Changes in physicochemical properties and fatty acid composition of pork following long-term frozen storage

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Abstract

The objective of this study was to investigate the effect of long-term frozen storage (2 years) of pork on colour stability, lipid and protein oxidation, drip/thawing losses and intramuscular fatty acid composition, as well as to examine the relationships among them. Pork quality parameters were significantly altered by frozen storage during 2 years, causing an increase in pH values, drip and thawing losses and metmyoglobin percentage, and a decrease in colour indices (a* and b* values) and oxymyoglobin percentage. The percentage of intramuscular fat and total saturated and monounsaturated fatty acids were significantly higher in pork after 2 years of storage at −20 °C, whereas it had the lowest values of polyunsaturated fatty acids, especially n-3 long chain fatty acids. Furthermore, the relationships among colour stability, lipid and protein oxidation, thawing losses and fatty acid profile revealed the complex interrelations generated during frozen storage.

Keywords

Freezing · Colour stability · Fatty acid composition · Protein and lipid oxidation

Introduction

The global meat export industry is currently worth more than US$ 13 billion. Freezing plays an essential role in ensuring the safety of the meat products being supplied to all regions of the world [1]. In fact, freezing is the most

frequently used technology to preserve fresh meat during long-term storage, enabling its nutritive value to be maintained. However, experimental evidence shows that long-term frozen-stored pork may have quality problems, with poorer colour and rancid smell and taste [2].

Despite microbial spoilage being effectively inhibited by freezing, quality deterioration cannot be avoided due to the formation of ice crystals, which leads to mechanical damage, distortion of tissue structure and protein denaturation [3]. Ice crystals damage the ultrastructure and concentrate the solutes in the meat which, in turn, lead to biochemical alterations and influence the physical properties of the meat [1]. One of these alterations is the decrease of water-holding capacity. The fluid purge has a direct impact on meat weight and reduces its tenderness and the overall eating quality, thus affecting its commercial value [4]. In addition, protein oxidation reduces the quality of meat by promoting protein fragmentation or aggregation and decreasing protein solubility [5]. Carbonylation is an irreversible modification of proteins induced by oxidative stress and other mechanisms [6]. Carbonyls (aldehydes

and ketones) can be formed in proteins by direct oxidation of susceptible amino acid side chains, which has been highlighted as the most potent oxidative attack to proteins [7].

Lipids of pork can also suffer alterations during frozen storage, owing to lipolysis and oxidation, which results in quality loss [8]. Phospholipids, which have a high content of polyunsaturated fatty acids, constitute the lipid fraction most susceptible to oxidation. Therefore, it can lead to radical secondary lipid oxidation upon thawing [9], resulting in adverse changes in colour, odour, flavour and healthfulness [1].

Colour changes can occur, too, due to pigment degradation. The biochemical reactions responsible for myoglobin and lipid oxidation generate products that can further

accelerate oxidation in a reciprocal manner. The oxidation of the iron atom within the heme group is responsible for discoloration, a change from red oxymyoglobin to brownish metmyoglobin [10]. It has also been reported that denaturation of the globin moiety of the myoglobin molecule may occur at some stage during freezing, frozen storage and thawing [11]. Denaturation results also in an increased susceptibility of myoglobin to autoxidation and subsequent loss of fresh meat colour [1]. Keeping meat under frozen storage enables the meat industry to (a) adapt its offerings to consumers' demand, (b) adjust the meat supply to the processing rate and (c) transport meat to distant countries [12]. In fact, significant pork stocks are steadily being stored frozen for varying periods before being thawed to be consumed. Since storage time is dependent on varying market demands, it may easily occur that those pork stocks be stored for long periods. Therefore, a thorough understanding of the physical and chemical changes induced by frozen storage is of utmost importance for the meat industry [13]. Notwithstanding, there are a limited number of studies available in the literature on the relationships among fatty acid composition, lipid and protein oxidation and meat quality in long-term frozen storage. It seems necessary to bring into focus the effects of these substantial changes brought about by freezing, which would allow predicting frozen pork quality. With this purpose, the goal of this study was to investigate the effect of long-term frozen storage (2 years) on colour stability, lipid and protein oxidation, drip/thawing losses and fatty acid composition, as well as to examine the relationships among them in M. Longissimus thoracis et lumborum in pork.

Materials and methods

The pigs used for this trial were cared in accordance with the guidelines from the Spanish Ministry of Agriculture [14].

Animals, preparation of frozen samples and storage

The experiment was conducted with 43 entire male pigs from the mating of Pietrain sires to Landrace × Large White dams. At the beginning of the study trial, all pigs were subjected to the same standard pelleted diet (control diet) and management during 44 days. Thereafter, pigs were randomly assigned to one of the five dietary fat groups with the individual animal as the experimental unit. All diets were iso-proteic [17 % crude protein (CP)], and pigs had ad libitum access to feed and water during 64 days. All the diets contained the same proportions of raw materials (barley grain, wheat grain and soybean meal

44 % CP), except the proportion of corn grain that was different depending on the percentage of added fat. The whole composition of the diets has been reported in Alonso et al. [15]. The five diets differed in their fat sources: (1) control diet (without added fat); (2) animal fat (tallow–lard mix that had a 3/5 acidity grade) at 1 %; (3) animal fat at 3 %; (4) soybean oil at 1 %; and (5) calcium soaps of palm oil

fatty acids at 1 % (MAGNAPAC®, NOREL S.A., Madrid, Spain). All groups of pigs entered into the growing period at the same time and were slaughtered at the same day. All animals were slaughtered in a commercial facility (stunning with CO2) at 83.8 ± 6.3 kg carcass weight [16]. The experiment was carried out from April 2010 to April 2012. M. Longissimus thoracis et lumborum (LTL) was removed from each carcass 48 h after slaughter. After 24 h, at 4 ± 1 °C, the M. LTL was divided in halves and the caudal portion was sectioned into 2-cm-thick boneless chops for: lipid oxidation, fatty acid composition, drip loss and muscle colour measurements. Caudal samples (except those for colour and drip loss) and the cranial portion of M. LTL were placed in vacuum (−900 mbar of pressure) polyethylene–polyamide bags (water vapour permeability of 2.8 g/ m2/day at 23 °C and an O2 permeability of 50 cm3/m2/day at 23 °C; Irma, Zaragoza, Spain) and stored at −20 °C ± 2 °C in the dark in a freezer (static air chamber) until analysis (caudal samples for lipid oxidation and fatty acid profile: maximum 1 month; one caudal 2-cm-thick boneless chop: 12 months; and cranial portion: 24 months). The samples from the caudal portion will be named 'control' throughout the manuscript. Twelve months later, one 2-cm-thick boneless chop was thawed in tap water for 4 h before the vacuum was broken and only lipid oxidation analysis was carried out. Two years later, the cranial portion of M. LTL was thawed in tap water for 4 h before the vacuum was broken and sectioned into 2-cm-thick steaks for lipid and protein oxidation, fatty acid composition, drip loss and muscle colour measurements. The samples from the cranial portion will be named '2 years frozen' throughout the manuscript.

pH measurement

The pH values of control [at 72 h post-mortem (p.m.)] and 2-year frozen/thawed pork were measured using a portable pH meter equipped with a glass electrode Crison PH 25 (Crison instruments, Barcelona, Spain). Each value was the mean of four random measurements that were carried out on loin before slicing.

Instrumental measurement of colour and myoglobin forms

A Minolta CM-2002 (Osaka, Japan) spectrophotometer was used to measure colour at the surface of a 2-cm-thick

LTL chop from control (at 72 h p.m.) and 2-year frozen/ thawed pork exposed to air for 2 h. The illuminant used was D65, and the standard observer position was 10°. The parameters registered were CIE L* (lightness), a* (redness) and b* (yellowness). Each value was the mean of ten observations on the same chop, avoiding areas with excess fat. The relative contents of deoxymyoglobin (DMb), oxymyoglobin (OMb) and metmyoglobin (MMb) were calculated from the reflectance curve according to Krzywicki [17] using 730 nm as maximum wavelength. The instrument measured the reflectance between 400 and 740 nm at 10-nm intervals. Reflectance values at wavelengths not given by the instrument (473, 525 and 572 nm) were calculated using linear interpolation. Also, the extent of oxygen saturation of myoglobin on meat surface was measured by the parameter Iso2 [18].

Thawing loss and drip loss

After 2 years stored at −20 °C, the cranial portion of M. LTL was thawed in tap water for 4 h before the vacuum was broken and excess moisture removed before meat was weighed. Thawing loss was expressed as a percentage of the initial weight (at 72 h p.m.). Furthermore, a 2- cm-thick LTL chop from control (at 72 h p.m.) and 2-year frozen/thawed pork were weighed and placed on a supporting mesh in a sealed plastic container (with no contact between sample and container). After a storage period of 24 and 48 h at 4 ± 1 °C, the samples were taken out of the container, dabbed lightly on filter

paper and weighed again. Drip loss was expressed as a percentage of the initial weight, based on Honikel [19].

Intramuscular fat and fatty acid analysis

After LTL samples were thawed in tap water (without losing vacuum), they were ground and 10 g of sample were weighted. The fat was extracted in chloroform–methanol (1:1 v/v), with 2,6-di-tert-butyl-4-methylphenol (BHT) (1 g/10 ml methanol) as antioxidant [20]. One millilitres of chloroform phase was used to assess the percentage of intramuscular fat (IMF) by drying at 100 °C for 20 min; the results were expressed as the weight percentage of wet muscle. The rest was evaporated in a sand bath under nitrogen gas at 50 °C. The methyl esters from fatty acids (FAMES) were formed using a KOH solution in methanol and collected in hexane for analysis by gas chromatography following the method described by Carrilho et al. [21]. The FAMES were analysed in a gas chromatograph HP-6890 II (Hewlett-Packard, Waldbronn, Germany) using a capillary column SP-2380 (100 m × 0.25 mm × 0.20 μm) and oven temperature programming as follows: column temperature was set at 140 °C, then raised at a rate of 3 °C/

min from 130 to 158 °C, and 1 °C/min to 165 °C, kept for 10 min, raised at 5–220 °C and kept constant for 50 min. Nitrogen was used as a gas carrier at a constant flow rate of 0.8 ml/min with an injected volume of 1 μl. The methyl esters were identified using retention times of Supelco® 37 Component FAME Mix. Data regarding FAMES composition were expressed as area percentage of total identified FAMES.

Lipid oxidation

Lipid oxidation was measured by the 2-thiobarbituric acid (TBA) method of Pfalzgraf et al. [22]. Meat samples of 10 g were taken and homogenized with 10 % trichloroacetic acid using an Ultra-Turrax T25 (Janke & Kunkel, Staufen, Germany). Samples were centrifuged at 2680 g-force for 30 min at 10 °C, and the supernatants filtered through quantitative paper. Two millilitres of the filtrates were taken and mixed with 2 ml of TBA (20 mM), homogenized and incubated for 20 min in boiling water. Absorbance was measured at 532 nm. The TBA-reactive substances (TBARS) values were calculated from a standard curve of malondialdehyde and expressed as mg malondialdehyde (MDA)/kg sample.

Protein oxidation

Protein oxidation was measured by estimation of carbonyl groups formed during incubation with 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl following the method described by Ventanas et al. [23]. Carbonyl concentration was measured on the treated sample by measuring DNPH incorporated on the basis of absorption of 21.0 mM−1/cm at 370 nm for protein hydrazones. Results were expressed as nmol of DNPH fixed per milligram of protein. Protein oxidation was expressed as nmol carbonyls/mg protein. Protein concentration was calculated by spectrophotometry at 280 nm using bovine serum albumin (BSA) as standard.

Statistical analysis

All data were statistically analysed by the General Linear Model (GLM) procedure of SPSS, version 19 [24]. The model included dietary fat supplementation and frozen storage as main effects as well as their interaction. However, only the results related to the effect of frozen storage were presented in this manuscript. Duncan's post hoc test was used to assess differences between mean values when $P \leq 0.05$. Mean values and standard deviation (SD) are reported in tables. Relationships among meat quality parameters and intramuscular fatty acid composition were evaluated by calculating Pearson's correlation coefficients.

Results and discussion

Physicochemical parameters

As described in the 'Materials and methods' section, the whole set of animals were used for a study on the influence of dietary fat on meat properties [15]. Since no significant interaction was observed between dietary fat supplementation and frozen storage for pH values, colour measurements (L*, a* and b* values), meat pigment proportions, Iso2, drip loss and lipid oxidation, only the effect of frozen storage is presented in Table 1.

Table 1 Mean values ($\bar{x}$) and standard deviation (SD) for physicochemical properties in *M. Longissimus thoracis et lumborum* for control and 2-years frozen/thawed pork

	Control		2 Years frozen		Sign.
	$n = 43$		$n = 43$		
	$\bar{x}$	SD	$\bar{x}$	SD	
pH	5.51	0.13	5.65	0.15	***
Colour					
*L**	45.92	2.84	45.04	4.02	ns
*a**	6.48	1.72	4.05	1.66	***
*b**	12.55	1.58	9.05	1.68	***
% OMb	29.46	12.85	1.76	3.96	***
% DMb	65.63	15.49	80.87	10.99	***
% MMb	4.91	3.13	17.37	8.40	***
Iso_2 (%)	21.78	1.16	10.08	2.28	***
Water loss					
24-h drip loss (%)	1.79	0.61	5.04	1.71	***
48-h drip loss (%)	2.54	0.85	6.18	1.95	***
Thawing loss (%)	–	–	3.90	1.36	–
TBARS values (mg malondialdehyde/kg)	0.090	0.019	0.083	0.016	ns
Protein oxidation (nmol carbonyls/mg protein)	–	–	1.10	0.35	–

OMb oxymyoglobin, *DMb* deoxymyoglobin, *MMb* metmyoglobin, *Iso_2* oxygen saturation of myoglobin on meat surface and *TBARS* TBA-reactive substances

ns $P > 0.1$; *** $P \leq 0.001$

Results showed that the values of pH were slightly higher ($P \leq 0.001$) in 2 years frozen than in control pork, which agreed with Brewer and Harbers [25] who observed a slightly increase in pH in raw ground pork frozen during 39 weeks. In contrast, Leygonie et al. [26] found a decrease in pH of ostrich meat stored frozen for 1 month most likely arose from the loss of minerals and peptides as exudates due to freezing and thawing, thereby changing the ionic balance in the meat. It is possible that long-term

frozen storage had changed the iso-electrical point of the proteins (denaturation of the proteins) resulting in increased pH values. Anyway, the pH values after freezing were normal by swine. There was no effect ($P > 0.05$) of frozen storage on L* values, which agreed with the reports of Hansen et al. [2] for pork and Muela et al. [27] for lamb. However, a* and b* values were significantly lower ($P \leq 0.001$) in 2-year frozen pork. Similarly, Hansen et al. [2] reported that redness was lower for 30-month frozen pork chops than for fresh chops. These authors explained that the lower redness of the frozen pork may have been caused by processes occurring in the frozen state such as myoglobin cold denaturation [28]. In contrast, these same authors found that frozen pork chops had significantly higher b* values than fresh chops. The significant decrease in a* and b* values could translate into a product that is browner and duller in appearance, overall less attractive to the consumer [26].

Table 1 also shows the results of meat pigment proportions [oxymyoglobin (OMb), deoxymyoglobin (DMb) and metmyoglobin (MMb)] and the parameter Iso2. Frozen storage had a statistically significant effect on the three meat pigment forms ($P \leq 0.001$). The MMb proportion increased significantly after 2 years of frozen storage, whereas the proportion of OMb decreased. Myoglobin is one of the proteins that denatures during freezing and thawing, being the globin fraction which denatures leading to a loss in colour stability [3, 11]. On the other hand, the metmyoglobin-reducing enzyme (MRE) system continually reduces MMb back to DMb and then to OMb in fresh muscle [29]. However, as meat ages or is frozen, the activity of the MRE decreases and MMb begins to accumulate on the surface of the meat at a rapid rate [30]. Also, MRE and/or co-factors, such as NADH, could be 'lost' from the post-mortem sarcoplasmic environment by leaching as exudate during thawing, and/or due to oxidation, and/or be used by reactions unrelated to MRE, which would all contribute to accelerated oxidation and loss of bloom [1, 30]. Moreover, the oxygen saturation on the meat surface, assessed by Iso2, decreased ($P \leq 0.001$) after 2 years of frozen storage. The transformation of DMb into OMb was slowed after vacuum packaging had been broken and thus produced a high percentage of DMb, which agreed with Kim et al. [31]. These authors found that frozen/thawed meat exhibited a slower rate of oxygenation conversion from DMb to OMb. Also, the MMb layer beneath the surface (located between superficial OMb and interior DMb) gradually thickens and moves towards the surface [32].

In conclusion, pork had a lower blooming capacity and a higher percentage of MMb after 2 years of frozen storage. Both 24- and 48-h drip loss percentages were higher ($P \leq 0.001$) in 2 years frozen than in control chops. Loss of fluid as exudate is a key problem in the meat processing industry, and 2-year frozen samples could loss 8.94 % fluid accounting for thawing loss and 24-h drip loss (Table 1).

During freezing, ice crystals formed among and within the fibres physically damage the ultrastructure of the meat since as water freezes out; it leads to an increase in the concentration of the solutes surrounding the sensitive protein structure. The ice crystals that form draw water from the intracellular spaces to the intercellular spaces lead to excessive moisture loss during thawing [11]. The moisture loss further increases as the damage to the ultrastructure of the meat fibres does, not allowing uptake of moisture into the intracellular spaces upon thawing. In addition, the increase in solute concentration during freezing and frozen storage also leads to the denaturing of proteins, which influence the water-holding capacity [33]. In this respect, a significant positive correlation was found between thawing loss percentage and protein oxidation (nmol carbonyls/mg protein) (0.37, $P = 0.016$) at 2 years of frozen storage (Table 1), which agreed with Estévez et al. [12]. The loss of protonable amino groups as a result of carbonylation may impair electrical arrangement of the proteins. As a direct consequence of these chemical modifications, a shift of the balance between protein intramolecular interactions and protein–water interactions would take place, causing a loss of protein solubility and a decreased water-binding capacity (see review of Estévez [34]). Regarding the oxidative behaviour, no significant differences

were observed for lipid oxidation values among control, 1-year (0.090 ± 0.012 mg MDA/kg; data not shown in Table 1) and 2-year (0.083 ± 0.016 mg MDA/kg) frozen pork. Therefore, lipid oxidation remained stable throughout frozen storage for 24 months. Those results agreed with Hansen et al. [2], who found that TBARS values were not significantly higher after of 30-month frozen storage. During frozen storage (−20 °C), a portion of the water, termed unfrozen water, does not freeze and is available for chemical reactions. Generally, lipid oxidation increases rapidly post-thawing as peroxidation (primary lipid oxidation) occurs during frozen storage, giving rise to rapid and severe secondary lipid oxidation (thiobarbituric acid forming) and resulting in increased TBARS values [9, 26]. In all these previous studies, only the secondary lipid oxidation products using the TBARS method were measured immediately after thawing; therefore, probably primary lipid oxidation products (reactive oxygen species) could have been accumulated during frozen storage, but were not measured. In addition, samples used in our study were placed in vacuum bags to be stored in freezing. It is known that the exclusion of oxygen through the vacuum packaging prevents the oxidative degradation of muscle lipids during meat freezing [35]; therefore, it could have helped to produce the low values of TBARS that we found after frozen storage. On the other hand, the interaction between lipid and protein oxidation presumably may lead to a lower TBARS value, as malondialdehyde (MDA) acts as a substrate in one of the

pathways of protein oxidation [36]. Proteins might create a covalent binding to non-protein carbonyl compounds such as 4-hydroxy-2-nonenal or MDA [37]. Leygonie et al. [26] explained that a decrease in the level of MDA in the frozen meat could explain the lack of a significant difference in TBARS, an argument that was supported by the higher carbonyl level they found. Therefore, several factors could have contributed to find this low level of lipid oxidation in our results. In any case, it was clearly found that the secondary lipid oxidation remained fairly stable during 2 years of frozen storage.

Intramuscular fatty acid profile

As shown in Table 2, differences between control and 2-years frozen pork were significant when comparing the percentage of intramuscular fat (IMF) and concentrations of most individual fatty acids. The intramuscular fat of loin was significantly ($P \leq 0.001$) higher in 2 years frozen than in control pork. This could be due to a relative increase in fat concentration in muscle due to water losses that occur after thawing. However, Hernández et al. [8] did not find any significant difference in the total lipids of M. Longissimus dorsi during 180 days of frozen storage in pork. On the other hand, the proportion of palmitic (C16:0), stearic (C18:0) and total saturated fatty acids (SFA) were significantly greater ($P \leq 0.001$) in 2 years frozen than in control pork; the proportion of oleic (C18:1n-9) ($P \leq 0.01$) and total monounsaturated fatty acids (MUFA) ($P \leq 0.05$) were also higher. However, proportions of n-6 polyunsaturated fatty acid (PUFA) ($P \leq 0.01$) such as linoleic acid (C18:2) ($P \leq 0.05$) and arachidonic acid (C20:4) ($P \leq 0.001$) of pork stored frozen for 2 years were significantly lower. The n-3 PUFA, such as α-linolenic (C18:3), eicosapentaenoic, EPA (C20:5), docosahexaenoic, DHA (C22:6), and other long chain fatty acids were significantly lower ($P \leq 0.001$) in 2-years frozen pork. Therefore, the total PUFA percentage decreased during the 2-years frozen storage, producing a relative increase in SFA and MUFA percentage. Hernández et al. [8] found a decrease in PUFA percentage of the phospholipid (PL) fraction after 6 months of frozen storage in pork; in particular, linoleic and α-linolenic acids and non-polar lipid fatty acids remained unchanged.

Table 2 Fatty acid composition of intramuscular fat (% of total fatty acids) in *M. Longissimus thoracis et lumborum* for control and 2-years frozen/thawed pork

	Control		2 Years frozen		Sign.
	$n = 43$		$n = 43$		
	$\bar{x}$	SD	$\bar{x}$	SD	
IMF (%)	2.17	0.53	3.01	0.99	***
C12:0	0.066	0.01	0.073	0.01	***
C14:0	1.11	0.12	1.26	0.15	***
C16:0	22.72	0.82	23.54	0.87	***
C16:1	3.31	0.50	3.33	0.41	ns
C18:0	10.50	0.81	11.19	0.82	***
C18:1n-9	39.86	2.47	41.32	2.24	**
C18:1n-7	4.45	0.36	4.34	0.28	ns
C18:2n-6	10.50	2.39	9.39	2.25	*
C18:3n-6	0.087	0.02	0.074	0.02	**
C18:3n-3	0.40	0.06	0.36	0.08	***
C20:1n-9	0.73	0.08	0.75	0.07	ns
C20:2n-6	0.34	0.06	0.34	0.06	ns
C20:2n-3	0.11	0.03	0.08	0.03	***
C20:3n-6	0.35	0.08	0.25	0.07	***
C20:3n-3	0.07	0.01	0.07	0.02	ns
C20:4n-6	2.23	0.70	1.58	0.65	***
C20:5n-3	0.12	0.04	0.08	0.03	***
C22:5n-3	0.38	0.11	0.27	0.09	***
C22:6n-3	0.16	0.05	0.10	0.04	***
∑SFA	35.17	1.42	36.82	1.49	***
∑MUFA	48.90	3.16	50.29	2.70	*
∑PUFA	14.78	3.36	12.62	3.12	***
∑n-6	13.52	3.10	11.65	2.90	**
∑n-3	1.25	0.26	0.96	0.23	***
C18:2n-6/C18:3n-3	25.98	3.62	26.24	3.78	ns
P/S ratio	0.42	0.10	0.35	0.09	***
n-6/n-3 ratio	10.79	0.53	12.15	0.49	***

IMF percentage of intramuscular fat extracted by Bligh and Dyer method, *SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids and *P/S ratio* PUFA/SFA ratio

ns $P > 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Similarly, Igene et al. [38] reported changes in the unsaturated fatty acids of PL in beef and poultry meat during frozen storage, especially up to the 8th month. Awad et al. [39] found a considerable decrease in the PL content during an 8-week storage period of bovine muscle at −4 °C. This general decrease in PUFA of PL could be accounted for the enzymic hydrolysis of PL concentration during frozen storage, because lipolytic enzymes remain active at freezing temperatures. That hydrolysis of PL occurred during frozen storage of muscle with the formation of free fatty acids and water-soluble decomposed phospholipids. These results were confirmed by Hernández et al. [8], who reported a significant increase in free fatty acids during storage, which arose mostly from phospholipids

hydrolysis, being phosphatidylethanolamine the most affected PL. However, these authors only found a total increase in free fatty acids content of 50.6 mg/100 g dry matter in 6 months. Taking into account that our results included the total percentage of fatty acids of neutral lipids and phospholipids together, even so we found an important decrease of PUFA percentage (14.61 % with respect to initial percentage). Therefore, a small part of this decrease could be due to the release of free fatty acids, but the rest is to be related to the formation of new components coming from lipid oxidation.

No significant differences were observed for the C18:2n-6/C18:3n-3 ratio between frozen and control pork, while the n-6/n-3 ratio was ($P \leq 0.001$) greater in frozen pork. Consequently, this difference could be due to a higher decrease in n-3 long chain fatty acids in stored frozen pork. This was confirmed by a greater percentage of decrease in n-3 PUFA (23.2 %) than in n-6 PUFA (13.83 %), specifically in EPA (33.3 %) and DHA (37.5 %). The n-3 fatty acids are more easily oxidized, taking into account that unsaturated fatty acids react more rapidly, and the more double bonds that a fatty acid contains, the more reactive it is [40]. Some fatty acid ratios related to human health are shown too in Table2. The 2-year frozen pork had the lowest P/S ratio ($P \leq 0.001$) and produced meat with a lower nutritional value, under the nutritional guidelines for P/S ratio >0.4 [41]. A maximum value of 4 for the intake of n-6/n-3 PUFA has been recommended [41] because it is a risk factor for coronary heart disease. Pork is characterized by a high content of C18:2n-6 following a cereal-based diet, which leads to acceptable P/S ratio, but the high content in n-6 PUFA usually results in undesirably high n-6/n-3 ratio from a human health perspective. After 2 years of frozen storage, the n-6/n-3 ratio increased significantly.

Relationship among physicochemical parameters and IMF fatty acid composition

The principal Pearson's correlations among physicochemical parameters and IMF fatty acid composition in 2-year frozen/thawed pork are summarized in this section (Table 3). There were negative significant correlations between the percentage of SFA (−0.48; $P = 0.001$) and IMF (−0.31; $P = 0.042$) and TBARS values of frozen samples. Therefore, pork with less proportion of saturated fatty acids could have an enhanced lipid oxidation during storage. This could be related to the fact that meat with a lower percentage of SFA had a higher percentage of PUFA. This statement is based on the fact that the proportion of SFA and MUFA increase faster with increasing intramuscular fat, while the relative proportion of PUFA decreases [42], and vice versa. In this regard, a significant ($P = 0.001$) correlation was found between percentages of SFA (−0.54) and PUFA and among the percentages of SFA (0.46), MUFA (0.67) and PUFA (−0.77) and IMF after frozen storage. There is a close relationship between lipid oxidation and the composition of fatty acids. Lipid oxidation is a complex process in which molecular oxygen reacts with unsaturated fatty acids, particularly with PUFA [43]. A higher ratio of PUFA to SFA in meat is more susceptible to oxidative damage [44]. Therefore, a lower amount of PUFA, especially n-3 long chain fatty acids, could be indirectly related to the increase in lipid oxidation. The percentage of SFA was negatively correlated with protein oxidation as well (−0.34, $P = 0.025$). Also, protein oxidation was positively correlated (0.29; $P = 0.055$) with the n-6/n-3 ratio after 2 years in frozen storage. Again, meat with greater percentage of PUFA, especially n-3 long chain fatty acids, could have a higher carbonyl content after 2 years of frozen storage.

Table 3 Relationship among physicochemical parameters and IMF fatty acid composition in 2-years frozen/thawed pork ($n = 43$)

	pH	*L**	*a**	*b**	TBARS	24-h DL	48-h DL	MMb	DMb	OMb	Iso_2
pH	1										
*L**	**−0.51****	1									
*a**	−0.13	−0.25	1								
*b**	**−0.33***	**0.43****	−0.07	1							
TBARS	0.05	−0.15	0.17	−0.21	1						
24-h DL	−0.22	0.02	0.11	−0.16	−0.16	1					
48-h DL	−0.21	0.02	0.10	−0.16	−0.19	**0.99****	1				
MMb	−0.26	0.09	**0.75****	−0.17	0.08	0.22	0.22	1			
DMb	0.24	−0.14	**−0.76****	0.04	0.01	−0.15	−0.16	**−0.95****	1		
OMb	−0.12	0.19	**0.52****	0.26	−0.19	−0.04	−0.03	**0.52****	**−0.76****	1	
Iso_2	**−0.38***	0.18	**0.38***	**0.51****	0.02	0.19	0.19	0.23	**−0.37***	**0.52****	1
TL	**−0.55****	**0.42****	0.24	0.16	0.15	0.28	0.25	**0.31***	−0.30	0.16	**0.36***
P-OX	0.01	−0.14	**0.32***	0.01	0.20	0.08	0.05	0.14	−0.13	0.05	0.27
IMF	0.01	0.29	−0.18	**0.50****	**−0.31***	−0.23	−0.21	−0.17	0.07	0.17	0.17
SFA	**0.40****	0.18	**−0.33***	0.17	**−0.48****	−0.21	−0.18	−0.14	0.09	0.05	−0.27
MUFA	−0.01	**0.40****	**−0.36***	**0.32***	0.02	−0.29	−0.28	**−0.33***	0.22	0.10	0.10
PUFA	−0.18	**−0.42****	**0.46****	**−0.34***	0.20	**0.35***	**0.32***	**0.34***	−0.22	−0.11	0.04
n-3	−0.21	**−0.40****	**0.43****	**−0.37***	0.17	**0.36***	**0.34***	**0.33***	−0.22	−0.10	0.04
n-6	−0.17	**−0.42****	**0.46****	**−0.34***	0.20	**0.34***	**0.32***	**0.34***	−0.22	−0.11	0.04
P/S	−0.20	**−0.42****	**0.47****	**−0.34***	0.24	**0.35***	**0.32***	**0.34***	−0.22	−0.10	0.07
n-6/n-3	0.09	−0.15	0.28	0.09	0.23	0.02	0.02	0.17	−0.11	−0.05	0.06

	TL	P-OX	IMF	SFA	MUFA	PUFA	n-3	n-6	*P/S*	n-6/n-3
pH										
*L**										
*a**										
*b**										
TBARS										
24-h DL										
48-h DL										
MMb										
DMb										
OMb										
Iso_2										
TL	1									
P-OX	**0.37***	1								
IMF	−0.15	−0.13	1							
SFA	**−0.47****	**−0.34***	**0.46****	1						
MUFA	−0.02	0.01	**0.67****	0.08	1					
PUFA	0.23	0.15	**−0.77****	**−0.54****	**−0.89****	1				
n-3	0.25	0.11	**−0.78****	**−0.53****	**−0.87****	**0.99****	1			
n-6	0.23	0.16	**−0.77****	**−0.53****	**−0.89****	**0.99****	**0.99****	1		
P/S	0.27	0.19	**−0.77****	**−0.62****	**−0.83****	**0.99****	**0.98****	**0.99****	1	
n-6/n-3	0.01	0.29	−0.15	−0.15	−0.26	0.30	0.14	**0.31****	0.29	1

TBARS TBA-reactive substances, *24-h DL* 24-h drip loss, *48-h DL* 48-h drip loss, *MMb* metmyoglobin, *DMb* deoxymyoglobin, *OMb* oxymyoglobin, *Iso_2* oxygen saturation of myoglobin on meat surface, *TL* thawing loss, *P-OX* protein oxidation, *IMF* percentage of intramuscular fat extracted by Bligh and Dyer method, *SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids and *P/S ratio* PUFA/SFA ratio

Bold numbers indicate that Pearson's correlations are significant: * $P \leq 0.05$; ** $P \leq 0.01$

The increase in carbonyls is expected as the freezing and thawing treatments lead to structural changes in the proteins through oxidative modification of the amino acid side chains, presumably initiated by the peroxidation of the polyunsaturated fatty acids [34, 36]. On the other hand, the percentage of MMb was significantly (P = 0.025) correlated with the proportion of MUFA (−0.33), PUFA (0.34), n-6 (0.34), n-3 (0.33) and P/S ratio (0.34) after 2 years of frozen storage. Metmyoglobin formation is generally

greater in the presence of unsaturated aldehydes than their saturated counterparts of equivalent carbon chain length. In addition, increasing chain length of aldehydes results in an increased MMb formation. Moreover, aldehydes alter myoglobin redox stability by increasing OMb oxidation, decreasing the MMb reduction via enzymatic process, and enhance the prooxidant activity of MMb (see review of Chaijan [45]). Therefore, the biochemical reactions directly responsible for myoglobin oxidation and lipid oxidation each generate products that can further accelerate oxidation in a reciprocal manner [10]. Nevertheless, in our study, a non-significant correlation was found between the percentage of MMb and secondary lipid oxidation (TBARS values). The unique relationship between pO2 and myoglobin redox forms such that low nonzero pO2 that favours met–heme formation [46] provides conditions in which the oxidative interaction is not tightly interconnected. The extent of lipid oxidation in meat is proportional to the concentration of oxygen present and would thus be expected to be minimal in low pO2 environments. Therefore, atmospheres containing very low concentrations of oxygen (vacuum packing in our case) provide conditions in which the oxidative interaction between lipid and myoglobin is not tightly linked [10]. It seems relevant to point out that there was a significant correlation (−0.39; P = 0.009) between pH and TBARS values, as well as between pH values and the percentage of MMb (−0.52; P = 0.001) and thawing loss (−0.66; P = 0.001) in control pork (data not shown). Also, pH values were correlated with the percentage of MMb (−0.26; P = 0.087) and thawing loss (−0.55; P = 0.001) after long-term frozen storage. The significant correlations found between pH values and some of the parameters analysed

could indicate a higher oxidative susceptibility of meat with lower post-mortem pH. The autoxidation of OMb is acid catalysed; consequently, a decrease in pH accelerates the autoxidation of OMb, decreasing colour stability and increasing lipid and protein oxidation [47]. Moreover, it is generally accepted that water-holding capacity decreases as muscle pH ultimately decreases, resulting in increased muscle surface exudate or drip loss and a higher thawing loss in our case.

Conclusions

Pork quality parameters were significantly altered by frozen storage during 2 years, causing an increase in pH values, drip and thawing losses and a decrease of colour stability (lower a* and b* values, lower blooming capacity and higher MMb percentage). However, secondary lipid oxidation remained stable during frozen storage when pork was immediately analysed after thawing. The intramuscular fatty acid profile was significantly affected by frozen storage, resulting in a decrease in the percentage of polyunsaturated fatty acids, especially n-3 long chain fatty acids, which modified the other fatty acid percentages and made lipid profile less unhealthy for human consumption. Furthermore, the Pearson's correlations found in this study showed that PUFA decrease, especially n-3 long chain fatty acids, was indirectly related to the increase in lipid and protein oxidation. Also, the PUFA percentage was positively correlated with the amount of MMb. The significant correlations found between pH values and MMb percentage and lipid oxidation could indicate a higher oxidative susceptibility of meat with lower post-mortem pH. Based on the present study, the relationships among colour stability, lipid and protein oxidation, thawing losses and fatty acid profile revealed the complex interrelations generated during frozen storage.

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Compliance with ethical standards

Conflict of interest None.

Compliance with ethics requirements Research involving animals.

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