presence or depletion of antioxidants, particularly phenolics, plays a central role in regulating these transitions.

13-HODE is a non-volatile and relatively stable oxidation product which makes it a very good and reliable biomarker for lipid oxidation, complementing fewer stable markers, including PV and volatile compounds (Spiteller & Spiteller, 1997). Experimental analysis of PPI isolated from the seeds subjected to various RF and conventional heating treatments revealed distinct trends in the formation of 13-HODE, as shown in Fig. 7b. Specifically, RF treatment at 8 cm for 30 min resulted in the highest concentration of 13-HODE (0.81 μg g^{-1}). In contrast, more intense RF treatments, either extended exposure (8 cm, 45 min) or higher energy input by increasing sample thickness (15 cm, 30 min), led to significantly lower 13-HODE levels (0.14 μg g^{-1} and 0.10 μg g^{-1}, respectively), while concurrently increasing PV and hexanal concentrations (see Fig. 7c).

This inverse relationship between 13-HODE, PV and hexanal in these samples suggests a shift in the oxidative pathway depending on the treatment intensity. This behavior aligns with established lipid oxidation mechanisms reported in plant systems. Earlier studies show that 13-HODE is a relatively stable hydroperoxide-derived product that forms preferentially when antioxidants are available to reduce 13-HpODE (Aranda-Rivera et al., 2022; Spiteller & Spiteller, 1997; Yoshida et al., 2015). Conversely, when antioxidant capacity declines, hydroperoxides are more likely to undergo cleavage into aldehydes via non-enzymatic pathways (Vangaveti et al., 2010). Since phenolic content decreased sharply under the harsher RF treatments in our study, the observed decline in 13-HODE together with increased hexanal is consistent with a shift from hydroperoxide reduction to thermal or oxidative cleavage. During the oxidation process in peas, linoleic acid undergoes initial hydrogen abstraction at its 1,4-pentadiene part, a process catalyzed by 13-LOX, resulting in the formation of 13-hydroperoxyoctadecadienoic acid (13-HpODE). This hydroperoxide can follow two major fates: enzymatic cleavage by 13-hydroperoxide lyase (13-HPL) to yield volatile aldehydes such as hexanal, or reduction to a stable hydroxyl derivative i.e. 13-HODE (see Fig. 1). A similar oxidation pathway is observed for α-linolenic acid (Xiang et al., 2023). HODE formation reaction may proceed enzymatically or non-enzymatically and is facilitated by the presence of antioxidants, which donate electrons to reduce the hydroperoxide (-OOH) part to a hydroxyl group (-OH), stabilizing the molecule (Aranda-Rivera et al., 2022; Vangaveti et al., 2010; Yoshida et al., 2015). Therefore, under optimal RF conditions (8 cm, 30 min) where LOX activity was effectively reduced, which decelerated peroxide formation during the protein extraction and the high levels of total phenolics observed may have enhanced the reduction of the formed hydroperoxides to stable compounds like 13-HODE, thereby limiting the formation of volatile products such as hexanal. Conversely, under harsher RF conditions, the depletion of phenolic antioxidants likely favors hydroperoxide cleavage and auto-oxidation processes, leading to increased production of hexanal and other volatiles. 13-HODE, as a stable terminal product of lipid oxidation, acts as a "sink" that halts the propagation of the oxidation chain reaction. Its accumulation under optimal conditions limits the formation of reactive intermediates, which is consistent with the low levels of volatile off flavors observed.

In the remaining RF treatments, as well as the conventional heating and untreated samples, low hexanal concentrations coincided with relatively higher 13-HODE levels, further supporting the link between oxidative conditions, hydroperoxide stability, and volatile formation.

Thus, the data suggest that moderate RF treatments may inhibit lipid peroxidation pathways by promoting the accumulation of stable lipid oxidation products, while intense treatments may disrupt antioxidant systems and enhance the formation of undesirable volatiles through non-enzymatic routes.

3.2.7. Effects of RF treatment on the formation of beany volatile compounds in pea protein isolate

Volatile compounds are critical determinants of off-flavor in PPI, particularly those generated through lipid oxidation and related biochemical pathways (Xiang et al., 2023). The application of RF and conventional thermal treatments significantly influenced the volatile profile of PPI, with distinct outcomes depending on the specific conditions applied (see Table 1).

Hexanal is a LOX-derived aldehyde from the degradation of

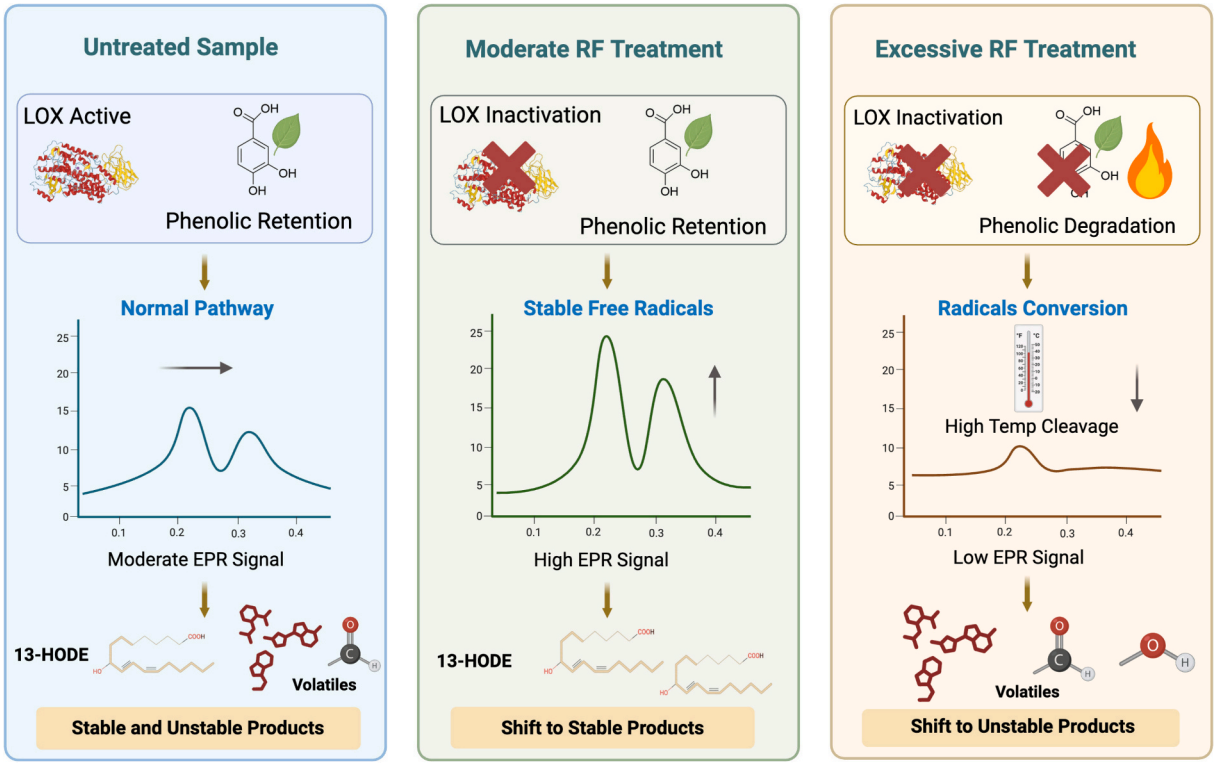

Fig. 6. Schematic illustration of the pattern of free radicals (FRs) detected by EPR induced by LOX inactivation and phenolic retention and its relationship with lipid oxidation pathways and off-flavor formation.

hydroperoxides produced from the oxidation of linoleic acid, contributing to grassy, beany, and green flavors (Rackis et al., 1979). It was present at 3.74 μg g^{-1} in the untreated PPI but decreased to 2.40 μg g^{-1} following the optimum RF treatment (8 cm, 30 min). This outcome is consistent with earlier findings that optimal RF exposure can efficiently reduce LOX activity while preserving phenolic compounds, thus decelerating enzymatic hexanal formation and accumulation of HODE during the protein extraction, aligning with observed reductions corresponding to oxidation markers, see Section 3.2.6. (Manouel et al., 2024). However, more intense or uneven RF heating (8 cm, 45 min; 15 cm, 30 min) caused hexanal levels to surge (7.98 and 9.01 μg g^{-1}, respectively), implicating non-enzymatic routes such as thermal degradation of hydroperoxides, a pathway known to accelerate under higher temperatures or longer heating times, see also Section 3.2.4., (Jiang et al., 2018).

Degradation of hydroperoxides from oleic acid could produce nonanal, another aldehyde associated with fruity, floral and green flavors (Ebert et al., 2022). Formation of nonanal in the PPI was also reduced under the optimum RF condition (0.01 μg g^{-1}) compared to the untreated sample (0.03 μg g^{-1}), whereas its levels increased with more intense RF treatments (0.07 μg g^{-1} for 8 cm, 45 min and 0.13 μg g^{-1} for 15 cm, 30 min), both significantly higher than the untreated control. It seems uneven heating can promote non-enzymatic oxidation, leading to increased nonanal as well. Cao et al. (2020) demonstrated that heating oleic acid induces the formation of aldehydes, including nonanal, through the homolytic cleavage of lipid hydroperoxides. Furthermore, they observed that the concentration of these aldehydes increases progressively with rising temperature, highlighting the temperature-dependent acceleration of lipid oxidation processes. Jiang et al. (2021) also reported that the concentration of nonanal in soy milk produced from soybeans treated with RF heating significantly decreased compared to untreated control samples.

Alcohols are a class of compounds generated by linking hydroxyl groups and aliphatic carbon atoms. 1-Hexanol, one of the most representative beany flavor compounds, contributes a lemon, grassy, green odor, and is converted from hexanal under the action of alcohol dehydrogenase (ADH). Alcohols, including 1-hexanol and 1-pentanol, are less influential than aldehydes among off-flavor components of pea protein due to their higher odor threshold. Alcohol dehydrogenase facilitates the conversion of fatty aldehydes to alcohol compounds (X. Wang et al., 2022). 1-hexanol was decreased in the 8 cm, 30 min treatment (0.08 μg g^{-1}) compared to the untreated sample (0.10 μg g^{-1}), but its concentration increased substantially under more intense RF treatments (0.48 μg g^{-1} for 8 cm, 45 min and 0.53 μg g^{-1} for 15 cm, 30 min). A similar trend was observed for 1-pentanol (grilled and dust), where the optimum RF treatment resulted in a significantly lower concentration (0.69 μg g^{-1}) compared to untreated (1.33 μg g^{-1}) and oven-treated (0.94 μg g^{-1}) samples. Jiang et al. (2018) reported that the analysis of volatile compounds in soy milk showed a significant reduction in 1-pentanol concentration from 87 to 4 μg L^{-1} and a decrease in 1-hexanol levels from 271 to 6.99 μg L^{-1} following RF treatment. These changes suggest that RF treatment effectively reduces off-flavor compounds, thereby improving the sensory quality of soy milk.

Benzaldehyde, which can contribute a bitter almond-like flavor (Utz et al., 2022), showed a general increase following both conventional heating and RF treatments, with the highest concentration observed after oven treatment (0.17 μg g^{-1}) and elevated levels in the most intense RF treatments (0.05 μg g-1 for 8 cm, 45 min and 0.17 μg g^{-1} for 15 cm, 30 min), compared to the untreated sample (0.02 μg g^{-1}). However, in the 8 cm, 30 min treatment, benzaldehyde remained low (0.024 μg g^{-1}) and close to the untreated sample. The formation of this aldehyde compound is commonly linked to the Strecker degradation, which is a side reaction of the Maillard reaction and is mainly associated with high-temperature processing (Yaylayan, 2003).

The concentration of 3-methyl-1-butanol was also reduced by RF treatment with 8 cm gaps and 30 min duration (0.03 μg g-1) relative to both the untreated (0.02 μg g-1) and oven-treated (0.14 μg g-1) samples, with even lower values observed under intense RF conditions. Pentanal (valeraldehyde), with optimum RF treatment yielding a lower

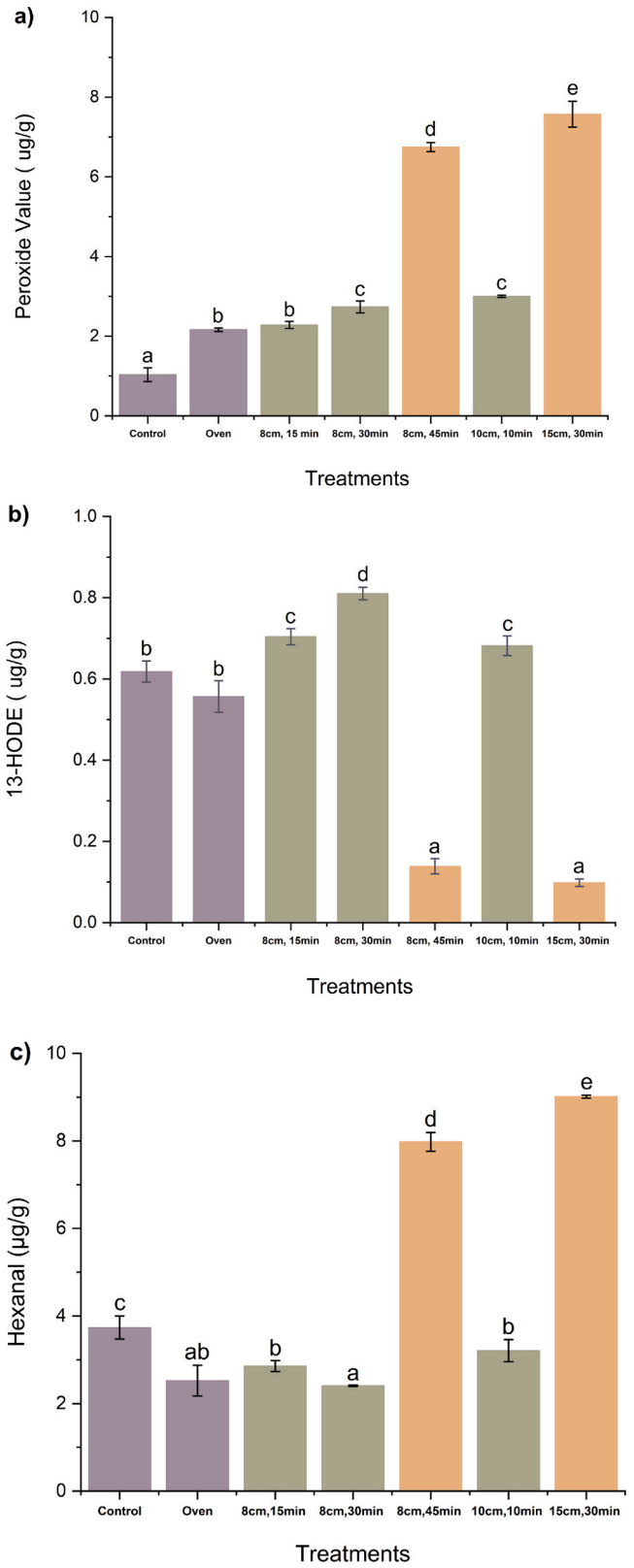

Fig. 7. Primary oxidation products (peroxide value) (a), secondary oxidation products, 13-HODE (b) and hexanal (c) in proteins isolated from yellow peas subjected to various radio frequency (RF) conditions (8 cm, 15 min; 8 cm, 30 min; 8 cm, 45 min; 15 cm, 30 min; 10 cm, 10 min), conventional heating (oven) and untreated sample (control). Bars labeled with different letters indicate statistically significant differences between treatments ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

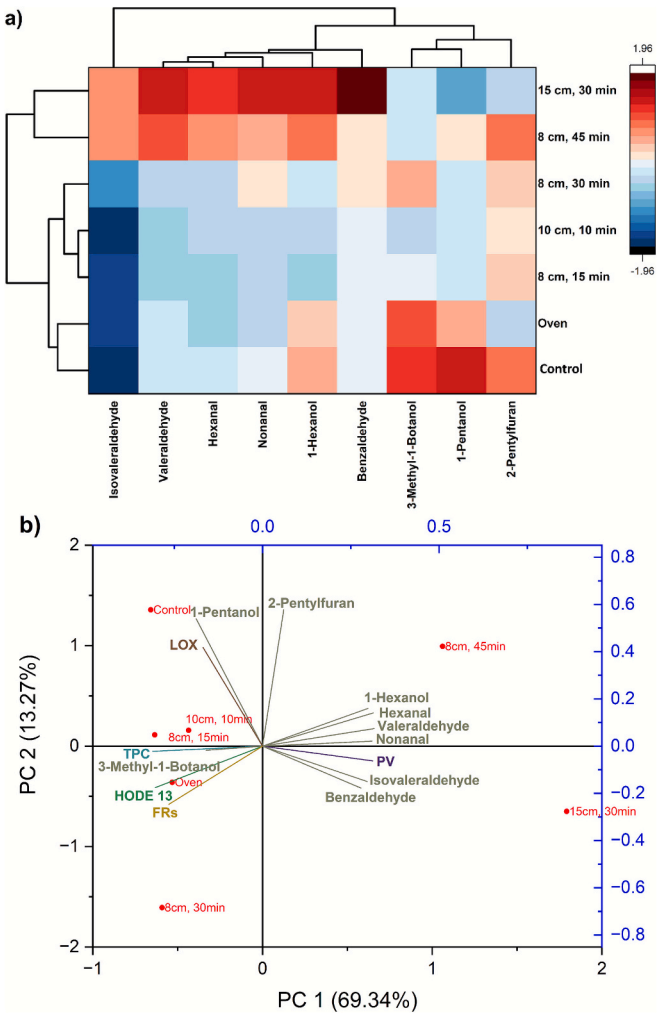

Fig. 8. Hierarchical cluster analysis heatmap of selected bean volatile compounds (a) and principal component analysis (PCA) biplot (b) illustrating the relationships between RF treatments, conventional heating, and the control sample in relation to peroxide value (PV), 13-HODE, free radicals (FRs), total phenolic content (TPC), lipoxygenase (LOX) activity, and key volatile compounds in yellow pea protein isolate (PPI). Positive PC1 represents intense oxidation (high PV and aldehydes), whereas negative PC1 reflects enzyme-dominated oxidation (high LOX activity, TPC, FRs, HODE-13). PC2 indicates heating uniformity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentration (0.08 µg g-1) than both untreated (0.15 µg g-1) and oven-treated (0.11 µg g-1) samples, while intense RF treatments led to significant increases (0.31 µg g-1 and 0.41 µg g-1 for 8 cm, 45 min and 15 cm, 30 min, respectively).

Overall, these results demonstrate that the application of RF treatment of pea seeds under optimum conditions (8 cm, 30 min) is effective in minimizing the formation of key volatile compounds associated with off-flavors in yellow pea protein isolate. In contrast, more intense RF treatments tend to increase the concentration of these volatile compounds. This highlights the importance of optimizing RF processing parameters to improve the sensory quality of PPI.

4. Multivariate analysis of the effect RF treatments on off-flavor formation in the proteins

The hierarchical cluster analysis (Fig. 8a) revealed distinct grouping patterns in the abundance of key volatile compounds associated with the beany flavor profile of PPIs obtained from seeds subjected to different RF

Table 1
Quantitative comparison of volatile compounds (expressed in μg.g^{-1} DW) in proteins isolated from yellow peas subjected to various RF (8 cm, 15 min; 8 cm, 30 min; 8 cm, 45 min; 15 cm, 30 min; 10 cm, 10 min), conventional heating (oven) and untreated sample (control).

Selected beany volatile compounds	Control	Oven	8 cm. 15 min	8 cm. 30 min	8 cm. 45 min	10 cm. 10 min	15 cm. 30 min
Isovaleraldehyde	0.43 ± 0.00^{d}	0.40 ± 0.03^{d}	0.33 ± 0.02^{d}	0.76 ± 0.10^{c}	1.06 ± 0.16^{b}	0.36 ± 0.00^{d}	1.30 ± 0.08^{a}
Valeraldehyde	0.15 ± 0.03^{c}	0.11 ± 0.03^{cd}	0.09 ± 0.02^{d}	0.10 ± 0.02^{d}	0.31 ± 0.00^{b}	0.12 ± 0.00^{cd}	0.40 ± 0.01^{a}
3-Methyl-1-Butanol	0.06 ± 0.02^{bc}	0.14 ± 0.00^{a}	0.08 ± 0.02^{b}	0.03 ± 0.03^{cd}	0.01 ± 0.00^{d}	0.05 ± 0.05^{bcd}	0.03 ± 0.01^{bcd}
1-Pentanol	1.33 ± 0.08^{a}	0.94 ± 0.13^{bc}	1.01 ± 0.15^{b}	0.69 ± 0.11^{c}	1.01 ± 0.19^{b}	0.78 ± 0.26^{bc}	0.35 ± 0.04^{d}
Hexanal	3.74 ± 0.36^{c}	2.53 ± 0.55^{de}	2.86 ± 0.23^{de}	2.41 ± 0.02^{e}	7.98 ± 0.41^{b}	3.21 ± 0.65^{cd}	9.01 ± 0.03^{a}
1-Hexanol	0.23 ± 0.03 ^{c}	0.20 ± 0.02 ^{c}	0.06 ± 0.00 ^{d}	0.09 ± 0.03 ^{d}	0.47 ± 0.04^{b}	0.10 ± 0.00^{d}	0.53 ± 0.00^{a}
2-Pentylfuran	0.03 ± 0.00^{ab}	0.02 ± 0.00^{bc}	0.03 ± 0.00^{ab}	0.02 ± 0.00^{c}	0.04 ± 0.01^{a}	0.04 ± 0.01^{a}	0.03 ± 0.00^{abc}
Benzaldehyde	0.02 ± 0.01^{b}	0.03 ± 0.01^{b}	0.03 ± 0.02^{b}	0.02 ± 0.00^{b}	0.05 ± 0.04^{b}	0.03 ± 0.00^{b}	0.16 ± 0.03^{a}
Nonanal	0.03 ± 0.00 ^{c}	0.02 ± 0.01^{cd}	0.03 ± 0.01^{c}	0.01 ± 0.00^{d}	0.07 ± 0.00^{b}	0.03 ± 0.01^{c}	0.13 ± 0.00^{a}
Total	6.02	4.4	4.51	4.13	11	4.72	11.95

Data is represented as average ± SD. Values with different letters in a row represent significant differences ($p < 0.05$) ($n = 2$).

treatments. The control (untreated) and oven-treated samples clustered together and were characterized by high relative levels of 3-methyl-1-butanol, 1-pentanol, and 2-pentylfuran, compounds widely recognized as contributors to grassy, green, and fatty notes in legumes (Xiang et al., 2023). These samples also contained moderate amounts of isovaleraldehyde and valeradehyde, both branched-chain aldehydes linked to amino acid degradation during seed processing (Setiabasa., 1982).

Mild RF treatments with short durations (8 cm, 15 min; 10 cm, 10 min) formed an intermediate cluster, showing reduced levels of most aldehydes and alcohols compared to the control. This reduction likely reflects partial inactivation of LOX and related hydroperoxide lyase activity during RF heating, thereby slowing the enzymatic conversion of PUFAs into C6 and C9 aldehydes and alcohols (Jiang et al., 2018). The decrease is consistent with the lower EPR-detected radical levels and preserved TPC (Fig. 5a and b) and the small increase in HODE in these treatments, suggesting that early suppression of radical initiation and shifting the oxidation towards HODE pathways limits the formation of downstream volatile oxidation products. In contrast, the 8 cm, 30 min treatment was characterized by a volatile profile intermediate between mild and severe RF conditions, with moderate levels of aldehydes and alcohols. This aligns with its intermediate peroxide values, very high free radical and HODE (Figs. 5 and 6), indicating moderate lipid oxidation and potential secondary transformations during extraction but mostly shifted towards HODE pathways with limited formation of volatile oxidation products.

The most severe RF treatments (8 cm, 45 min; 15 cm, 30 min) clustered together and showed a pronounced increase in straight-chain aldehydes (valeraldehyde, hexanal, nonanal) while displaying lower levels of alcohols and 2-pentylfuran. The elevated aldehyde concentrations in these treatments likely arise from the thermal decomposition of pre-existing hydroperoxides formed during earlier oxidation stages, a process accelerated under high dielectric heating (Domínguez et al., 2019; Frankel, 2012). The concurrent reduction in alcohol could reflect the volatilization or degradation of more labile compounds during prolonged heating, or their conversion into secondary oxidation products. Notably, the strong accumulation of hexanal, a primary oxidation marker of linoleic acid, points to advanced lipid oxidation, consistent with the low EPR radical signals and HODE but high PV and loss of natural inhibitors (i.e., TPC) observed for these treatments.

To better understand and discover how RF and conventional heating conditions influenced lipid oxidation, antioxidant retention, enzyme activity, and volatile formation in PPI, PCA was carried out (Fig. 8b). Together, the first two principal components (PC1 and PC2) accounted for 82.61% of the total variance. PC1 (69.3%) was primarily driven by oxidation intensity, where positive PC1 scores aligned with high PV and aldehydes (hexanal), while negative PC1 scores corresponded to enzyme-dominated oxidation characterized by high LOX activity, TPC, FRs, and HODE-13. PC2 (13.3%) reflected heating uniformity across treatments.

Looking at the biplot, clear clustering patterns emerged based on the intensity of thermal treatment. The control sample, though unheated, was characterized by high LOX activity, reflecting enzymatic oxidation, but still retained the highest phenolic content. The untreated control, oven-treated sample, and milder RF conditions (8 cm for 15 min and 10 cm for 10 min) were grouped on the left side of PC1. These samples were associated with higher levels of TPC, 13-HODE, FRs, and 3-methyl-1-butanol, and lower PV and LOX activity. This suggests that these treatments helped to partially reduce LOX activity and preserve antioxidant capacity but had a limited effect on oxidative damage compared with the untreated samples. The sample treated at 8 cm for 30 min stood out in the lower-left quadrant, closely linked to the highest free radical concentration and elevated 13-HODE levels and its antioxidant system remained intact. This profile suggests that this treatment barricaded the oxidation in this sample into the propagation phase of lipid oxidation, where radicals and HODE accumulate, but avoided the occurrence of extensive lipid degradation and formation of volatile beany associate compounds. In contrast, the more intense RF treatments (8 cm, 45 min and 15 cm, 30 min) appeared on the far-right side of PC1. These samples were strongly associated with elevated PV, hexanal, nonanal, benzaldehyde, and isovaleraldehyde, all markers of advanced lipid oxidation and volatile degradation. Their lower FRs and TPC values suggest a weakened antioxidant barrier, which allowed for the rapid breakdown of hydroperoxides and the formation of volatile off-flavor compounds. The 8 cm, 45 min treatment also showed strong correlations with 2-pentylfuran and 1-pentanol, both known contributors to thermal and beany off-flavors, further indicating that oxidation had progressed into its later stages.

Overall, the PCA results clearly show how increasing RF intensity influences the oxidative and volatile profile of yellow pea protein. Moderate RF conditions, especially 8 cm for 30 min, offered a favorable balance by effectively reducing LOX activity and initiating mild oxidation without causing extensive heat-induced lipid autooxidation and off-flavor production. On the other hand, more aggressive RF treatments shifted the system towards uncontrolled oxidation, compromising both nutritional and sensory quality.

5. Conclusions

The efficiency of radio frequency (RF) heating as a pea seed pretreatment to mitigate the formation of lipid oxidation-related beany volatile compounds in their protein isolated using wet fractionation and its underlying mechanism was evaluated and optimized. RF treatments providing heating >110 °C for more than 20 min completely inactivated LOX in the seeds but caused seed darkening and markedly reduced protein yield (up to 50%).

Further optimization of RF treatment by adjusting sample thickness, electrode distance, and heating duration enabled >90% reduction in LOX activity of the seeds at below 100 °C, while fully preserving seed

colour and protein yield, and outperforming conventional oven heating in efficiency. Moderate RF treatment (8 cm electrode distance, 30 min, 2.5 cm thickness, ~75 °C) reduced LOX activity by 80% in pea seeds, preserved natural antioxidants (i.e., phenolic compounds), and significantly lowered beany volatile formation in the resulting protein isolates compared with untreated seeds. This condition retarded lipid oxidation during protein extraction, leading to the accumulation of EPR-detected free radicals and HODE, while preventing extensive lipid degradation and beany off-flavor formation. In contrast, more intense RF treatments more effectively inactivated LOX, but depleted antioxidant phenolic compounds, reduced free radical levels, and accelerated lipid breakdown, resulting in both higher formation of hydroperoxide and a sharp rise in off-flavor volatiles compared with the control.

Overall, RF heating of pea seeds under carefully optimized conditions (70–80 °C) can effectively reduce LOX activity without compromising protein extractability or natural antioxidants, thereby limiting lipid oxidation during protein extraction and lowering the accumulation of beany-associated volatile compounds in the protein isolate.

CRediT authorship contribution statement

Elahe Sharifi: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **José Villacís-Chiriboga:** Writing – review & editing, Methodology, Investigation. **Kubra Kahraman:** Writing – review & editing, Software, Methodology. **Eda Coskun:** Writing – review & editing, Methodology. **Ferruh Erdoğdu:** Writing – review & editing, Supervision, Resources, Methodology, Data curation. **Mehdi Abdollahi:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

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.

Acknowledgements

We gratefully acknowledge the Lantmännen Research Foundation for the research grant within the TastyPeas project (project number: 2022H038) and Swedish Research Council Formas for research grant within the SwedPea project (project number: 2023-02041). We also extend our sincere thanks to Lantmännen AK for their valuable contributions, particularly for providing samples and sharing industrial insights. Special thanks are given to Rikard Fristed for his excellent technical assistance in developing and implementing the methodology for HODE determination.

Data availability

No data was used for the research described in the article.

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