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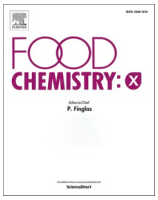

Paprika extract as a natural antioxidant in cold-stored pork patties: Effect on oxidative stability and heterocyclic amines inhibition

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ABSTRACT

In this study, we compared the degree of oxidation of pork patties refrigerated at 7 °C for 0, 7, and 14 days and the content of 10 types of heterocyclic amines (HCAs) after heating. The pork patties used in the study were added with 0.7 mg sodium nitrite (SN) and 5 mg paprika extract (PE), respectively. IQx (2-Amino-3-methyl-imidazo[4,5-*f*]-quinoxaline), MeIQx (2-Amino-3, 8-dimethyl-imidazo[4,5-*f*]-quinoxaline), PhIP (2-Amino-1-methyl-6-phenyl-imidazo[4,5-*b*]-pyridine), and Harman (1-Methyl-9H-pyrido[4,3-*b*]-indole) contents increased with increasing storage periods of treatment. On the other hand, HCAs production in SN and PE treatments were suppressed over the storage period, with IQ (2-Amino-3-methyl-imidazo[4,5-*f*]-quinoline) and Aαc (2-Amino-9H-dipyrido[2,3-*b*]-indole) being suppressed significantly ($P < 0.05$). The control's pH, cooking loss, lipid, and protein oxidation were higher than SN and PE-treated patties at 14 d ($P < 0.05$). These differences affect the formation of HCAs. PLS-DA showed a strong correlation between protein oxidation and IQx, Harman, 4,8-DiMelQx (2-Amino-3, 4, 8-trimethyl-imidazo[4,5-*f*]-quinoxaline), PhIP, and MeIQx, while lipid oxidation correlated with IQx, Harman, and PhIP. Both SN and PE showed HCAs inhibitory activity and exhibited oxidative stability during storage.

1. Introduction

Meat is a vital food resource that provides humans with several high-quality nutrients and essential amino acids. Typically, meat is consumed after heating or processing, which improves the flavor, digestibility, and commercial value of the meat (Shabbir, Raza, Anjum, Khan, & Suleria, 2015). However, depending on the method and conditions used for heating, several harmful substances can be produced from the meat products (Shabbir et al., 2015). For example, heterocyclic amines (HCAs) are harmful products formed when meat and processed foods are heated at high temperatures. They are formed by the Maillard reaction and Strecker degradation from precursors such as amino acids, creatinine, creatine, and glucose (Kang et al., 2022). These are primarily categorized into amino imidazoazaarenes (AIAs) and amino carbolines (ACs). AIAs are also known as 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) type compounds, and include polar HCAs, such as IQ, 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]-pyridine (PhIP), 2-amino-3,8-dimethyl-imidazo [4,5-*f*]-quinoxaline (MeIQx), 2-amino-3-methyl-imidazo[4,5-*f*]-quinoxaline (IQx), 2-amino-3,4,8-trimethyl-imidazo[4,5-*f*]-quinoxaline (4,8-DiMeIQx), and 2-amino-3,7,8-trimethyl-imidazo[4,5-*f*]-quinoxaline (7,8-DiMeIQx). On the other hand, ACs are known as non-IQ-type compounds, with non-polar HCAs, such as 2-amino-9H-dipyrido[2,3-*b*]-indole (Aαc), 2-amino-3-methyl-9H-dipyrido[2,3-*b*]-indole (MeAαc), 1-methyl-9H-pyrido[4,3-*b*]-indole (Harman), and 9H-pyrido[4,3-*b*]-indole (Norharman) belonging to this category (Gibis, 2016). These HCAs are the main HCAs formed when meat is heated (Syeda & Cannon, 2022).

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IQ is classified into Group 2A, whereas MeIQx, PhIP, Aαc, and MeAαc are classified into Group 2B (IARC, 1993). These HCAs caused cancer in the colon, mammary gland, and prostate in animals, and showed mutagenesis targeting the lung, liver, skin, and clitoris (Kang et al., 2022). Although the content of HCAs observed in food is minimal, regular consumption of even small amounts is harmful to the human body, so research is being actively conducted on ways to suppress HCAs production (Kang et al., 2022).

Oxidation during meat storage and processing degrades quality, with lipid and protein oxidation during meat storage adversely affecting its functional, nutritional, flavor, texture, and meat color characteristics (Bao & Ertbjerg, 2019; Domínguez et al., 2019).

Lipid oxidation that occurs during storage of meat is mainly autooxidation and is a major process that causes oxidative deterioration of meat and meat products through the interaction between unsaturated fatty acids and oxygen, causing rancid odor, discoloration, nutrient losses, decrease in shelf life, and accumulation of harmful compounds. This can cause quality deterioration (Domínguez et al., 2019; Fan et al., 2019). Protein oxidation causes chemical changes, such as changes in the composition of creatine, creatinine, and free amino acids present in meat, and physical changes, such as structural changes in the protein, negatively affecting the water-holding capacity (WHC), texture, and meat color (Bao et al., 2019).

The inhibition or promotion of HCAs production depends on the antioxidants added during processing, the type of ions, the number of precursors, the type of amino acids within the meat, and the meat's pH (Gibis, 2016). Precursors of HCAs include creatinine, creatine, glucose, and free amino acids, and changes in these substances affect HCAs production. Furthermore, the difference in oxidation within meat caused by the addition of antioxidants is relevant to the production of HCAs (Parvin, Seo, Eom, Ahamed, & Yang, 2023).

Antioxidants are added to meat to inhibit oxidation during processing, storage, and cooking. Typical synthetic antioxidants include phenolic compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and sodium nitrite. These substances' chemical stability, strong antioxidant capacity, and low cost have been emphasized as their significant advantages (Aziz & Karboune, 2018). However, with the increasing research on the potential carcinogenic properties of BHA and BHT, along with harmful derivatives of sodium nitrite, such as *N*-nitroso compounds, their use is now being reduced (Falowo, Fayemi, & Muchenje, 2014; Šojić et al., 2020; Wang et al., 2015). Studies have been conducted to replace synthetic antioxidants by developing antioxidants derived from natural substances containing polyphenols and carotenoids (Falowo et al., 2014).

Paprika (*Capsicum annuum L.*), an annual plant belonging to the *Solanaceae* family, contains an abundance of carotenoids, including capsanthin, capsorubin, and capsanthin 3,6-epoxide (Kim, An, Park, Lim, & Kim, 2016). The double bonds within these carotenoids terminate the oxidation reaction, protecting the tissues from damage by peroxides and free radicals, even at low oxygen levels. Capsanthin and capsorubin are effective scavengers of reactive oxygen species (ROS) as well as superoxide and nitric oxide produced by lipid oxidation (Britton, 2020).

Therefore, the objective of this study was to investigate changes in HCAs production and oxidation in pork patties during storage. In addition, we hypothesized that the paprika extract could actively inhibit lipid and protein oxidation, along with HCAs formation, when compared to sodium nitrite in pork patties during storage.

2. Materials and methods

2.1. Materials and chemicals

The ten types of HCAs (S. 2) used in the external standard method were purchased from Toronto Research Chemicals (Toronto, Canada). The reagent used in this experiment and six types of chemical compounds (neoxanthin, violaxanthin, capsanthin, lutein, zeaxanthin, and β-carotene) of paprika used in the external standard method were supplied by Sigma-Aldrich (Saint Louis, MS, USA). All standards were ≥90 % pure. The organic solvent (HPLC grade or higher) was purchased from Merck (Darmstadt, Germany). The pork patties were prepared using pork hind legs purchased from a local butcher shop (Jinju-si, Korea). These hind legs were frozen at −20 °C after purchase. The paprika extract was prepared using red paprika (Special, grown in Jinju-si, harvested around Nov.) purchased from a local agricultural produce market (Jinju-si, Korea). The produced paprika extract was then refrigerated at −75 °C.

2.2. Preparation of paprika extract

Paprika was washed under running water while the wick and seeds were removed and then diced. The diced paprika was then dried in a hot-air drying oven at 60 °C. The dried paprika was pulverized subsequently and passed through a 1 mm sieve to obtain the powdered form. The paprika powder and hexane were mixed at a 1:10 (w/v) ratio to obtain a fraction after shaking at room temperature for 24 h. Then, the powder was centrifuged for 10 min at 3,000 *g* using a centrifuge (LaboGene 1736R, LABOGENE, Lillerød, Danmark), and the supernatant was separated using Whatman No. 1 filter paper. Next, it was concentrated, followed by concentration under reduced pressure in a water tank at 40 °C to produce a blackish-red, viscous liquid paprika extract. This extract was then stored at −75 °C until used in further experiments.

2.3. Preparation of pork patty and cooking conditions

Frozen pork hind legs were defrosted, with excess connective and fat tissues removed. The meat was then ground with a grinder plate (6-mm diameter plate holes, GG 22, German Knife, CA, USA) to produce a porcine patty. Pork patties were standardized into an identical round shape of 90 (±2) mm in diameter and 100 (±5) g in weight using a petri dish. The materials and proportions used in patty production are shown in S. 3. Common ingredients in the pork patty included lean pork meat (78.99 %), water (9.83 %), back fat (9.83 %), salt (1.48 %), and complex phosphate (0.20 %). The pork patties were subsequently divided into those that did not include antioxidants (control), those that included 0.70 mg of sodium nitrite (SN), and those that included 5 mg paprika extract (PE). The pork patties were then refrigerated at 7 °C for 0, 7, and 14 days in air-flushed packaging, and the pH, protein oxidation, and lipid oxidation were measured for each treatment.

Patties were pan-roasted on an electric hot plate at 250 °C for 10 min (5 min on each side). Before roasting, the hot plate was pre-calibrated by monitoring the surface temperature at 200 °C and 250 °C using a laser infrared thermometer (Bluetec, BO-350, No:04732728, China). The core temperature of each patty was determined using a probe thermometer (RS components, Beauvais, France) to record the end point of cooking at 75 °C. The pork patties were then cooled to room temperature, and the cooking loss was measured for each treatment. Subsequently, the patties were processed for HCAs extraction and identification.

2.4. Identification and quantification of chemical compounds of paprika extract

For analysis, paprika extract was re-dissolved in 5 mL of solvent (methyl *tert*-butyl ether (MTBE): methanol (MeOH), 1:1, v/v) and filtered with a 0.45 μm micro syringe filter. Identification and quantification of paprika extract chemical compounds were done using high-performance liquid chromatography (HPLC, Shimadzu, Japan). The HPLC column used in the analysis was a Prontosil C_{18} EPS (250×4.6 mm; 5 μm i.d., Bischoff, Germany), and the column temperature was 30 °C. The UV detector was operated at a wavelength of 450 nm. The sample injection volume was 10 μL; Mobile phase A was water + acetonitrile (ACN) (1:9), and B was MTBE + MeOH (4:6); initially, the

solvent contained (100 % A), at 5 min (90 % A), at 40 min (10 % A), and the flow rate was 1 mL/min. External standard methods determined the identification and quantification of 6 types of chemical compounds (neoxanthin, violaxanthin, capsanthin, lutein, zeaxanthin, and β-carotene). Their identification was made by comparing the retention times and peak shape with external standard chemical compounds; Quantification was done using a calibration curve equation (S.4.).

2.5. Physicochemical evaluation of pork patty

2.5.1. pH and cooking loss determination

About 27 mL of distilled water was added to 3 g of pork patty. It was then homogenized at 10,000 RPM for 30 s using a homogenizer (ULTRA-TURRAX T-25, IKA, Staufen, Germany). The pH of the homogenized sample was measured using a pH meter (S20 SevenEasy^{TM} pH, Mettler-Toledo, Columbus, OH, USA) calibrated with three standard buffers of pH 4.01, 7.0, and 10.01 at 20 °C.

The pork patties were weighed and heated in a frying pan at 250 °C for 10 min, 5 min on each side. After that, the cooked pork patty was cooled down at room temperature for 30 min, and the weight was measured. After that, the measured weight values were substituted into Eq. (1) to calculate the cooking loss.

$$\text{Cooking loss}\,(\%) = \frac{uncooked\,Pork\,patty\,weight_{(g)} - cooked\,Pork\,patty\,weight_{(g)}}{uncooked\,Pork\,patty\,weight_{(g)}} \times 100 \quad (1)$$

2.5.2. Lipid oxidation determination

The 2-thiobarbituric acid (TBA) extraction method was used to measure the lipid oxidation levels of pork patties, following the method of Cherian, Orr, Burke, and Pan (2013). The absorbance of each sample was measured at 531 nm against a blank containing 2 mL of deionized distilled water and 2 mL of TBA solution in a spectrophotometer (Cary 60 UV–Vis, Agilent Technologies, Santa Clara, CA, USA). The TBARS value was calculated as mg of MDA per kg of sample.

2.5.3. Protein oxidation determination

The measurement method was performed by modifying the methods of Soglia, Petracci, and Ertbjerg (2016). Cold 0.15 M potassium chloride (KCl) was added to 3 g of pork patty and homogenized with a homogenizer (ULTRA-TURRAX T-25, IKA, Staufen, Germany) at 10,000 PRM for 30 s. One mL of cold 10 % trichloroacetic acid (TCA) was added to the homogenized solution and centrifuged at 5000 *g* for 5 min with a centrifuge (LaboGene 1736R, LABOGENE, Lillerød, Danmark). After removing the supernatant, 0.4 mL of 5 % sodium dodecyl sulfate (SDS) was added and the pellet was separated using a high-speed sonication system (VC750, S/N: 98166AR-10–17; Sonic & Materials Inc., Newtown, CT, USA). After pellet separation, 0.8 mL of 2 M hydrochloric acid (HCl) containing 10 mM dinitrophenylhydrazine (DNPH) was added to one of the two samples. About 0.8 mL of 2 M HCl was added to the other sample as a control. Then, the sample was left at room temperature for 30 min, 0.2 mL of cold 20 % TCA was added, centrifuged at 5,000 *g* for 5 min, and the supernatant was removed. Ethanol-ethyl acetate (1:1, v/v) was added to the sample to which 2 M HCl containing 10 mM DNPH was added, and after vortexing, centrifuged at 5000 *g* for 10 min to remove residual DNPH. This process was repeated until all remaining DNPH was removed. After that, 1.5 mL of 6 M guanidine-HCl in 20 mM phosphate buffer (pH 6.5) was added to the DNPH-containing sample and control; and left for 24 h at room temperature. After 24 h, centrifugation at 10,000 *g* for 10 min was done, and absorbance at 280 nm and 370 nm was measured using a spectrophotometer (Microplate Spectrophotometer, BioTek, Winooski, VT, USA) and 96 well-plates (UV Max^{TM} 33096, SPL, Pocheon -si, Korea). After that, the absorbance value was substituted into Eq. (2) to calculate the carbonyl content of the pork patty, and the degree of protein oxidation was confirmed through this.

$$\text{Carbonyl content}\,(nmol/mg\,protein) = \frac{A_{370} - A_{370(blank)}}{22,000 \times [A_{280} - (A_{370} - A_{370(blank)})] \times 0.43} 10^6 \quad (2)$$

2.6. Hcas determination

2.6.1. Extraction scheme

The extraction of HCAs was carried out by modifying the method of Parvin et al. (2023). About 30 g patty sample was mixed with 120 mL of 2 M sodium hydroxide and homogenized by a high-speed sonication system (VC750, S/N: 98166AR-10–17; Sonic & Materials Inc., New town, CT, USA) for 10 min. Then it was mixed well with 225 g diatomaceous. The mixed sample was filled in an empty extrelut column (350 mL), and 600 mL of dichloromethane flowed three times. The flowed sample was filtered with a quantitative filter paper. The filtered sample was concentrated in a water bath at 40 °C with a decompression concentrator to prepare a 40 mL sample. Then, the sample was passed through a pre-conditioned PRS cartridge (Chromatographic cartridge made with silica gel for polar and non-polar ion exchange) by flowing 0.1 M HCl (6 mL), 0.1 M HCl/MeOH (15 mL), and distilled water (2 mL) in that order. Adjust the vacuum of the C18 cartridge and pre-condition 5 mL of MeOH and 5 mL of distilled water. Subsequently, the PRS cartridge was placed on top of the C18 cartridge. The PRS cartridge was washed with 20 mL of 0.5 ammonium acetate (pH 8.5), then the PRS cartridge was removed. Finally, C18 was washed with 1 mL of 10 % ammonia solution in MeOH to dissolve the analyte. Next, the sample passes through the 0.45 μm micro syringe filter and dryness with N2 flow at 45 °C. Then, 200 μL MeOH was added to a dried sample, vortexed, melted, put into a micro glass vial, and stored until analysis at −20 °C.

2.6.2. Identification and quantification

HCAs were identified using high-performance liquid chromatography (HPLC, Ultimate 3000, column compartment, Dionex, Sunnyvale, CA, USA) connected to a UV detector and an autosampler. The HPLC column used in the analysis was a shim-pack VP-ODS column (150×4.60 mm; 5 μm i.d., Shimadzu, Japan), and the column temperature was 35 °C. The UV detector was operated at a wavelength of 250 nm. The sample injection volume was 20 μL; Mobile phase A was 50 mM ammonium acetate (pH 3.6), and B was 100 % acetonitrile (ACN); initially, the solvent contained (90 % A), at 15 min (40 % A), at 17 min (5 % A), at 20 min (5 % A), at 21 min (90 % A), at 28 min (90 % A), and the flow rate was 1 mL/min. The external standard methods determined the quantity of 10 types of HCAs. Their identification was made by comparing the retention times and peak shape with standard HCAs. Quantification was done using a calibration curve equation (S.5.).

2.7. Statistical analysis

This study was performed according to a completely randomized design in 4×3 (four independent batches × three treatments) roasted pork patties prepared independently, and four replicates were analyzed per treatment. All data were analyzed using the MIXED Model of SAS analysis using SAS 9.4. software (SAS Institute Inc., Cary, NC, USA). The effects of treatments and storage days were considered independent or fixed effects; cooking loss, pH, carbonyl content, TBARS, and HCAs were dependent variables. The treatment × storage days with batches were used as random effects.

The presence of HCAs in different treatments was expressed as mean ± standard error (SE). The predicted means and standard errors were generated from the Proc Mixed Model. Statistical significance was set at $P < 0.05$, as calculated using Duncan's multiple range test. Causation analysis between lipid and protein oxidation and HCAs production of patties using partial least squares-discriminant analysis (PLS-DA) of MetaboAnalyst 5.0 software.

3. Results and discussion

3.1. Identification and quantification of chemical compounds of paprika extract

The result of the identification and quantification of chemical compounds of paprika extract is shown in S. 1. The content of carotenoid substances such as neoxanthin, violaxanthin, capsanthin, lutein, zeaxanthin, and β-carotene were confirmed in paprika extract. Neoxanthin, violaxanthin, capsanthin, lutein, zeaxanthin, and β-carotene showed a content of 2.8±0.1, 5.1±0.2, 45.7±0.6, 0.6±0.1, 85.1±0.6, and 19.1 ±1.7, mg/kg respectively. Carotenoid substances are used as antioxidants, and their antioxidant capacity can also be expected in pork patties (Britton, 2020; Falowo et al., 2014; Kim et al., 2016). In addition, high antioxidant capacity is expected to suppress the occurrence of HCAs.

3.2. pH and cooking loss

The changes in pH and cooking loss of the pork patties (C, SN, and PE) based on their storage period (days 0, 7, and 14) are shown in Table 1. On day 0, the pH values of the control, SN, and PE were 5.94 ±0.03, 5.90±0.02, and 5.93±0.01, respectively, with no significant difference found among the values ($P > 0.05$). On day 7, the pH of the control was 6.06±0.02, which was significantly higher ($P < 0.05$) than that of SN or PE (5.91±0.01 and 5.98±0.00, respectively). On day 14, the pH levels of the control, SN, and PE all showed significant increases compared to those on day 7, which were 6.62±0.07, 6.12±0.09, and 6.44±0.01, respectively ($P < 0.05$). In addition, SN had significantly lower pH values than the control and PE ($P < 0.05$). During storage, there was a significant change in the pH values for all the treatment groups. These results could be attributed to ammonia production due to protein oxidation within the pork patties, the exposure of primary functional groups due to the amino acid degradation, accumulation of peroxides due to lipid oxidation, and charges incurred due to the production of aldehydes and ketones. (Chang et al., 2019; Choe, Kim & Kim, 2017).

Changes in cooking loss showed a similar pattern to the changes in pH values. On day 0, the cooking losses in the control, SN, and PE were 21.13±1.61, 20.77±0.4, and 20.89±0.65 %, respectively, with no significant differences being identified among the values ($P > 0.05$). On day 7, the cooking losses in the control, SN, and PE were 25.36±0.69, 21.13±0.47, and 22.29±0.41 %, respectively, while the cooking loss in the control was significantly higher than that in SN and PE ($P < 0.05$). On day 14, the cooking losses in the control, SN, and PE were 24.27 ±0.25, 20.77±0.23, and 20.75±0.38 %, respectively, with the cooking loss in the control being significantly higher than that in SN and PE, similar to what was observed on day 7 ($P < 0.05$). Additionally, with the increase in storage period, the cooking loss increased significantly only in the control ($P < 0.05$). The cooking loss represents the WHC of the meat, and the increase in cooking loss indicates a decrease in WHC. This decrease in WHC is attributable to the denaturation of myosin due to lipid and protein oxidation and reduced protein function (Delles & Xiong, 2014; Wang, Wang, Li, & Zhang, 2019). This means that significant changes in pH and cooking loss implied that chemical changes, such as oxidation, occurred within the pork patties. Additionally, the pH and cooking loss changes were less significant in SN and PE than in the control. Therefore, adding nitrite and paprika extract inhibited chemical changes, including lipid and protein oxidation.

Table 1
Effect of sodium nitrite and paprika extract on pH values and cooking loss of pork patty during storage time.

	Day	control[1)]	SN	PE
pH	0	$5.94±0.03^{c}$	$5.90±0.02^{b}$	$5.93±0.01^{c}$
	7	$6.06±0.02^{Ab}$	$5.91±0.01^{Cb}$	$5.98±0.00^{Bb}$
	14	$6.62±0.07^{Aa}$	$6.12±0.09^{Ba}$	$6.44±0.01^{Aa}$
Cooking loss (%)	0	$21.13±1.61^{b}$	20.77±0.45	20.89±0.65
	7	$25.36±0.69^{Aa}$	$21.13±0.47^{B}$	$22.29±0.41^{B}$
	14	$24.27±0.25^{Aa}$	$20.77±0.23^{B}$	$20.75±0.38^{B}$

[1)]Control: without antioxidant; SN: added with sodium nitrite; PE: added with paprika extract.
[A-C]Means with a different letter within a different raw are significantly different ($P < 0.05$).
[a-c]Means with a different letter within a different column are significantly different ($P < 0.05$).

3.3. Lipid and protein oxidation

Lipid oxidation that occurs during meat storage degrades the flavor, texture, and color of the meat (Domínguez et al., 2019). TBA can be used to measure the lipid oxidation occurring during storage; it reacts with malondialdehyde (MDA), a byproduct of lipid oxidation, to produce a red color. The darker this red color, the higher the MDA concentration. Hence, the intensity of the red color indicates the extent of lipid oxidation (Choe, Kim, & Kim, 2017). The changes in the levels of TBA reactive substances (TBARS) for each treatment group and storage period are shown in Fig. 1. On day 0, the TBARS values in the control, SN, and PE were 0.40±0.03, 0.37±0.04, and 0.40±0.03 mg MDA/kg meat, respectively, with no significant differences being observed among these values ($P > 0.05$). These values corresponded to the range of TBARS value (0.202–0.664 mg MDA/kg meat) for fresh pork meat (Pogorzelsre, Godziszewska, Brodowska & Wierzbicka,2018). The lipid in the pork patties on day 0 indicated that lipid oxidation did not occur. Meanwhile, on day 7, the TBARS values in the control, SN, and PE were 1.69±0.18, 0.60±0.02, and 0.89±0.08 mg MDA/kg meat, respectively, with a significant increase being observed compared to those on day 0 ($P < 0.05$). Furthermore, the TBARS value in the control was significantly higher than those in SN and PE ($P < 0.05$). On day 14, the TBARS values in the control, SN, and PE were 3.55±0.87, 1.28±0.56, and 1.83 ±0.47 mg MDA/kg meat, respectively, with a significant increase being identified compared with those on day 7 ($P < 0.05$). The difference between the control, SN, and PE was significant, with the control showing the highest value ($P < 0.05$). These results indicated lipid oxidation was inhibited in pork patties containing nitrite and paprika extract during storage.

During storage, protein oxidation negatively impacts functional or nutritional aspects of the meat by causing changes in the amino acid side chain, increasing carbonyl compounds, and altering amino acids (Delles et al., 2014; Domínguez et al., 2019). Protein oxidation was identified by measuring the carbonyl content using the 2,4-dinitrophenylhydrazine reagent, with the measurement results being shown in Fig. 2. On day 0, the protein carbonyl contents of the control, SN, and PE were 1.49 ±0.25, 1.64±0.39, and 1.56±0.18 nmol/mg protein, respectively, with no significant difference observed among these values ($P > 0.05$). The protein carbonyl content of unoxidized meat samples was one nmol/mg protein (Choe et al., 2017), indicating that pork patty carbonyl content was not oxidized on day 0. On day 7, the control, SN, and PE carbonyl contents were 3.04±0.36, 2.34±0.38, and 2.35±0.35 nmol/mg protein, respectively, with an observed elevated value ($P > 0.05$). On day 14, the control, SN, and PE carbonyl contents were 11.49±0.04, 8.36±1.19, and 8.76±0.08 nmol/mg protein, respectively. The carbonyl content among all treatment groups increased from day 7 ($P < 0.05$) and, in particular, was significantly higher in the control sample than in SN or PE ($P < 0.05$). These results were attributed to the effective inhibition of protein oxidation by adding nitrite and paprika extract to the pork patty.

The addition of paprika extract and sodium nitrite to pork patties inhibited protein and lipid oxidation during storage. Additionally, we observed that the antioxidant capacity of the paprika extract was similar to that of sodium nitrite. This antioxidant effect of the paprika extract was attributed to the presence of antioxidant compounds, such as carotenoids, including capsanthin and capsorubin in the extract, which

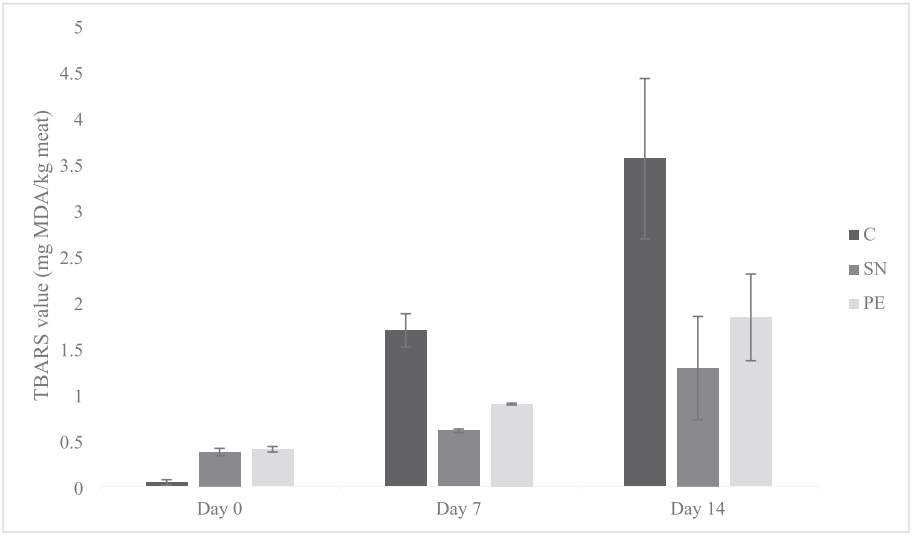

Fig. 1. Effect of sodium nitrite and paprika extract on TBARS value (mg MDA/kg meat) of pork patty during storage time. [1)]Control: without antioxidant; SN: added with sodium nitrite; PE: added with paprika extract. [A-B]Means with a different letter within a different raw are significantly different ($P < 0.05$). [a-b]Means with a different letter within a different column are significantly different ($P < 0.05$).

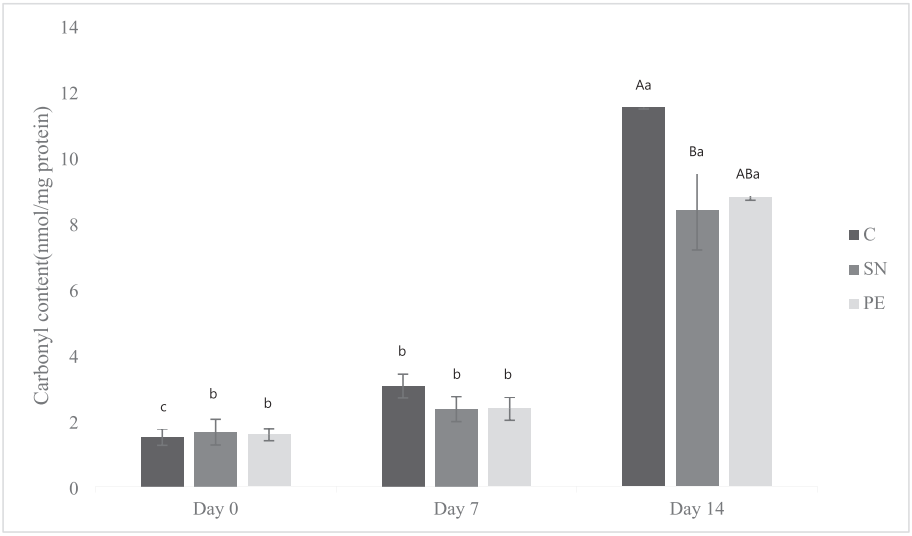

Fig. 2. Effect of sodium nitrite and paprika extract on carbonyl content (nmol/mg protein) of pork patty during storage time. [1)]Control: without antioxidant; SN: added with sodium nitrite; PE: added with paprika extract. [A-B]Means with a different letter within a different raw are significantly different ($P < 0.05$). [a-c]Means with a different letter within a different column are significantly different ($P < 0.05$).

inhibited the formation of the oxidation products (free radicals, superoxide, and nitric oxide) and scavenged ROS (Britton, 2020).

3.4. HCAs content of pork patty stored for 0, 7, and 14 days

The changes in HCAs production according to the storage period of each treatment group are summarized in Table 2. In control, the production of most polar HCAs tended to increase as the storage period increased, with increased production of Harman, Aαc, and MeAαc among the non-polar HCAs. The production of IQx, MeIQx, PhIP, and Harman significantly increased alongside the increase in the storage period ($P < 0.05$). In the SN treatment sample, a lower content of HCAs production was observed compared to the control on days 0, 7, and 14. In particular, IQ, PhIP, and Aαc on day 0, IQx and Norharman on day 7, and IQ, IQx, PhIP, and Harman on day 14 were all found to be significantly lower ($P < 0.05$). Furthermore, for the PE treatment sample, the production of IQ and Aαc on day 0, IQx, Norman on day 7, and IQ, IQx, PhIP, Harman, and Aαc on day 14 were all significantly lower than that of the control ($P < 0.05$).

The addition of sodium nitrite and paprika extract to the pork patties significantly inhibited the production of IQ and Aαc ($P < 0.05$). Additionally, a prolonged storage period led to a significant increase in the production of IQx, MeIQx, PhIP, and Harman ($P < 0.05$), which was inhibited by adding sodium nitrite and paprika extract. HCAs are produced through the Maillard reaction and Strecker degradation (Kang et al., 2022). The Maillard reaction is a non-enzymatic browning reaction in which reducing sugars and free amino acids react during heating to form pigments and flavors. Along with the Strecker degradation, this produces flavoring substances and melanoid compounds (Kang et al., 2022). During this process, the flavoring substances produced act as precursors for ROS generation and influence the production of carcinogenic aromatic compounds, such as imidazoles, pyridines, pyrazines, and pyrrole. Pyridines can also combine with imidazoles to form AIAs or amino groups to form ACs (Adeyeye & Ashaolu, 2021).

Table 2
Effect of sodium nitrite and paprika extract on heterocyclic amines formation (ng/g meat sample) of pork patty during storage time.

		Day	control[1)]	SN	PE
Polar HCAs	IQ	0	2.58^{A}	1.19±0.04^{C}	2.12^{B}
		7	2.35±0.39	1.50±0.46	2.62±1.35
		14	3.85±0.03^{A}	1.65±0.23^{B}	2.70±0.35^{B}
	IQx	0	2.25±0.25^{b}	1.27±0.14	2.98±1.81
		7	7.69±1.35^{Aab}	1.95±0.32^{B}	4.24±0.06^{B}
		14	10.90±2.81^{Aa}	3.50±1.10^{B}	5.20±1.11^{B}
	MeIQx	0	2.35±0.13^{Bb}	1.54±0.14^{Bb}	9.08±0.04^{A}
		7	1.92±0.09^{Bb}	2.08±0.03^{Bb}	9.51±1.82^{A}
		14	3.45±0.77^{a}	4.83^{a}	6.99±2.86
	7,8-DiMeIQx	0	1.70±0.44^{B}	1.20±0.19^{B}	3.79±0.05^{A}
		7	1.02±0.55	1.42±0.47	2.88±0.65
		14	1.06±0.50^{B}	0.90±0.17^{B}	3.09±0.47^{A}
	4,8-DiMeIQx	0	3.12±0.57	5.24±1.69	3.68±0.38
		7	4.31±3.38	7.68±2.00	8.37±1.69
		14	6.49±0.56	8.30±3.25	7.74±1.69
	PhIP	0	3.20^{ABb}	2.34^{B}	5.63±0.14^{Ab}
		7	3.84±2.07^{b}	1.63	5.35±0.86^{b}
		14	14.92±0.72^{Aa}	5.81±2.49^{B}	8.96±0.91^{ABa}
Non-Polar HCAs	Harman	0	1.89±0.35^{b}	1.52±0.08^{b}	1.93±0.55
		7	2.22±1.38^{b}	1.27±0.25^{b}	1.08
		14	18.09±0.52^{Aa}	5.77±0.62^{Ba}	6.50±1.90^{B}
	Norharman	0	2.59±0.78	1.48±0.17^{ab}	2.26
		7	1.74±0.24^{AB}	2.24±0.13^{Aa}	1.34±0.07^{B}
		14	1.54±0.43	1.29±0.23^{b}	1.94±0.73
	Aαc	0	6.23±0.08^{A}	5.31±0.37^{AB}	4.15^{B}
		7	6.26	5.75	5.88±0.14
		14	6.24±0.18^{A}	5.83±0.08^{A}	4.62±0.52^{B}
	MeAαc	0	4.09±0.04	2.95±0.39^{b}	3.28±0.53
		7	4.61±0.20	3.44±0.45^{b}	4.59±0.57
		14	4.59±0.54	4.89±0.39^{a}	4.73±0.27

[1)]Control: without antioxidant; SN: added with sodium nitrite; PE: added with paprika extract.
Data were presented as means ± standard error of all treatments (n = 4).
[A-C]Means with a different letter within a different raw are significantly different ($P < 0.05$).
[a-b]Means with a different letter within a different column are significantly different ($P < 0.05$).

Additionally, free radicals and free amino acids in meat can affect the formation of HCAs (Gibis, 2016; Kang et al., 2022). These are produced from the lipid and protein oxidation of meat during storage. Therefore, the more extended storage period of meat promotes the production of HCAs. These results were consistent with those of a previous study by Szterk and Jesionkowska (2015), which analyzed the number of HCAs produced based on the long-term aging of pork meat.

Protein oxidation in meat mainly results in loss and modification of amino acids, reduction of WHC, and carbonylation. When protein oxidation progresses in meat, changes occur in amino acids, such as histidine, tryptophan, and leucine. (Bao et al., 2019; Delles et al., 2014; Domínguez et al., 2021). Histidine reduces the production of PhIP, Harman, and Norharman; leucine reduces the production of PhIP, IQx type HCAs, Harman, and Norman (Xue et al., 2020). Tryptophan also reduces the production of PhIP and IQx-type HCAs (Linghu et al., 2020). Therefore, the changes in histidine, tryptophan, and leucine induced by protein oxidation diminution the inhibition of HCAs production. The reduction in the WHC of meat also increases cooking loss during heating. This facilitates HCAs production, as water-soluble HCAs precursors, such as creatinine, creatine, glucose, and amino acids, migrate with water to the surface of the meat. Moreover, it eases the production of MeIQx, PhIP, and other HCAs (Gibis & Weiss, 2017; Vangnai, Houser, Hunt, & Smith, 2014). The process of protein carbonylation occurs through the direct oxidation of lysine, arginine, proline, and threonine (Yu, Morton, Clerens, & Dyer, 2016). The carbonyl compound produced through oxidation affects the formation of HCAs, ROS, and certain Maillard intermediates (Barzergar, Kamankesh & Mohammadi, 2019). The correlation between the content of HCAs produced and protein oxidation, which could significantly affect the formation of HCAs was identified through partial least squares-discriminant analysis (PLS-DA). The pork patties were categorized into those in which the protein was oxidized (OP) and those in which the protein was not oxidized (NP), as shown in Fig. 3A – C. Fig. 3A and B show the difference in the HCAs produced from OP and NP; moreover, IQx, Harman, 4,8-DiMeIQx, PhIP, and MeIQx with VIP scores of ≥1, as in Fig. 3C, were determined as important variables related to protein oxidation (Cáceres-Nevado, Garrido-Varo, De Pedro-Sanz, Tejerina-Barrado, & Pérez-Marín, 2021).

Lipid oxidation facilitates the formation of Strecker aldehyde, whereas the peroxy-radical induced by lipid oxidation forms aldehydes and ketones; this consequently promotes the formation of pyrazines (Gibis, 2016; Lu, Kuhnle, & Cheng, 2017), which influence the formation of IQx-type HCAs (Zamora & Hidalgo, 2020). Additionally, malondialdehyde produced directly from lipid oxidation can cause carbonylation of myofibrillar proteins and promote protein oxidation (Wang, He, Emara, Gan, & Li, 2019). Thus, the relationship between lipid oxidation and HCAs production was identified through PLS-DA. Pork patties were categorized into those in which the lipids were oxidized (OL) and those in which lipids were not oxidized (NL), as shown in Fig. 4A – C. The difference between OL and NL in terms of HCAs production was evaluated using IQx, PhIP, and Harman with VIP scores being ≥1, as shown in Fig. 4A, 4B, and 4C, which were determined to be important variables related to lipid oxidation (Cáceres-Nevado et al., 2021).

Therefore, the relationship between the storage period of pork patties and HCAs generation can be summarized as follows: i) The production of HCAs increases significantly with more extended storage periods for pork patties. In particular, the production of IQx, MeIQx, PhIP, and Harman was significantly increased ($P < 0.05$), ii) a change in amino acid composition could have caused the increase in HCAs due to protein oxidation, and a decrease in WHC, and carbonylation: mainly, the increase in IQx, PhIP, Harman, and Norharman was significantly influenced by protein oxidation, and iii) the production of pyrazines and

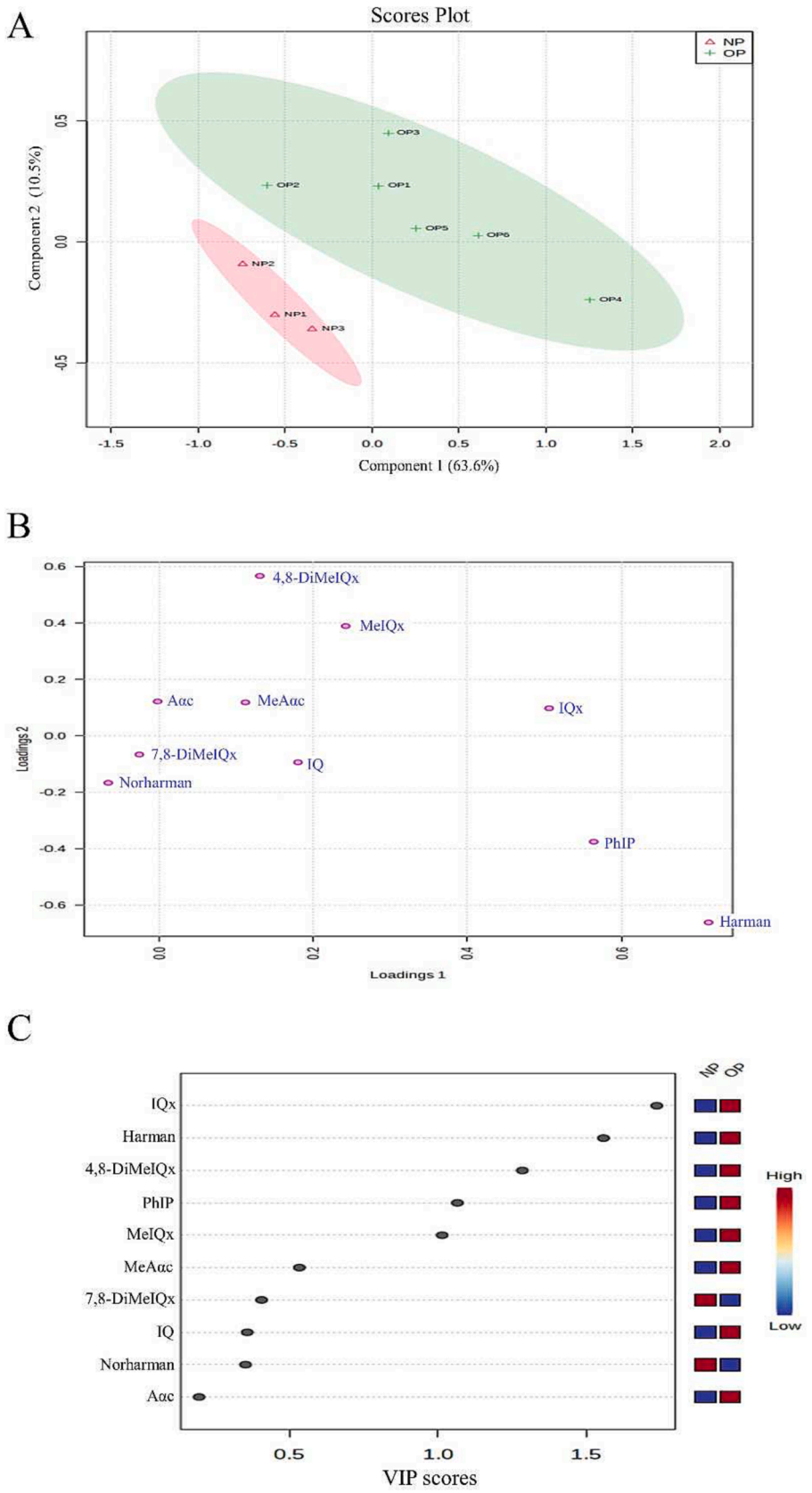

Fig. 3. Partial Least Squares–Discriminant Analysis (PLS-DA); (A) Score plot between selected PCs (PC1 versus PC2) of non-oxidized protein and oxidized protein of pork patty based on their analyzing parameters, (B) Loading plots between the selected PCs considering polar and non-polar HCAs, (C) VIP scores between the selected PCs. Non-oxidized protein (NP): Carbonyl content in range of 1 nmol/mg protein; Oxidized protein (OP): Carbonyl content of more than 2 nmol/mg protein.

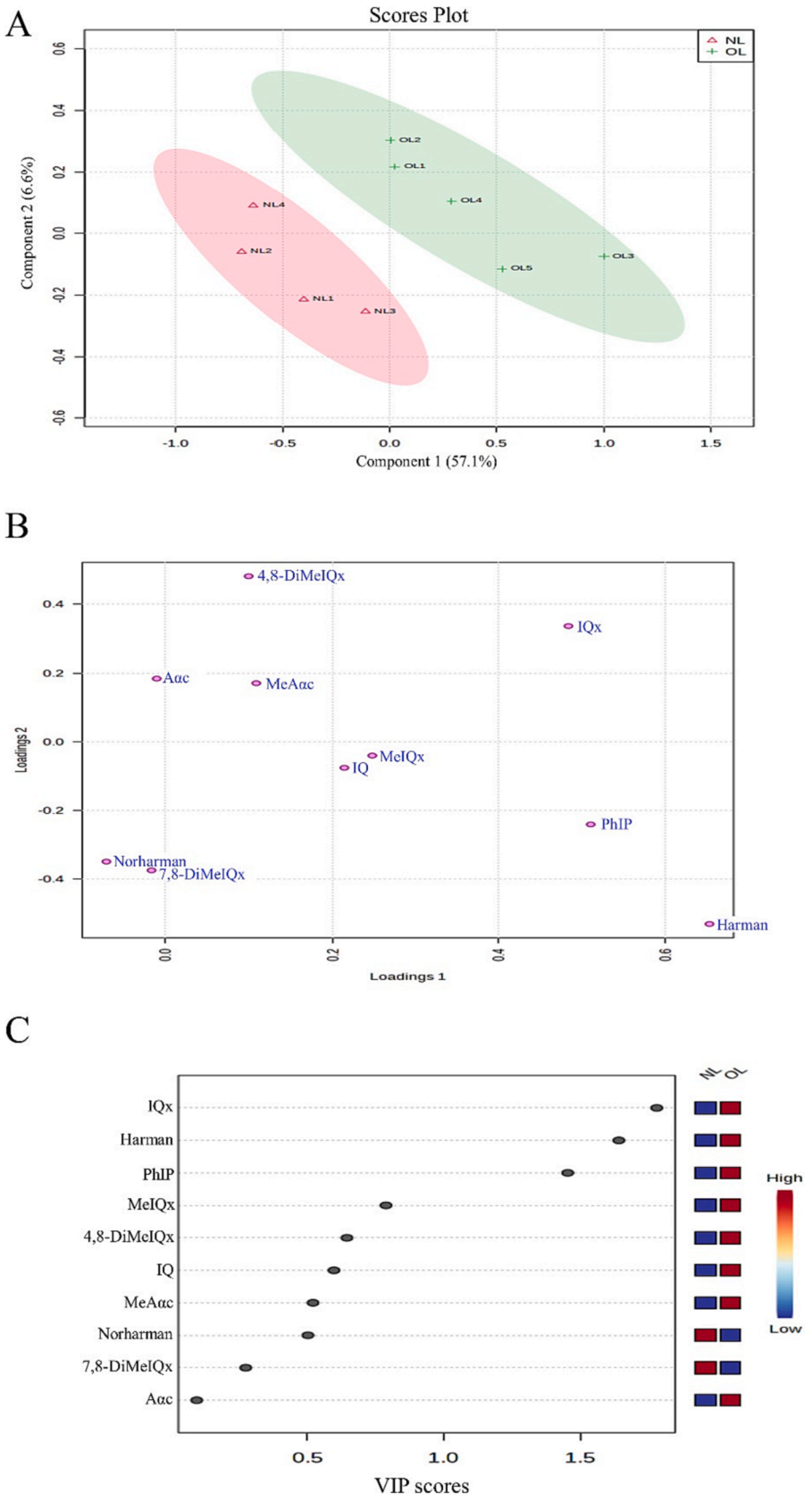

Fig. 4. Partial Least Squares–Discriminant Analysis (PLS-DA); (A) Score plot between selected PCs (PC1 versus PC2) of non-oxidized lipid and oxidized lipid of pork patty based on their analyzing parameters, (B) Loading plots between the selected PCs considering polar and non-polar HCAs, (C) VIP scores between the selected PCs. Non-oxidized lipid (NL): TBARS value in range of 0.2 ~ 0.6 mg MDA/kg meat; Oxidized lipid (OL): TBARS value of more than 0.6 mg MDA/kg meat.

reactive radicals facilitated by lipid oxidation could have caused an increase in HCAs, whereas the increase in IQx, PhIP, and Harman was significantly affected by lipid oxidation.

The addition of nitrite and paprika extract inhibited protein and lipid oxidation, thereby inhibiting the HCAs production, although paprika extract induced several side effects. In PE-added patties, the contents of MeIQx, 7,8-DiMeIQx, and PhIP produced on day 0, MeIQx produced on day 7, and 7,8-DiMeIQ produced on day 14 were all significantly higher than those in the control and SN ($P < 0.05$). This is thought to be due to the carotenoids, which are abundant in paprika, becoming unstable due to exposure to oxygen or light. Carotenoids effectively inhibit 4,8-DiMeIQx and MeIQx at certain concentrations, but the inhibitory effect is lost at other concentrations (Vitaglione, Monti, Ambrosino, Skog & Fogliano, 2002). These properties of carotenoids were not considered during the preparation of paprika extract, which could have limited the inhibitory properties of HCAs against the formation of certain HCAs.

4. Conclusions

Similar to sodium nitrite, paprika extract effectively inhibited protein and lipid oxidation, changes in pH, and cooking loss that had occurred during storage. Likewise, the addition of sodium nitrite and paprika extract inhibited the production of IQ and Aαc. In contrast, the production of IQx, MeIQx, PhIP, and Harman increased with the storage period. Additionally, protein and lipid oxidation significantly correlated with HCAs production, and of the HCAs, IQx, PhIP, and Harman were more significantly affected by protein and lipid oxidation. This effect was inhibited by sodium nitrite and paprika extracts. However, adding paprika extract did not inhibit the production of MeIQx, Di-MeIQx, or PhIP. This was considered to be due to the properties of the carotenoids present in paprika. Therefore, paprika extract is a natural antioxidant that can be used to substitute nitrite; however, further studies are required to explore its potential for effectively inhibiting HCAs.

CRediT authorship contribution statement

Kang-Jin Jeong: Methodology, Investigation, Writing – original draft. **Jin-Kyu Seo:** . **Zubayed Ahamed:** Validation, Writing – review & editing. **Youn Su Lee:** Investigation. **Han-Sul Yang:** Project administration, Supervision.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100936.

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