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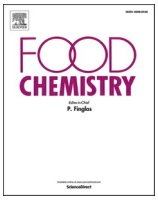

Influence of processing of seitan, tempeh, and firm regular tofu on protein and lipid oxidation and Maillard reaction products formation

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ABSTRACT

The impact of production and cooking on protein oxidation, lipid oxidation and Maillard reaction products (MRPs) generation was evaluated for firm regular tofu, tempeh, and seitan, three plant-based alternatives to meat products. Tofu showed higher content of protein-bound carbonyls (25.5 ± 5.4 nmol/mg protein) indicating higher degree of protein oxidation, while seitan exhibited the lowest content of MRPs like N(6)-carboxymethyllysine, as revealed by LC-MS/MS. Through determination of the peroxide value and thiobarbituric acid reactive substances as well as analysis of volatile oxidation compounds by GC–MS, an overall higher lipid oxidation level was found in tofu. Likewise, a mean 92.2 % depletion of naturally occurring antioxidant tocopherol, assessed by HPLC-DAD, was observed in tofu. Furthermore, acrylamide was detected in tofu at 196.0 ± 15.4 μg/kg after frying at 190 °C. This study, therefore, concluded that practices used in tempeh and seitan processing are promising strategies to limit oxidation and Maillard reactions in food.

1. Introduction

Over the past decades, awareness about animal welfare and sustainability has been increasing, leading to an exponential rise of vegetarian and vegan diets and a growing demand for plant-based meat substitutes or analogues. This trend is expected to keep growing in the upcoming years (Andreani et al., 2023; Flint, Bowles, Lynn, & Paxman, 2023). The popularity of plant-based diets additionally lies on their correlation with a lower risk of lifestyle-associated diseases, as they involve a lower intake of total fat, cholesterol, and sodium, as well as a higher content of polyunsaturated fatty acids (PUFA), antioxidants, fiber, and magnesium (Li, 2014). However, sufficient protein intake might be challenging for vegans (Bakaloudi et al., 2021). The availability of different plant-based protein sources is therefore of concern for the vegan population and for the food industry. While tofu, a source of plant-based protein as well as omega-3 fatty acids (Pal, Devrani, & Ayele, 2019), has well made its way to Western countries, other alternatives, even those that are part of the traditional Asian culture for decades, are still on the way of being introduced to the Western world (Zhao, Wang, Hu, & Zheng, 2023).

Food processing occurs in both industry and households to prepare food products for preservation or consumption (Jadhav, Annapure, & Deshmukh, 2021). Processing techniques often involve heat treatments, which promote different chemical reactions that can positively influence

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the quality of the food by generating desirable aromas or flavors (Jadhav et al., 2021). On the other hand, heat exposure can negatively impact food quality and digestibility as well, by allowing procedures such as protein denaturation or reactions between food constituents, and even by leading to the formation of harmful substances such as carcinogens or toxins (Hellwig, 2019).

One of the most concerning side reactions during food processing for the industry is the promotion of oxidation, as minimizing its progress remains a challenge (Domínguez et al., 2019). Meat substitutes are affected by protein oxidation, decreasing the digestibility and the functionality of their proteins (Estévez, Díaz-Velasco, & Martínez, 2022). Moreover, lipid oxidation can have a significant role in the quality of products derived from fat-rich plant-based sources as well, resulting in off-flavors and a decrease in shelf-life (Medic, Atkinson, & Hurburgh, 2014; Pignitter & Somoza, 2012). Furthermore, lipid and protein oxidation can interact, adding complexity to the task of limiting their progress (Estévez et al., 2019). Maillard reactions also take place during food processing (Tamanna & Mahmood, 2015). While some Maillard reaction products (MRPs) are highly valued by the industry as they provide desirable properties on food, such as flavor, other products, especially advanced glycation end products (AGEs), including N(6)-carboxymethyllysine (CML), are associated with health issues. Therefore, it is recommended to avoid their formation (Poojary et al., 2020; Sun et al., 2022). Acrylamide, another MRP, is classified as a class 1B carcinogen and mutagen (IARC, 1994). Therefore, it is essential to understand how specific food processing techniques influence these chemical reactions to limit the formation of potentially hazardous byproducts and ensure food safety. While meat and fish products have been extensively studied in this regard (Armenteros, Heinonen, Illilainen, Toldrá, & Estévez, 2009; Domínguez et al., 2019; Nguyen, van der Fels-Klerx, & van Boekel, 2014), certain plant-based products, not as common in traditional Western diets, have only received attention from a limited number of studies. For instance, Goerke et al. (2019) reported higher acrylamide content in vegetarian and vegan diets compared to omnivore individuals, while highly processed meat alternatives have been associated with health issues (Flint et al., 2023). This further highlights the need of investigating the impact of processing techniques on specific meat substitutes.

Given its implication on the quality and safety of protein- and fat-rich foods, the aim of the current study was to determine the impact of processing (production and cooking) on lipid and protein oxidation and the formation of selected MRPs in plant-based products that can be used as meat substitutes. Due to these chemical reactions potentially taking place in a wide variety of food products, our main goal was to evaluate them simultaneously during processing, as this may uncover potential interrelations and emphasize the importance of assessing them together routinely. We hypothesize that production techniques involving milder heating or addition of antioxidants may help hinder these processes, even during the subsequent cooking of the products. In addition, we theorize these effects may be more or less pronounced depending on the starting material. To this end, two soy-based products (tofu and tempeh) and one wheat-based alternative (seitan) were studied and compared at three different processing stages (raw plant material, raw product, and fried product) to assess the influence of each processing step on lipid and protein oxidation as well as MRPs generation. These three products are popular sources of protein (Andreani et al., 2023), and were chosen as a mean to study the influence of processing when only production differs (tofu and tempeh) and when both production and starting material are different (seitan).

2. Materials and methods

2.1. Materials

The soybeans used in the present study were bought from Heuschen & Schrouff Oriental Foods (Landgraaf, The Netherlands). Food grade magnesium chloride, also called Nigari, was purchased from Nature & Partage (Gornac, France), while tempeh starter culture was obtained from Tempehstation (Edinburgh, Scotland). Wheat flour (type W840), apple cider vinegar and canola oil were acquired from a local supermarket (Vienna, Austria). Chemicals for the different experiments were acquired from Sigma-Aldrich (Vienna, Austria), VWR International (Vienna, Austria), Carl Roth (Karlsruhe, Germany), AppliChem (Darmstadt, Germany), Fisher Scientific (Vienna, Austria), Büchi AG (Flawil, Switzerland), Iris Biotech (Marktredwitz, Germany), and Abchem (Olsztyn, Poland).

2.2. Preparation and cooking of plant-based products

Firm regular tofu was produced according to Li et al. (2015) with slight modifications. After soaking 250 g soybeans in water for 24 h, they were separated in three portions and mixed in a HR2195 900 W kitchen blender (Philips, Vienna, Austria) with double the amount (*w/v*) of hot water. The content of the blender was then squeezed through a cotton cloth. The resulting soymilk was collected and subsequently boiled on a gas stove for 10 min with constant stirring. The milk was then cooled until it reached 80 °C (approximately 4 min). For each liter of soymilk, 45 mL magnesium chloride solution (1 g/mL) were added. After 10 min, the mix was transferred to a tofu press (Tofuture, Amersham, United Kingdom) and pressed at the highest setting for 8 min, obtaining firm tofu.

Tempeh was produced as described in Esaki, Onozaki, Kawakishi, and Osawa (1996). After soaking 250 g soybeans for 24 h, they were cooked at medium heat in 2 L fresh tap water for 60 min. Soybeans were then cooled and dried in a kitchen towel at room temperature and subsequently peeled and separated into three bowls equally. Apple cider vinegar (17 g) and *Rhizopus oligosporus* tempeh starter culture (0.6 ± 0.1 g) were added to each bowl, after which the beans were thoroughly mixed and packed tightly in 1 L plastic bags. Afterwards, holes every 2 cm along the bags were punctured with a scalpel. The bags were then left at 33 ± 1 °C in an incubator (Heraeus Instruments, Hanau, Germany) for 3 days, until the mass was evenly coated with white mold.

Seitan production was adapted from Anwar and El-Chaghabi (2019). Briefly, 250 g flour were kneaded with 150 mL lukewarm water for 20 min using a household stand mixer. The resulting dough was left covered at room temperature for 20 min, after which it was washed through a sieve under running water to remove the starch. The washing process was repeated six times, until the running water remained clear. The resulting product was blanched in boiling water for 10 min.

All preparations were done in triplicate. The three products were cut into 1 cm^3 cubes and frozen at −20 °C until use. All products were stored the same amount of time and thawed at room temperature to minimize the impact of storage time and re-heating.

Frying was chosen as the common cooking procedure, as it is a fast method often used for these products (Goerke et al., 2019; Jeleń, Majcher, Ginja, & Kuligowski, 2013; Pal et al., 2019). Hence, samples were fried at 130 °C for 3.5 min in 25 mL canola oil in a 20 cm diameter frying pan on an induction plate. An additional triplicate of each product was fried at 190 °C for 1.5 min for the determination of acrylamide (section 2.8). For the raw material stage analysis, soybeans were freshly homogenized before sample preparation, while no additional prior step was required for flour.

2.3. Determination of protein content

The protein content of the plant-based products was determined by the Kjeldahl method adapted by Marcó, Rubio, Compañó, and Casals (2002). A total of 1 g homogenized sample was placed in a 300 mL Kjeldahl flask along with two 5 g Kjeldahl tablets and 20 mL concentrated sulfuric acid. The flask was then placed in a pre-heated digestion block (Digest Automat K-438, Büchi AG, Flawil, Switzerland) at 420 °C for 150 min. Subsequently, 90 mL sodium hydroxide 32 % (*w/v*) were

added to the digested solution after a 30 min cooldown period. Steam distillation (Distillation unit K-355, Büchi AG, Flawil, Switzerland) was performed for 4 min. The distillate was collected in a titration flask containing 60 mL boric acid 4 % (*v*/v). Finally, 5 drops of Tashiro indicator were added and the sample was titrated with 0.2 mol/L hydrochloric acid. Protein content was calculated according to Eq. 1:

$$\%P = \frac{V_{sample} - V_{blank}}{m_{sample} \bullet 1000} \bullet z \bullet M \bullet PF \bullet 100 \quad (1)$$

where *%P* represents the percentage of protein, V_{sample} is the volume of titrant used for the sample (mL), V_{blank} is the volume of titrant used for the blank titration (mL), m_{sample} is the exact weight of the sample (g), *c* is the concentration of the measuring solution, *M* is the molar mass of nitrogen (14.007 g/mol) and *PF* is the protein conversion factor (6.25).

2.4. Determination of fat content

Lipid extractions were performed according to Folch, Lees, and Stanley (1957) with some modifications. Briefly, lipids were extracted by mixing 10 g homogenized soybeans or raw soy-based product with 60 mL chloroform:methanol 2:1 mix (v/v) and subjecting them to magnetic stirring under argon atmosphere for 1 h. Afterwards, samples were filtered into a separation funnel containing 20 mL potassium chloride 8.8 g/L. After vigorous shaking and a 10 min settling period, the lower organic phase was collected and dried under nitrogen. Fat content was obtained by the differential weighing before phase collection and after drying. Flour and seitan had insufficient fat content to be successfully extracted.

2.5. Determination of the fatty acid profile by gas chromatography-flame ionization detector (GC-FID)

Fatty acids were extracted from the oil obtained in section 2.4 according to Lall, Proctor, and Jain (2009), with modifications by Pignitter et al. (2014). In short, fatty acids were derivatized into fatty acid methyl esters (FAME) and transferred into GC vials. GC-FID analysis was carried out as described by Grüneis et al. (2019). Further details about the extraction procedure and GC-FID analysis can be found in Supplementary method 1.

2.6. Determination of carbonyl content by 2,4-dinitrophenylhydrazine (DNPH) assay

Protein-bound carbonyls were analyzed in the plant-based products as an indicator of protein oxidation, following the modified version of the DNPH assay described by Soglia, Petracci, and Ertbjerg (2016). The optimal food:buffer ratios (*w/v*) were determined to be 1:2 for tofu, 1:5 for tempeh, 1:3 for seitan and 1:4 for flour and soybeans. Further method details are described in Supplementary method 2.

2.7. Determination of AGEs by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Selected lysine-derived AGEs were extracted and analyzed following an acidic protein hydrolysis method adapted from Teerlink, Barto, Ten Brink, and Schalkwijk (2004). Prior to extraction, 1–3 g of tofu, tempeh or seitan samples were freeze-dried in a VaCo 5-II freeze dryer (Zirbus Technology, Bad Grund, Germany) to exclude differences in AGEs content exclusively due to different water content (Table S2). A total of 20 mg freeze-dried product or raw material was subsequently dissolved in 100 µL bidistilled water and 500 µL 100 mmol/L sodium borohydride dissolved in 200 mmol/L sodium borate buffer (pH 9.2), following a 2 h incubation at 25 °C. Afterwards, samples were mixed with 1 mL 40 % trichloroacetic acid (TCA), for protein precipitation, and centrifuged (16,000 × *g*, 10 min), removing the supernatant afterwards. The step was repeated with 1 mL 10 % TCA and 500 µL 6 mol/L hydrochloric acid were added to the pellet, following protein hydrolysis for 20 h at 110 °C in an incubator (VWR International, Vienna, Austria). After drying the samples under vacuum at 60 °C in an Eppendorf Concentrator Plus (Vienna, Austria), residues were dissolved in 500 µL 5 mmol/L perfluoropentanoic acid and passed through a 0.20 µm nylon syringe filter into HPLC vials.

Samples (10 µL) were then injected into a LCMS-8040 system (Shimadzu, Korneuburg, Austria) and separated on a Luna HILIC 200 Å column (100 mm × 3 mm × 3 µm, Phenomenex, Aschaffenburg, Germany), equipped with a precolumn (4 mm × 2 mm × 3 µm, Phenomenex). Mobile phase A consisted of 6 mmol/L ammonium formate and 0.1 % formic acid (*v*/v) in water (pH 2.4), while B was 80 % acetonitrile with 0.1 % formic acid (v/v) and 20 % A (v/v). The HPLC gradient was: 0–2 min 90 % B; 3–5 min 87 % B; 9–15 min 30 % B; 20–30 min 90 % B. Flow rate was 0.4 mL/min and the oven temperature was 25 °C.

The ESI-triple quadrupole MS settings were: nebulizing gas flow (N_2) 3 L/min, drying gas flow (N_2) 13 L/min, desolvation line temperature 170 °C, heat block temperature 350 °C and argon as collision-induced dissociation (CID) gas (230 kPa). Targeted MS analyses were performed by multiple reaction monitoring (MRM) in positive mode, with the transitions shown in Table S3. Standard curves of CML and L-lysine were measured (Table S1), while N(6)-carboxyethyllysine (CEL) and pentosidine were relatively quantified by area under curve (AUC).

2.8. Determination of acrylamide by LC-MS/MS

Extraction of acrylamide was performed according to Gökmen, Palazoglu, and Senyuva (2006), with slight modifications. Briefly, 1 g ground samples were mixed with 500 µL Carrez I solution and 500 µL Carrez II solution, and 0.2 mmol/L acetic acid was added until a total volume of 6 mL was reached. After vigorous mixing, samples were centrifuged (2540 × *g*, 10 min, −5 °C) and the clear supernatants were collected and pressed through 0.20 µm nylon syringe filters into HPLC vials.

A total of 10 µL sample was injected into a LCMS-8040 system (Shimadzu, Korneuburg, Austria) and separated on a Shim-pack VP-ODS reversed-phase column (150 mm × 4.6 mm × 5 µm, Shimadzu), equipped with a Shim-pack GVP-ODS precolumn (10 mm × 4.6 mm × 5 µm, Shimadzu), with an isocratic mixture of water with 0.2 % formic acid (*v*/v) and 0.01 mmol/L acetic acid at a flow rate of 0.4 mL/min and 25 °C.

MS settings were as described in section 2.7, except for a drying gas flow (N_2) of 12 L/min and a desolvation line temperature of 250 °C. MS analyses were performed in positive mode, with the MRM transitions shown in Table S3. An acrylamide calibration curve was measured for quantification (Table S1).

2.9. Analysis of lipid oxidation products by spectrophotometric assays and gas chromatography coupled to mass spectrometry (GC–MS)

The extent of lipid oxidation in the soy-based products was evaluated either directly from the products or from the extracted fat samples obtained in section 2.4. Primary lipid oxidation products, namely hydroperoxides, were determined in the extracted soy-based fat samples, according to the ferrous oxidation-xylenol orange (FOX) method described by Shantha and Decker (1994). In-detail description of the procedure used is available in Supplementary method 3.

Analysis of malondialdehyde (MDA), a representative secondary lipid oxidation product, was carried out in the soy-based products by the thiobarbituric acid reactive substances (TBARS) assay, as described by Mendes, Cardoso, and Pestana (2009) and Papastergiadis, Mubiru, Van Langenhove, and De Meulenaer (2012), with the modifications listed in Supplementary method 4.

Lastly, analysis of volatile oxidation compounds was performed directly from the food samples according to Alberdi-Cedeño, Ibargoitia,

and Guillén (2020), by transferring a total of 2.5 g soy-based product to a 20 mL headspace brown glass vial prior to analysis by solid phase microextraction (SPME) followed by GC–MS, with the parameters described in Supplementary method 5.

2.10. Determination of tocopherols by high performance liquid chromatography coupled to diode array detection (HPLC-DAD)

The content of natural vitamin E was evaluated as described in Pignitter et al. (2014). Extracted fat samples (50 mg) were dissolved in 1 mL isopropanol and 1 μL tocol (5 mg/mL) was added as internal standard. Samples were then filtered through a 0.22 μm PVDF syringe filter into HPLC vials and injected (10 μL) into a LC-20 system (Shimadzu, Korneuburg, Austria), with the settings described in Supplementary method 6. For quantification, external standard curves of α-, γ- and δ-tocopherol were measured (Table S1).

2.11. Data processing and statistical analysis

Integration of chromatographic data was performed with LabSolutions 5.0 (Shimadzu, Korneuburg, Austria) and, in the case LC-MS/MS data, additionally with MacCoss Lab Software Skyline 22.0 (MacLean et al., 2010). Further data processing and statistical analysis were carried out in GraphPad Prism 9.1.0 (Boston, MA, USA).

All experiments were carried out with at least three independent replicates ($n \geq 3$). Significant outliers ($p < 0.05$) were excluded according to Grubbs, while normal distribution and equal variances were confirmed by performing Shapiro-Wilk and Brown-Forsythe tests, respectively. Significant differences ($p < 0.05$) between sample groups were tested by one-way analysis of variance (ANOVA) followed by Šídák's multiple comparisons post-hoc test. In addition, correlation between different parameters was analyzed by a two-tailed Pearson's correlation test. Limits of detection (LOD) and quantification (LOQ) were determined by a signal-to-noise ratio greater than 3 and 10, respectively (Harmonised Tripartite Guideline, 2005).

3. Results and discussion

3.1. Impact of processing on the protein and lipid composition of plant-based products

The protein content of the studied soybeans was 36.6 ± 0.7 g/100 g food, and was significantly reduced ($p < 0.001$) after the preparation of tofu and tempeh, being significantly higher ($p < 0.001$) in tempeh than in tofu (Table 1). These results were expected considering the manufacturing of both tofu and tempeh strongly rely on water uptake to acquire the desired consistency (Esaki et al., 1996; Li et al., 2015). Additionally, the smaller decrease of protein content in tempeh can be attributed to the fact that the whole soybeans were cooked, resulting in a lower exposure than for tofu processing. Regarding seitan, an inverse trend was observed: protein content increased from 10.8 ± 0.2 g/100 g food to 24.1 ± 0.3 g/100 g food when producing seitan from flour (Table 1). This enrichment can be explained by the leaching of starch that occurs during flour processing, decreasing the starch content while proteins such as gluten are retained (Anwar & El-Chaghabi, 2019).

Table 1
Fat and protein content of plant-based products and their respective raw materials. Data is shown as mean ± SD (n ≥ 3). Different lower-case letters (a, b, c, d) show, for each parameter, statistically significant differences ($p<0.05$) between products.

Product	Fat content (g/100 g food)	Protein content (g/100 g food)
Tofu	5.5 ± 0.4^{a}	12.5 ± 2.1^{a}
Tempeh	6.6 ± 0.5^{b}	18.5 ± 1.7^{b}
Seitan	-*	24.1 ± 0.3^{c}
Soybean	9.7 ± 0.3^{c}	36.6 ± 0.7^{d}
Flour	-*	10.8 ± 0.2^{a}

* Insufficient fat content recovered.

The composition of soybeans, especially protein and amino acid composition, is known to differ between varieties (Kudełka, Kowalska, & Popis, 2021). To evaluate changes exclusively due to different processing techniques, tofu and tempeh were made from the same batch of soybeans. In comparison to values reported previously in meat, the protein content of the studied tofu (12.5 ± 2.1 g/100 g) was similar to that of Frankfurt sausages (12.4 g/100 g), while in tempeh (18.5 ± 1.7 g/100 g) it was comparable to that of chicken drumsticks (18.2 g/100 g) (Andersen, Souci, Forschungsanstalt, & für Lebensmittelchemie., 2011). The protein content of the studied seitan was close to values reported for turkey breasts (24.1 g/100 g), which are regarded as high-protein meat (Andersen et al., 2011). Consequently, consumption of seitan may contribute to prevent the protein deficit associated with vegan diets (Bakaloudi et al., 2021). However, soy-based products are known for their rich amino acid profile and might still be needed to ensure the uptake of complete protein (Kudełka et al., 2021).

The lipid content of soybeans experienced a significant decrease ($p < 0.001$) in total fat when tofu or tempeh were produced (Table 1). Soybeans have been reported to consist of 8.1 to 24 % (*w*/w) lipids, evidencing the soybean variety and the growing conditions can greatly impact their lipid content (Medic et al., 2014). The soybeans used for this study had a relatively low fat percentage within this range. The lipid content of tofu (5.5 ± 0.4 g/100 g food) and tempeh (6.6 ± 0.5 g/100 g food) was also in line with results previously reported (Haron & Raob, 2014; Li, Chen, et al., 2015). The significantly lower fat loss observed for tempeh may be attributed to protective effects conferred by cooking whole beans. The fatty acid profile of soybeans was not notably altered by the preparation of tofu and tempeh (Fig. S1). PUFAs, mainly linoleic acid, represented 62.31–65.17 % of the fatty acid composition, highlighting soybeans as a source of beneficial fats in vegan diets (Pignitter & Somoza, 2012).

3.2. Effects of processing on the extent of protein oxidation in plant-based products

The degree of protein oxidation was evaluated for each plant-based product in each processing stage by analyzing their carbonyl content (Fig. 1). Production of firm tofu seemed to promote protein oxidation, as evidenced by the significant increase ($p < 0.001$) in carbonyl content. Frying tofu further increased the carbonyl content value, experiencing a total 3.7-fold increase between the raw material stage (soybeans) and the fried product stage. Tempeh production showed the opposite trend, as shown by the significant decrease ($p < 0.05$) in carbonyl content when comparing soybeans to the raw tempeh samples. Since production of tempeh involved a significant decrease ($p < 0.001$) of protein (Table 1), it is likely that heating of the whole beans caused the loss of mainly already oxidized protein, especially in the outer layer, protecting most of the protein in its non-oxidized state. A significant increase ($p < 0.05$) in carbonyl content was observed after frying, although it did not reach the levels of carbonyl content in soybeans. For seitan, production had no significant impact ($p > 0.05$) on protein oxidation, although frying increased the carbonyl content ($p < 0.05$). Production of seitan is a softer processing method in comparison to the other substitutes, thus no impact was expected. The lack of lipid in flour likely contributed to this, as the progress of lipid oxidation has been stated to indirectly promote protein oxidation (Estévez et al., 2022). Frying under the selected conditions increased the content of carbonyls for all three of the studied products. Nevertheless, this increase was more pronounced in firm tofu than in tempeh or seitan. When comparing products of the same processing stage, this tofu consistently showed higher carbonyl content, whereas there was no significant difference ($p > 0.05$) between tempeh and seitan.

Protein oxidation has a negative impact on its quality and nutritional value since modification of protein-bound amino acids results in

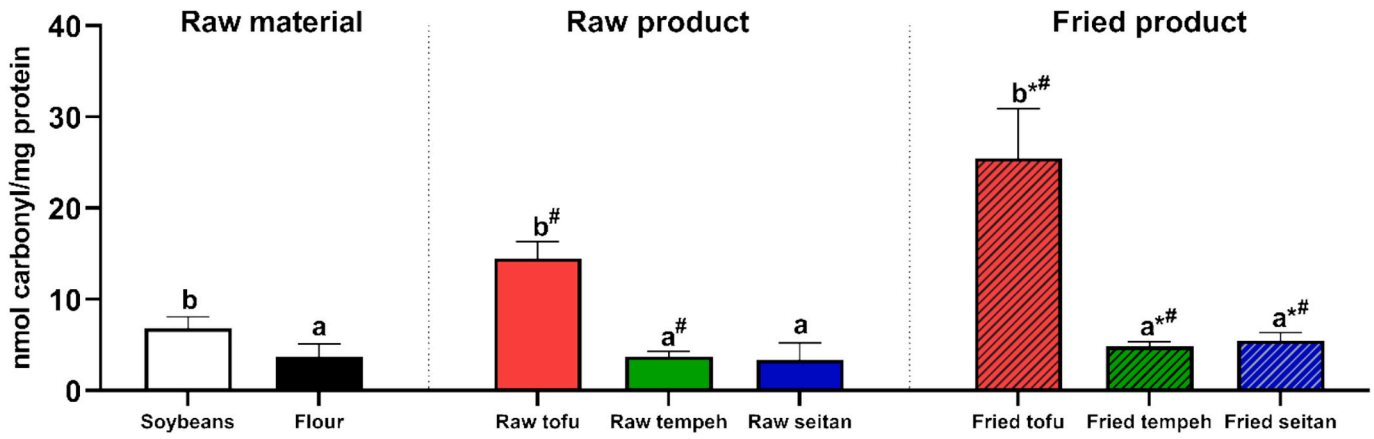

Fig. 1. Comparison of the carbonyl content between different preparation stages of tofu, tempeh, and seitan. Data is presented as mean + SD ($n \geq 9$). Different lowercase letters (a, b) show statistically significant difference ($p < 0.05$) between products within the same production stage, whereas # and * indicate statistically significant difference of each product compared to their raw material and to their raw products stages, respectively.

decreased digestibility and loss of essential amino acids (Lund, Heinonen, Baron, & Estévez, 2011). In addition, the intake of oxidized proteins has been stated to contribute to *in vivo* protein oxidation and has been associated with cytotoxic effects and health issues such as increased diabetes risk (Estévez & Luna, 2017). Several studies have analyzed the carbonyl content as a mean of monitoring protein oxidation in meat products and muscle foods (Estévez et al., 2022; Soglia et al., 2016). While the values greatly vary between different food systems, they are generally above the values obtained for tempeh and seitan in the present study, suggesting that these products contain a relatively low content of carbonyls. Considering that each carbonyl species may have a different effect in the human body, it is challenging to determine a total carbonyl value that can be considered safe. Furthermore, the extent to which dietary carbonyls contribute to *in vivo* protein oxidation is not yet fully understood (Estévez et al., 2022). While there is no established limit for carbonyls in the diet minimizing their consumption can be recommended. This study showed that tempeh and seitan might be an alternative to firm regular tofu in this regard.

The DNPH method is widely applied for protein oxidation determination and has been optimized to minimize interferences in the detection of protein-bound carbonyls in food (Soglia et al., 2016). However, it does not provide exact information on the nature of the detected carbonyls. Its intended aim is the determination of primary carbonyls such as α-aminoadipic and γ-glutamic semialdehydes, generated due to the oxidative deamination of alkaline amino acids, normally by a metal-catalyzed process or Maillard reaction dicarbonyls (Estévez et al., 2022; Levine et al., 1990). Nevertheless, it has been recently pointed out that DNPH is able to react with secondary protein-bound carbonyls that are formed due to interaction with lipid oxidation products, especially MDA (Estévez et al., 2019). While this is unlikely to happen in seitan production due to its low lipid content, it might be a contributing factor in the results obtained for the soy-based products and fried products in general. A previous study found carbonyl contents of 7–10 nmol carbonyls/mg protein in soy protein isolate, which involves more extensive processing than traditional tofu production (Zhang et al., 2017). Those results, however, were lower than the values obtained for firm tofu in the present study, which suggests that the presence of MDA might be generating protein-bound secondary carbonyls, leading to a higher number of carbonyl moieties detected. Therefore, it becomes clear that protein oxidation of soy-based products needs to be studied along with lipid oxidation, as in this study, to better understand oxidation processes during processing. Furthermore, while the DNPH assay is still being widely used by many researchers to measure protein oxidation (Soglia et al., 2016; Zhang et al., 2017), future studies should incorporate more specific methods to investigate the nature and origin of the carbonyls formed.

3.3. Impact of production and cooking on the occurrence of MRPs in plant-based products

Maillard reactions remain a controversial topic for the food industry, since they are responsible for the formation of compounds that positively influence the flavor, color, and texture of certain food products, but also compounds associated with health issues such as the promotion of diabetes and cardiovascular diseases (Starowicz & Zieliński, 2019; Tamanna & Mahmood, 2015). The present study focused on MRPs that are considered health detrimental.

3.3.1. Occurrence of lysine-derived advanced glycation end products (AGEs)

Lysine has been reported as one of the main essential amino acids in different soybean and wheat varieties (Kudełka et al., 2021; Laze et al., 2019). AGEs formation from this amino acid during processing was investigated, particularly CML content, which was expressed in relation to sample weight in order to reflect its uptake directly depending on product intake (Fig. 2A). Data was also expressed in relation to protein and lysine content (Table S4). Interestingly, this AGE was detectable in the raw materials, with a similar content for both soybeans and flour (Fig. 2A). Subsequently, it followed a different trend depending on the processing technique applied. For firm tofu, a significant increase ($p < 0.001$) in CML content was observed during production, but it did not change significantly ($p > 0.05$) after frying. An overall increase was found in the case of tempeh as well, although it developed in the opposite manner: an increase ($p < 0.001$) was only observed after frying, with no significant change ($p > 0.05$) between the raw material and raw product stages. As shown, the CML content was ultimately similar in both firm tofu and tempeh at the end of the processing. However, it is important to acknowledge the significantly lower amount of CML in raw tempeh in comparison to raw firm tofu ($p < 0.001$) since industries or consumers may choose to use other cooking conditions. Softer cooking techniques such as steaming or frying under milder conditions could limit the increase observed for tempeh in the present study, therefore reducing CML intake. Based on our results, this would not be a possibility for this specific tofu, even if eaten raw, as CML is known to be stable in food matrices once it is generated (Huang, Huang, & Dong, 2023). This suggests adopting strategies used in tempeh in traditional firm tofu production, such as the addition of organic acids, could be beneficial to limit the occurrence of the Maillard reaction, which agrees with previous evidence (Lund & Ray, 2017). In contrast, no overall increase in CML was detected in seitan due to processing (Fig. 2A). While

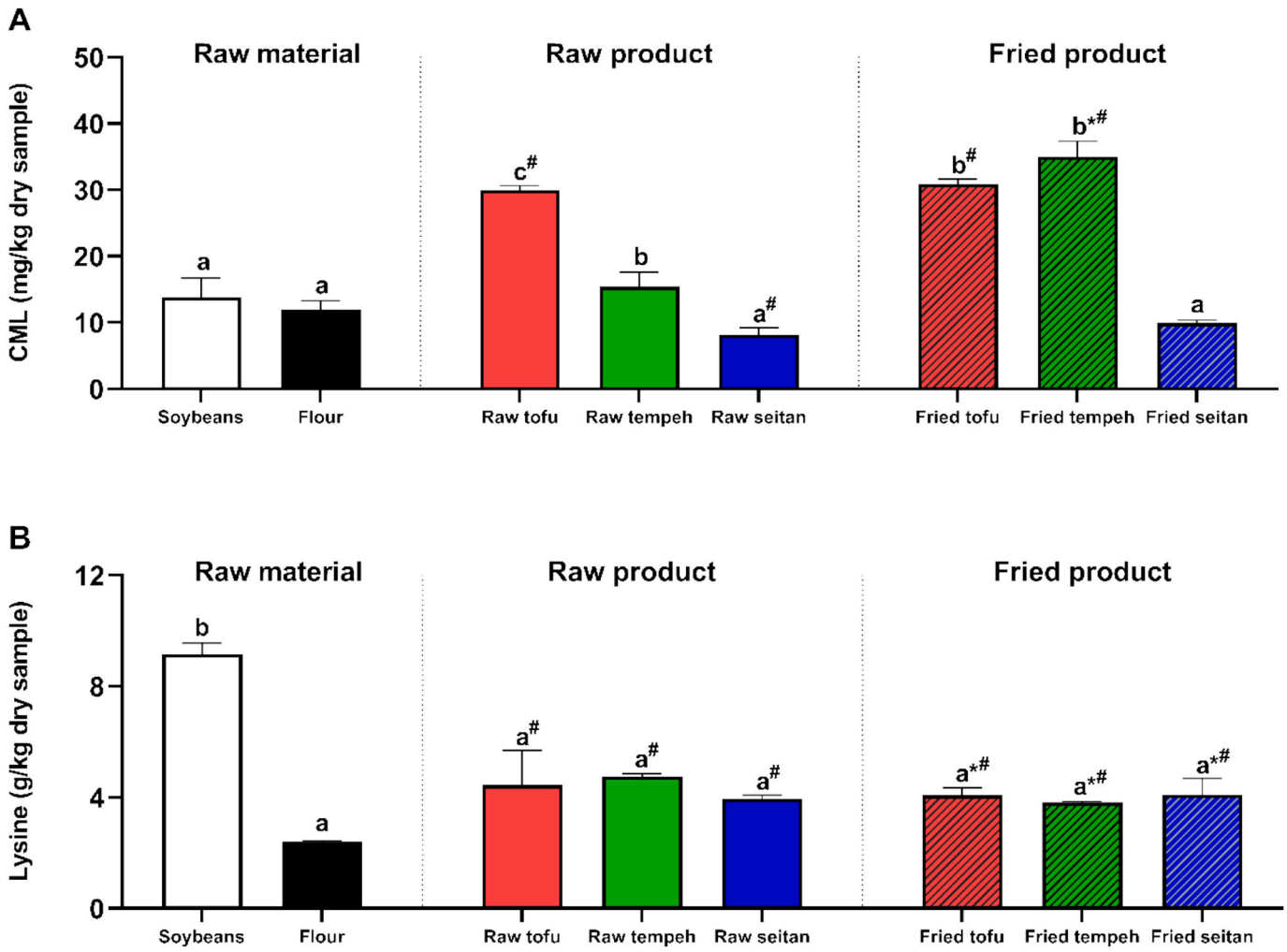

Fig. 2. Comparison of the (A) CML and (B) lysine contents between different preparation stages of tofu, tempeh, and seitan. Data is presented as mean + SD ($n = 3$). Different lower-case letters (a, b, c) show statistically significant difference ($p < 0.05$) between products within the same production stage, whereas # and * indicate statistically significant difference of each product compared to their raw material and to their raw products stages, respectively.

seitan production by starch leaching significantly lowered CML content ($p < 0.05$), frying in canola oil raised it back to concentrations not significantly different ($p > 0.05$) from the initial flour. As no significant differences ($p > 0.05$) between their dry weigh percentage were found within each stage (Table S2), seitan proved to be the product, in both raw and fried stages, with the lowest CML content out of the three plant-based products.

CML is one of the most used markers for AGEs due to its presence in several food products (Hull, Woodside, Ames, & Cuskelly, 2012). Upon intake, it can accumulate in certain tissues such as kidney and liver, along with the CML formed *in vivo* during aging (Li, Zeng, et al., 2015; Somoza et al., 2006). While a direct causality between CML intake and disease has not been demonstrated, it has been associated with the development of atherosclerosis, diabetes, and Alzheimer's disease, as well as promotion of oxidative stress and inflammation (Golchinfar et al., 2023; Nguyen et al., 2014). Lysine can be transformed into CML in food by oxidation of Amadori products formed *via* the Maillard reaction or by reacting with glyoxal (Poojary et al., 2020). Thus, lysine content of each plant-based product was monitored throughout processing (Fig. 2B). Low intake of this essential amino acid has been linked to several physiological disorders such as kidney stones, anemia, fatigue, or appetite loss. Lysine deficiency, while generally rare, can happen during vegetarian diets, emphasizing the importance of lysine-rich foods consumption when following this lifestyle (Căpriţă & Căpriţă, 2009). A daily intake of 12–45 mg lysine/kg body weight is recommended for human adults (Tomé & Bos, 2007). In the present study, all three plant-based products had a similar lysine content in each of the raw and fried product stages (Fig. 2B), making them a potential source of lysine. Nevertheless, it is important to take into consideration, apart from the final lysine content, the changes this amino acid experienced during processing, as these might hint towards the formation of modified lysine residues. In our study, both tofu and tempeh experienced a loss of lysine content with each processing stage, whereas seitan showed the opposite trend (Fig. 2B). In all cases the most pronounced changes occurred between the raw material and the raw product stages. These variations can be explained by the simultaneous changes on protein content, as they followed the same trend (Table 1). Therefore, lysine content was also expressed in relation to protein (Table S4). However, the protein content was partially influenced by the water uptake due to processing, whereas this possibility was excluded for the lysine analysis due to the use of dried samples. Therefore, it is likely that lysine modifications are playing an important role in the fate of this essential amino acid as well. A negative correlation ($r = -0.9999$, $p < 0.01$) was found between CML and lysine contents for tofu by Pearson's correlation analysis, while non-significant correlations ($p > 0.05$) were observed for tempeh and seitan. Furthermore, in the case of tempeh, a lysine content decrease was not accompanied by an increase in CML, suggesting the possibility of other lysine modifications being involved.

As a result, two other lysine-derived AGEs, CEL and pentosidine, were analyzed. CEL is formed from the reaction between lysine and

methylglyoxal and shares properties with CML, contributing in a similar manner to the promotion of chronic diseases. It represents an AGEs marker commonly found in meat products, albeit not to the same extent as CML (Zhu, Huang, Cheng, Khan, & Huang, 2020). Pentosidine is a cross-linking product generated by the interaction of lysine, arginine, and a sugar molecule, typically ribose, although other carbohydrates such as glucose and fructose have been shown to be able to form this AGE as well (Li & Yu, 2018). While its pathogenesis mechanism has not been elucidated and its overall contribution to the AGEs content in food has been stated to be lesser compared to CML or CEL, its association with potential health risks still makes it a relevant AGE to consider due to its presence in many food products (Li & Yu, 2018; Poojary et al., 2020). In the case of the present study, CEL showed the same trend for firm tofu and tempeh as CML throughout the processing steps, exclusively detecting significant increases ($p < 0.01$) after tofu production and tempeh frying, whereas seitan processing did not promote CEL formation at all (Fig. S2). Pentosidine formation was increased ($p < 0.01$) during both production and frying for both firm tofu and tempeh, without significant changes ($p > 0.05$) during seitan processing (Fig. S3). Unlike CML and CEL, the pentosidine content in the raw material stage (soybeans and flour) was not quantifiable, and the firm tofu revealed higher values than tempeh after frying. In addition, the significant pentosidine increase ($p < 0.05$) on tempeh production can partially explain the loss on lysine observed (Fig. 2B), although other lysine-derived AGEs, such as pyrraline, that were not measured in the present study due to their low stability under acidic conditions, might also be involved (Li & Yu, 2018). CML and CEL, which are generated through similar pathways, shared the same trend while pentosidine showed different changes, suggesting that processing may have a different impact on the content of an individual AGE depending on its formation pathway. Furthermore, lipid oxidation may have an impact, as certain α-dicarbonyls such as glyoxal and methylglyoxal, which are needed for the formation of CML and CEL, respectively, can originate not only from the Maillard reaction but from lipid peroxidation as well (Golchinfar et al., 2023). Considering the lack of lipids in seitan, this is likely the reason why this product displayed the lowest AGEs content. Even though lipid oxidation has been stated not to influence the formation of AGEs like pentosidine (Li & Yu, 2018), it becomes clear that, in order to fully understand the influence of processing techniques on food quality, oxidative processes such as lipid and protein oxidation as well as AGEs generation should no longer be evaluated as separate processes due to their complex interrelationships.

AGEs have been previously determined in several types of food products, especially meat products (Hull et al., 2012). However, meat substitutes have been underrepresented in these studies, with the existing evidence being limited to the analysis of CML as a reference for total AGEs in different tofu varieties (Uribarri et al., 2010). To our knowledge, this is the first time a study involving different plant-based protein sources has been carried out with emphasis on how processing influences individual AGE species. An overall AGEs intake limit has not been established, as the heterogeneity of these compounds and their complex interrelationships with other oxidative processes render it challenging. Nonetheless, it is generally recommended to minimize their intake (Zhu et al., 2020). The CML content in the three analyzed plant-based products was lower than previously reported in meat and fish (Hull et al., 2012). It is important to acknowledge that the analyzed AGEs were in protein-bound state, the main form in which they are present in several foods (Poojary et al., 2020). However, free AGEs could also exist within the samples, increasing the total AGEs content. Furthermore, AGEs have been stated to absorb more efficiently in the gastrointestinal tract when they are in free or dipeptide form (Golchinfar et al., 2023; Hellwig, Matthes, Peto, Löbner, & Henle, 2014). Therefore, the question whether free AGEs formation has a substantial impact on plant-based protein sources requires more research.

3.3.2. Acrylamide formation

Acrylamide content was determined for each plant-based product in each processing stage (Table 2). This contaminant was not detected in any stage in tempeh and seitan, and it could only be quantified in firm tofu after frying at 190 °C for 1.5 min. Surprisingly, an acrylamide amount below the limit of quantification could be already detected in raw firm tofu, and this did not change after frying at 130 °C for 3.5 min, indicating that higher heating temperatures greatly contribute to acrylamide formation in tofu rather than higher cooking times. Acrylamide is a class 1B carcinogen and mutagen formed mainly due to the Maillard reaction between a reducing sugar and an amino acid, mainly asparagine at temperatures above 120 °C (Rifai & Saleh, 2020). A temperature well above 120 °C (190 °C) was therefore selected for acrylamide analysis, to better evaluate the potential of the plant-based products to generate this compound, based on a previous study (Zyzak et al., 2003). However, our results show that a small non-quantifiable amount of acrylamide was generated during the boiling process for tofu production, albeit a significant acrylamide increase ($p < 0.05$) was only present after frying at 190 °C. Asparagine is not typically one of the main amino acids in soybeans, which might explain the low acrylamide formation at temperatures such as 130 °C (Panthee, Pantalone, Saxton, West, & Sams, 2006).

Since acrylamide was detected in food, it has been a topic of concern due to associations with different types of cancer. However, its mode of action in the human body has not been entirely elucidated, which has led researchers to point out limitations of these claims (Riboldi, Vinhas, & Moreira, 2014). While the World Health Organization (WHO) has published a limit of 0.5 μg/L for acrylamide in drinking water due to use of polyacrylamide in water treatments (World Health Organization, 2011), maximum limits for food products, despite especially discussed for bread and cereal-based products, have not been established yet. This is likely due to the uncertainty regarding acrylamide exposure and intake by consumers, as data on these parameters is commonly solely based on food frequency questionaries (FFQs), which tend to offer limited information, particularly because acrylamide formation has not been investigated for many food products yet (Rifai & Saleh, 2020).

The acrylamide content of fried firm tofu at 190 °C (196.0 ± 15.4 μg/kg food) was comparable to the up to 202 μg/kg food in meat products described by Goerke et al. (2019). However, this study also reported values of 50–100 μg acrylamide/kg food in plant-based products like tofu, which may indicate that the processing conditions used in the present study were especially harsh. Contrary to our results, that study also detected acrylamide in seitan-based meals (Goerke et al., 2019). Since the origin and prior treatment of tofu and seitan were not stated comparisons are difficult, further highlighting the importance of considering the impact of processing techniques on MRP generation. Kim et al. (2007) could not detect acrylamide in soy curd, a product similar to raw soft tofu, further confirming acrylamide formation during production of soy-based products is extremely low. To our knowledge there is no evidence on acrylamide analysis in tempeh prior to our work. Recently, consumption of tempeh has been associated with suppression of colon carcinogenesis in rats, suggesting a low amount of carcinogenic components (Divate et al., 2023).

Optimization of processing techniques to generate only beneficial

Table 2
Acrylamide content of meat substitutes and their respective raw materials. Data is shown as mean ± SD (n = 3).

Stage	Acrylamide (μg/kg)		
	Tofu	Tempeh	Seitan
Raw material (Soybeans/Flour)	< LOD		< LOD
Raw product	< LOQ	< LOD	< LOD
Fried product at 130 °C	< LOQ	< LOD	< LOD
Fried product at 190 °C	196.0 ± 15.4	< LOD	< LOD

LOD: Limit of detection; LOQ: Limit of quantification.

MRPs remains a challenge. It is therefore imperative to understand the influence of processing conditions and cooking methods on the individual products. For instance, microwave heating of soybeans was previously reported to result in higher acrylamide content when the temperature applied was lower, as higher temperatures started promoting its degradation, which differs from our results obtained after frying (Žilić et al., 2014). In this study, high temperature cooking was only performed after a considerable water uptake by the product, therefore hindering MRPs generation, as higher moisture content is known to slow down the Maillard reaction (Tamanna et al., 2015). Therefore, our results highlight the need to evaluate the impact of processing techniques applied by industry and consumers on MRPs formation.

3.4. Effects of processing on lipid oxidation in soy-based products

As only the soy-based products contained a considerable amount of fat (Table 1), lipid oxidation was monitored through the different processing stages. Flour and seitan were, therefore, excluded for the following analyses.

3.4.1. Occurrence of primary lipid oxidation products

The total lipid hydroperoxide content was determined by the PV (Fig. 3A). During both production and frying of tofu the hydroperoxide content increased notably ($p < 0.001$), evidencing an advance in lipid oxidation. The PV was significantly higher in tofu both raw and fried than in tempeh, where it only increased during frying. For refined and cold-pressed vegetable oils the established limit for PV are 10 and 15 meq O_2/kg, respectively (Codex Alimentarius Commission, 1999). For other foods no general limit has been established. Recently, a critical PV of 156 meq O_2/kg for foods has been suggested (Huang et al., 2022), far above all the PVs observed in the current study (Fig. 3A), deeming both products acceptable in this regard. A potentially higher unsaturated fatty acid intake can still be expected from tempeh consumption in comparison to the firm tofu, since both products showed a similar fatty acid composition while tempeh maintained a higher fat content (Fig. S1, Table 1). Nevertheless, analysis of secondary lipid oxidation products was necessary to ensure the lower hydroperoxides content in tempeh was not due to a more advanced lipid oxidation state.

3.4.2. Occurrence of secondary lipid oxidation products

TBARS content was analyzed in the soy-based products (Fig. 3B).

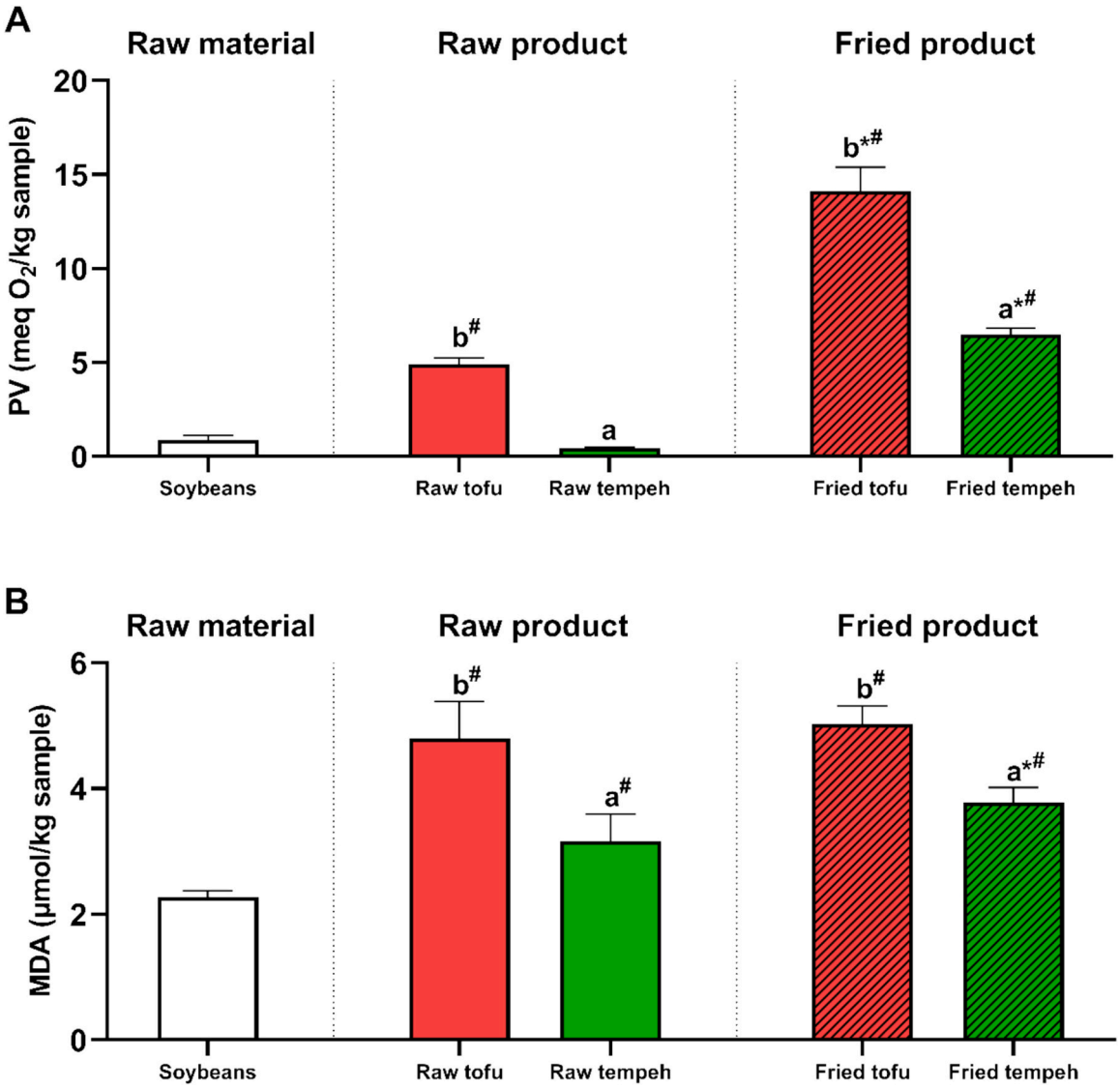

Fig. 3. Comparison of (A) the peroxide value and (B) malondialdehyde contents between different preparation stages of tofu and tempeh. Data is presented as mean + SD ($n \geq 3$). Different lower-case letters (a, b) show statistically significant difference ($p < 0.05$) between products within the same production stage, whereas # and * indicate statistically significant difference of each product compared to their raw material and to their raw products stages, respectively.

Firm tofu production led to an increase in MDA concentration, whereas frying did not. MDA content in tempeh increased during both processing steps, maintaining significantly lower values ($p < 0.001$) than tofu, once again proving a lower oxidative progress due to tempeh processing techniques. Interestingly, a base amount of MDA (2.27 ± 0.10 μmol MDA/kg) was detected in soybeans prior to processing (Fig. 3B), suggesting a preexisting degree of lipid peroxidation. A similar value was reported by Sohrabi, Heidari, Weisany, Golezani, and Mohammadi (2012) for chickpea plants (2.24 ± 0.11 μmol MDA/kg), which are commonly used for meat substitutes as well. A value of 20.8 μmol MDA/kg has been reported as a limit above which food products are perceived too rancid for consumption (Cong et al., 2020). In the present study all products showed values below 8 μmol MDA/kg, thus they can be considered fresh and safe to consume in terms of TBARS content (Cong et al., 2020).

While the TBARS method is widely used for evaluating the lipid oxidation state in different foods, certain limitations have been pointed out, such as the reactivity of other food components like sugars, certain lipids, oxidized proteins and other aldehydes and possible MDA generation during sample preparation (Pignitter & Somoza, 2012). The latter can potentially explain the TBARS increase found in raw tempeh while the PV showed no change. The PUFA to UFA and SFA ratio did not change due to tempeh production (Fig. S1), further supporting this claim as MDA is known to explicitly form from peroxidized PUFA (Pignitter & Somoza, 2012). Frying had little impact on MDA content in comparison to the other oxidation parameters measured. As discussed in section 3.2., MDA is capable of protein binding, generating adducts, which hinder the accessibility of TBA and lead to an underestimation of the MDA content (Estévez et al., 2019; Tsikas, 2017). This may explain the increasing hydroperoxides not being accompanied by increasing MDA during frying, especially in tofu, as well as the higher-than-expected carbonyl content detected by the DNPH assay (Zhang et al., 2017).

Volatile secondary lipid oxidation products were analyzed by GC–MS (Table S5). Three volatiles were identified in soybeans: 1-hexanol, 1-octen-3-ol and 2-pentylfuran, all of which were previously reported for these legumes (Boué, Shih, Carter-Wientjes, & Cleveland, 2003). These volatiles were also present in both processing stages of both soy-based products (Table S5). While firm tofu production either increased or did not impact their content, tempeh production caused an increase in 1-octen-3-ol and a decrease in 1-hexanol and 2-pentylfuran. 1-Hexanol, responsible for beany odor, was previously described to greatly diminish during tempeh fermentation, unlike 1-octen-3-ol and 2-pentylfuran, not previously known to be influenced by it (Feng, Larsen, & Schnürer, 2007). These results might be explained by the higher temperature used in tempeh production in comparison to prior literature covering volatiles in soy products (Feng et al., 2007).

Aside from these compounds, 12, 7, and 10 additional volatiles were identified in firm tofu (both raw and fried), raw tempeh, and fried tempeh, respectively, all of which were alkanals, alkenals, or alcohols. Common volatiles were compared between soy-based products and processing stages (Fig. 4). Frying of tempeh had a significantly lower impact ($p < 0.05$) on volatiles than frying of firm tofu (Fig. 4A and B), suggesting a higher protection against oxidation in the former. Frying of this tofu decreased the content of linoleic acid-derived aldehydes, such as 2-octenal, while promoted the formation of octanal, nonanal and 2-undecenal, which are associated with the oxidative degradation of oleic acid (Ludwig et al., 2021; Van Ba, Ryu, Lan, & Hwang, 2013). Therefore, it appears that oxidation of linoleic acid advanced during tofu production while oleic acid oxidation took place during tofu frying, in which linoleic acid-derived aldehydes further reacted or decomposed, 2,4-decadienal being the only exception. As PUFA are known to be more prone to oxidation than MUFA, this trend is expected (Pignitter & Somoza, 2012). Nevertheless, the changes during production were not enough to notably alter the fatty acid distribution found in soybeans (Fig. S1). Even though volatiles were only semi-quantified (AUC) in this study, their content was generally higher in firm tofu than in tempeh, which became even more apparent after frying (Fig. 4C and D). Especially striking was the 2,4-decadienal content in raw firm tofu, for which the mean area was more than 100 times higher than in tempeh. 2,4-Decadienal is an important lipid oxidation marker associated with frying

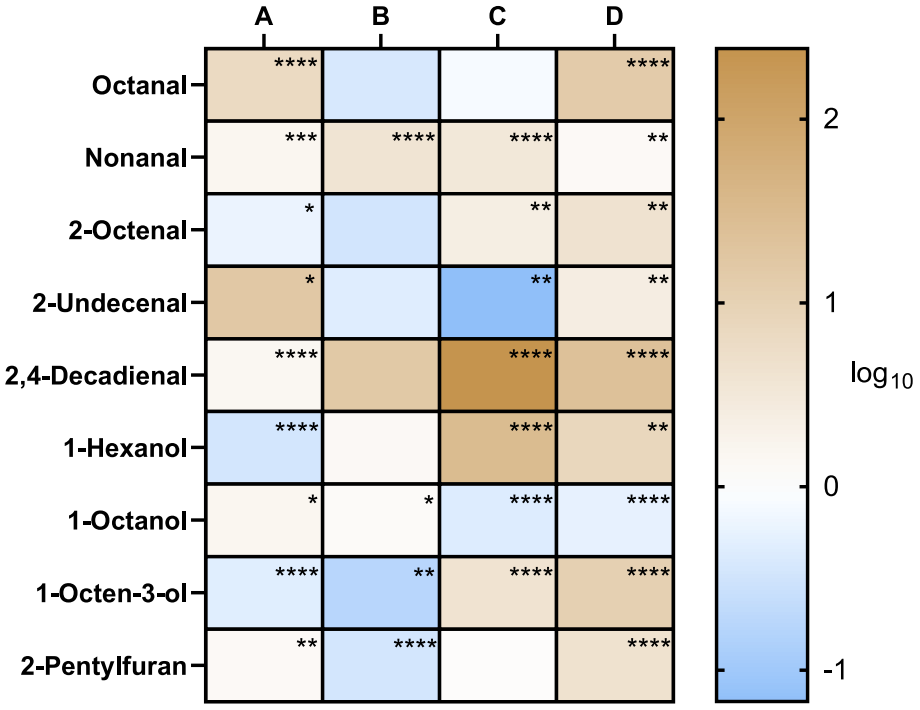

Fig. 4. Comparison of the peak areas of the common volatiles between tofu and tempeh in their raw and fried stages: (A) fried tofu compared to raw tofu, (B) fried tempeh compared to raw tempeh, (C) raw tofu compared to raw tempeh and (D) fried tofu compared to fried tempeh. Data is expressed as the decimal logarithm ($\log_{10}$) of the fold change between the mean areas of the compared groups. Statistically significant differences between the compared groups are indicated with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

processes and, while it has been previously identified in raw tofu, prior studies in tempeh could only detect it after frying (Jeleń et al., 2013; Lee, Cho, & Lee, 2014). This supports our results, which suggest that tempeh processing advances lipid oxidation to a significantly lower degree in comparison to firm regular tofu processing. However, exceptions to this trend such as 2-undecenal in the raw stage and 1-octanol in both stages were found, as the content of these volatiles were significantly higher in tempeh than in tofu. To our knowledge, neither 2-undecenal nor 1-octanol have been described as fermentation products. Consequently, the prior soaking and heating steps applied to the soybeans might be responsible for their formation instead, indicating the volatiles profile of soy-based products is highly specific to the processing technique.

In addition, the use of canola oil likely contributed to the increase of lipid oxidation products after frying, as its lipids may also oxidize and be absorbed into the foods. Therefore, the occurrence of volatiles not detected in the raw products, such as 2-nonenal and 2-decenal in tempeh, but also a certain increase of the already present ones may have originated from the canola oil lipids rather than the soy-based products themselves. With oleic and linoleic acids as its two main fatty acids, canola oil heating has previously been shown to generate several oleic- and linoleic acid-derived volatiles, some of which identified in this study (Multari, Marsol-Vall, Heponiemi, Suomela, & Yang, 2019). Hence, the further increase in 2,4-decadienal upon frying might be explained due to the presence of the oil, as well as the inversed trend observed for 2-undecenal when comparing tofu and tempeh in each stage (Fig. 4C and D), since the products' different consistencies could lead to a different oil uptake degree. Furthermore, oxidation of canola oil lipids can be expected to contribute to the total hydroperoxide content of the fried stage (Fig. 3A), as heating is known to notably impact the PV of the oil (Multari et al., 2019). Moreover, the interaction of the oxidized lipids of the oil with the plant-based products of study could have further increased the concentration of AGEs reflected in the fried stage (Section 3.3), as lipid oxidation has been associated with the promotion of the Maillard reaction as well (Zhu et al., 2020).

Overall, our findings highlight the protective effect against lipid oxidation that fermentation of whole soybeans brings over homemade firm regular tofu processing. Soaking has been described as a key step influencing lipid oxidation, its conditions being determinant for lipid radical formation (Feng et al., 2020). Production of tempeh, therefore, ameliorates this effect as less surface area is exposed during soaking along with the use of fermentation and the addition of organic acids, leading to better oxidative stability upon frying in comparison to firm regular tofu.

3.4.3. Stability of tocopherols during processing

Tocopherols were analyzed in the soy-based products to study the fate of these natural antioxidants (Fig. 5). Their content in soybeans was comparable to the one described by Lim, Woo, Kim, Jong, and Lee (2007). Total tocopherols experienced a decrease ($p < 0.001$) during processing for both soy-derived products (Fig. 5A). The individual contents of γ- and δ-tocopherol (Fig. 5B and C) followed the same trend, suggesting soybean processing does not impact each tocopherol form differently. In all cases, most loss of tocopherol (70.08–90.47 %) occurred during the production step. α-Tocopherol was only detected in raw soybeans (1.45 ± 0.26 mg/100 g) emphasizing how impactful production of soy-based foods on the content of this antioxidant is (Fig. S4).

Tocopherols content remained significantly higher ($p < 0.05$) in tempeh than in firm tofu for both raw and fried stages, once again evidencing the better resistance of tempeh to lipid oxidation. Fermentation processes have been stated to lead to the presence of antioxidants in tempeh, mainly isoflavones such as daidzein, genistein, 6,7,4′-trihydroxyisoflavone and 3-hydroxyanthranilic acid (Esaki et al., 1996; Murakami, Asakawa, Terao, & Matsushita, 1984), which may also play a role in the higher oxidative stability of this product. To our knowledge, this is the first time tocopherols were analyzed in tempeh. This is of special relevance, as a positive correlation between total tocopherols and linoleic acid, the main fatty acid in soybeans, has been described (Rani, Kumar, Verma, Shakya, & Chauhan, 2007).

Overall, our study analyzed the impact of processing of plant-based protein sources on lipid oxidation, protein oxidation and MRPs generation simultaneously for the first time, and provided evidence to suggest that these chemical reactions should be evaluated altogether during processing of protein-rich products, with the aim of finding strategies to limit these reactions more efficiently. Correlation analysis between different parameters measured (Fig. S5) showed some significant positive correlations ($p < 0.05$) between them, exhibiting their close relationship in plant-based protein alternatives. However, different products originated from the same type of reactions can behave differently, as shown for the different AGEs or the volatile lipid oxidation products detected. Therefore, the absence of direct correlations between certain products does not necessarily entail the lack of complex interrelations between them.

Under the conditions of this study, tempeh was the soy-based product that consistently showed superior resistance to oxidative processes, confirming our hypothesis that strategies such as fermentation and addition of antioxidants can be effectively used in plant-based protein sources to limit these processes. Nevertheless, this study explicitly evaluated freshly homemade products. Plant-based products are commonly stored at supermarkets for long periods of time, which only a few studies have investigated so far. Huang et al. (2022) evaluated the effect of tofu storage at different temperatures on the PV and TBARS content, while Lee et al. (2014) analyzed the formation of volatiles during storage at 4 °C. Future studies should compare the evolution of MRPs, protein and lipid oxidation products, and antioxidants between meat substitutes during storage, in order to obtain a full picture providing better understanding of the impact processing and storage have on the quality of these food products. Lastly, reducing sugars, key components in the Maillard reaction, might also be affected by processing (Poojary et al., 2020). Consequently, their content during production, storage, and cooking of plant-based products should be considered by future research, to further understand the extent to which MRPs formation originates from sugar moieties or from lipid-derived carbonyls.

4. Conclusion

Production of firm regular tofu promoted the formation of carbonyls and health detrimental MRPs to a higher extent than production of tempeh and seitan, as well as several lipid oxidation products in comparison to tempeh. Processing needed for this type of tofu also consistently showed a higher susceptibility to oxidative processes during frying, potentially leading to lower food quality in comparison to the other products. The combination of techniques involved in the production of tempeh generally showed a reduced progress on protein and lipid oxidation and MRPs generation, suggesting their implementation on the processing of other soy-based products might help preventing these chemical processes. Seitan contained the highest amount of non-oxidized protein, illustrating how the effects of processing can be crucially affected by the raw material and its nutrient composition. Therefore, our work highlighted, for the first time, the importance of simultaneously studying the influence of processing techniques of plant-based meat alternatives on lipid and protein oxidation and the Maillard reaction, hinting towards the need of adopting this side-by-side approach on a routine basis. Future studies should consider the research of alternative plant-based sources such as chickpeas or other soybean varieties as well as the influence of different storage conditions.

CRediT authorship contribution statement

Arturo Auñon-Lopez: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data

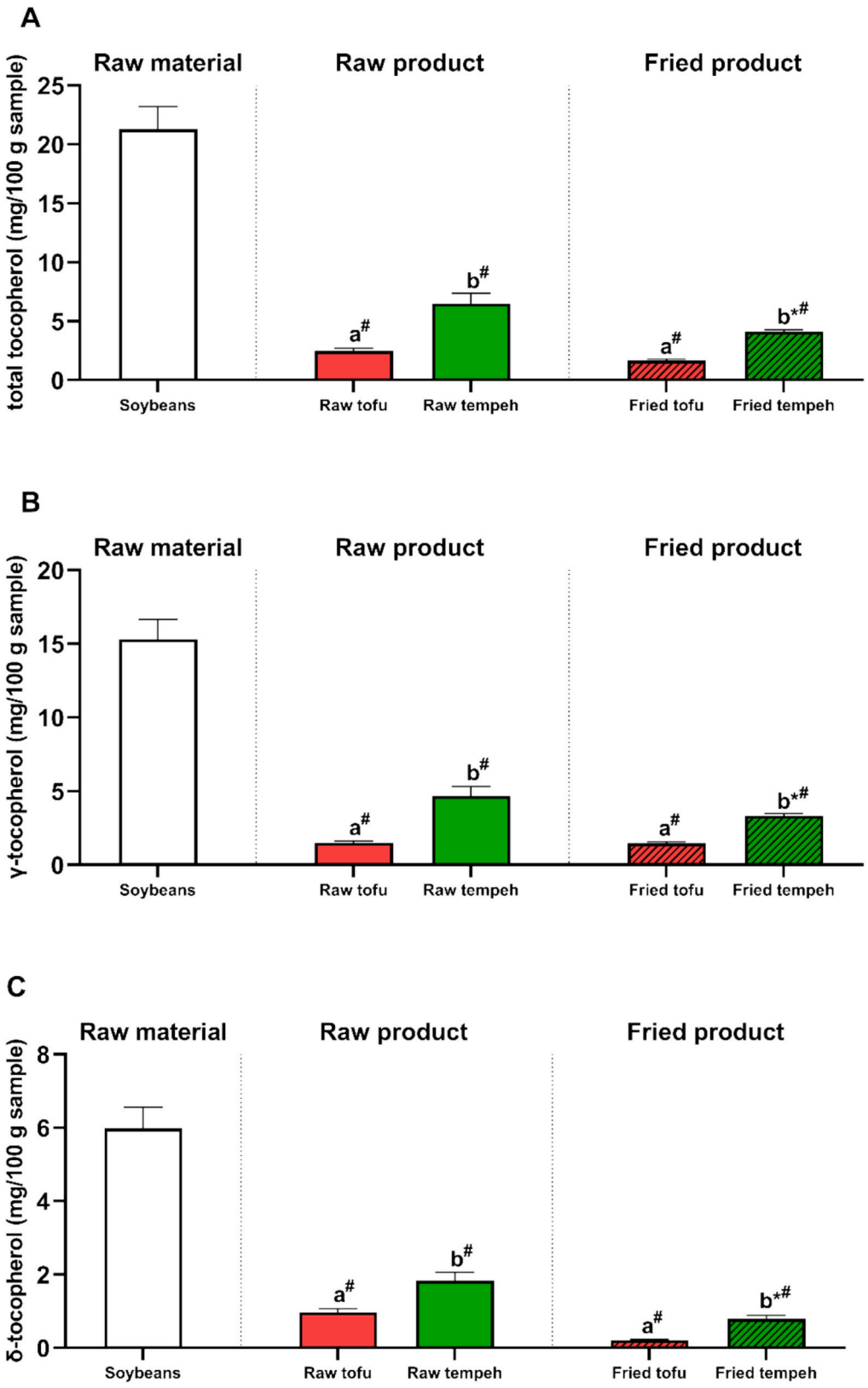

Fig. 5. Comparison of the (A) total tocopherol, (B) γ-tocopherol and (C) δ-tocopherol contents between different preparation stages of tofu and tempeh. Data is presented as mean + SD ($n \geq 3$). Different lower-case letters (a, b) show statistically significant difference ($p < 0.05$) between products within the same production stage, whereas # and * indicate statistically significant difference of each product compared to their raw material and to their raw products stages, respectively.

curation, Conceptualization. **Matthias Strauss:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Elena Hinterreiter-Kern:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Amelie Klein:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Elisabeth Varga:** Writing – review & editing, Methodology. **Marc Pignitter:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, 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.

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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.2024.142273.

Data availability

Data will be made available on request.

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