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Effect of plasma-activated water (PAW) soaking on the lipid oxidation of sardine (*Sardina pilchardus*) fillets

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ABSTRACT

The efficacy of plasma-activated water (PAW) as a chemical-free and environmentally friendly preservative has been documented for a variety of foods, but the onset of lipid oxidation induced by plasma-reactive species has been less extensively studied. In this work, global indices (peroxide value, UV specific absorbance) and direct analytical determinations of volatile and non-volatile oxidation products were performed on sardine lipids extracted from fish fillets immersed in PAW (treatments) and distilled water (controls) for 10–30 min. Evidence of PAW-induced lipid oxidation was provided by higher UV specific absorbances and higher levels of C5-C9 secondary volatile oxidation products in the treated samples. However, the degree of fatty acid oxidation was not sufficient to cause a significant reduction in nutritionally valuable eicosapentaenoic acid and docosahexaenoic acid. Twelve cholesterol oxidation products (COPs) were identified in the sardine lipids, but no significant differences in total COPs content were found between PAW processed and control samples.

1. Introduction

The sardine (Sardina pilchardus) is the most important species caught in the Mediterranean Sea (158,166 tonnes, representing 22.4 % of total landings, average 2018–2020) and the second most important species in terms of value (USD 187,606,195), after the European anchovy (Engraulis encrasicolus) (FAO, 2022). Sardines have been a staple food of the people of the Mediterranean region since ancient times, and they still play an important role in the culinary traditions of countries by the sea. In addition to the traditional canned, air-dried and dry-salted preserves, there is an increasing demand for minimally processed, ready-to-use and ready-to-eat products that can be used for household food preparation in an ever shorter time. Due to their chemical characteristics (abundant nutrients, high water content, near neutral pH), sardines are highly susceptible to microbiological, biochemical and chemical spoilage processes and provide an ideal environment for the growth of most foodborne pathogenic bacteria. The development of technologies and strategies to ensure the safety and extend the shelf life of seafood in the cold chain at low cost has therefore never ceased.

In the last decade, the potential of cold plasma technologies for microbiological and chemical decontamination of fresh and processed foods has been intensively studied (Ganesan, Tiwari, Ezhilarasi, & Rajauria, 2021). Cold plasma is a physical medium consisting of ions, radicals, free electrons, UV photons and neutral atoms/molecules in

Abbreviations: COPs, cholesterol oxidation products; DBD, dielectric barrier discharge; DHA, docosahexaenoic acid; EI, electron impact; EPA, eicosapentaenoic acid; FA, fatty acid; FAMEs, fatty acid methyl esters; GC, gas chromatography; MS, mass spectrometry; MUFA, monounsaturated fatty acid; PAW, plasma activated water; PUFA, polyunsaturated fatty acid; PV, peroxide value; RI, Kovats retention index; RONS, reactive oxygen and nitrogen species; RRT, relative retention time; SFA, saturated fatty acid; SPME, solid phase micro extraction.

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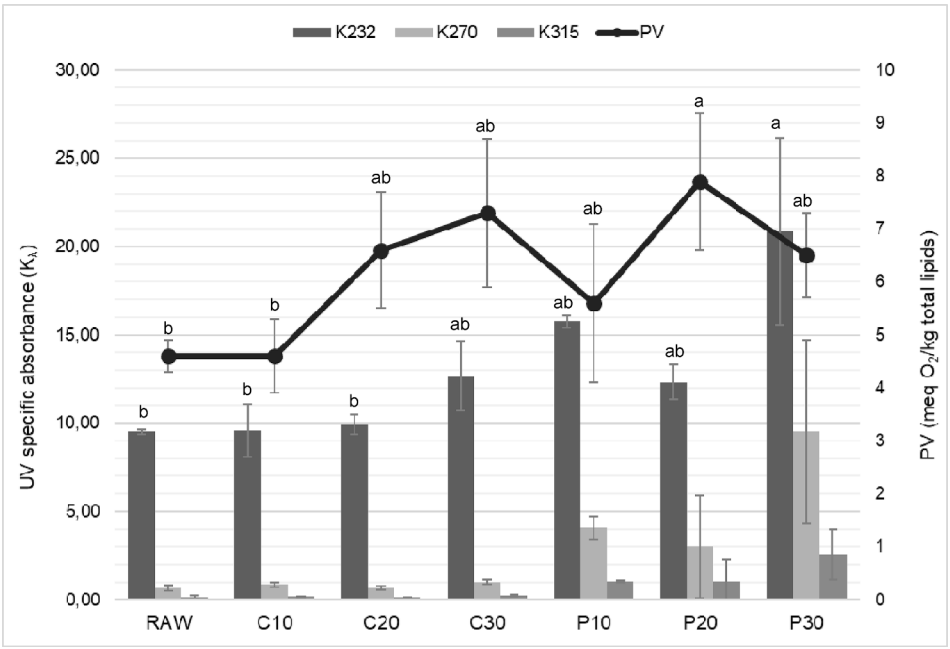

Fig. 1. Oxidation indices (peroxide value; UV specific absorbances at 232, 270, and 315 nm; mean ± SD, n = 3) of lipids extracted from PAW-processed sardine fillets and controls. P10-30 are PAW-dipped fillets for 10 to 30 min; C10-30 are fillets dipped in water for 10 to 30 min. RAW are the fresh, unprocessed fillets. Lower case letters indicate significant differences between treatments at an alpha level of 0.05 according to an HSD test.

both ground and excited states that can be generated under mild pressure (atmospheric pressure, mild vacuum) and temperature (room temperature or slightly above) conditions. Direct plasma treatments show efficacy in inactivating spoilage and pathogenic microorganisms of food interest and in degrading undesirable substances (mycotoxins, pesticides, food allergens) (Gavahian & Khaneghah, 2020), but their limited penetration and low surface coverage limit their use on a large scale. Indirect treatments with plasma-functionalised liquid media, created by the interaction between plasma and water or water solutions, are emerging as new chemical-free and environmentally friendly preservatives. In particular, plasma-activated water (PAW) is capable of treating the entire surface of solid foods, has an additional (mechanical) washing function and offers higher penetration and longer activity than direct cold plasma treatments. The decontamination capabilities of plasma-activated liquids have been attributed to the synergy between the acidic environment and a variety of long-lived reactive oxygen and nitrogen species (RONS), e.g. ozone, hydrogen peroxide, nitrites, which remain in solution for a long time after plasma treatment. Particularly, hydrogen peroxide and nitrite/nitric acid are stable species while the half-life of ozone is in the range of seconds to hours, depending on the quality of the water (Gao, Francis, & Zhang, 2022). However, several reports have emphasised the role of short-lived species peroxynitrous acid/peroxynitrite, superoxide and hydroxyl radicals, and singlet oxygen (Perinban, Orsat, & Raghavan, 2019). It was found that soaking with PAW was successful in inactivating *Shewanella putrefaciens* in fillets of Yellow River carp (*Cyprinus carpio*) (Liu, Zhang, Meng, Bai, & Dong, 2021) and *L. monocytogenes* and *S. Typhimurium* in grass carp (*Ctenopharyngodon idella*) (Esua, Cheng, & Sun, 2020), and extending the shelf life of Asian sea bass *(Lates calcarifer*) steaks (Chaijan et al., 2021). PAW ice (Liao et al., 2018) and PAW glazing (Herianto et al., 2022) have been suggested as possible preservation methods for fresh shrimps, while Zhao et al. (2021) investigated the decontaminating effect of a combination of PAW dipping and sonication on mackerel fillets.

The RONS involved in microbiological and chemical decontamination can also trigger the onset and/or accelerate the progress of numerous undesirable side reactions in food components, which can affect quality and, in the worst case, lead to the production of risky substances, limiting the use of plasma technologies in food production. Food lipids in particular are sensitive to oxidative degradation, especially when conditions are favourable, due to lipid characteristics (high degree of unsaturation) and/or substrate characteristics (high lipid content and/or low water activity). The depot (triacylglycerols) and structural lipids (phospholipids) of pelagic fish are extremely rich in n-3 polyunsaturated fatty acids (PUFAs), whose beneficial biological activities are supported by a large body of literature (Pacetti et al., 2015). However, oxidative degradation of tissue lipids can lead to the development of rancid off-odour and a reduction in nutritional value. The oxidation of tissue lipids induced by plasma reactive species has been recently reviewed and discussed by Perinban et al. (2019) and Herianto, Hou, Lin, and Chen (2021). As monounsaturated fatty acids (MUFAs), cholesterol can also be involved in autoxidation and photooxidation reactions, producing a range of polar (epoxy, hydroxy and keto derivatives) and non-polar (dienes, dienones, enones) products. Dietary intake of exogenous polar oxidation products leads to their accumulation in various organs and tissues, which can lead to health problems in humans (prostate and breast cancer, Parkinson's and Alzheimer's diseases, macular degeneration, cataracts, osteoporosis) (Maldonado-Pereira, Schweiss, Barnaba, & Medina-Meza, 2018). Therefore, clarifying the interactions between RONS and fish lipids (saponifiable and unsaponifiable) is imperative to assess the safety of the technology and the impact on nutritionally valuable substances (PUFAs). This is a crucial step for the successful introduction of PAW in food processing and the scale-up from pilot plant to industrial production.

To date, few reports have been published on the oxidative degradation of tissue lipids from seafood processed with PAW. Moreover, analytical data were limited to global indices of primary and secondary oxidation products (peroxide value, thiobarbituric acid reactive substances) (Chaijan et al., 2021, 2022; Herianto et al., 2022; Liao et al., 2018; Liu et al., 2021; Zhao et al., 2021) and, to our knowledge, more advanced analytical methods for direct quantification of volatile and non-volatile chemical species from FAs and cholesterol oxidation have not yet been performed.

For the above reasons, we intended to provide more detailed information on the oxidative state of sardine lipids after soaking the fish fillets in PAW for different periods of time. Global indices (peroxide value, UV specific absorbance) and direct analytical determinations of

Table 1
Fatty acid composition (% w/w as methyl esters; mean[a] ± SD, n = 3) of crude lipids extracted from sardine fillets soaked in PAW and distilled water. P10-30 are PAW-dipped fillets for 10 to 30 min; C10-30 are fillets soaked in water for 10 to 30 min. RAW are the fresh, unprocessed fillets.

FA[b]	RAW	C10	C20	C30	P10	P20	P30
C10:0	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.03	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
C12:0	0.11 ± 0.01	0.23 ± 0.04	0.32 ± 0.23	0.32 ± 0.11	0.12 ± 0.04	0.15 ± 0.04	0.15 ± 0.04
C13:0	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.02	0.07 ± 0.01
C14:0	7.04 ± 0.08	7.15 ± 0.58	7.46 ± 0.49	7.40 ± 0.25	8.54 ± 0.25	7.41 ± 1.35	7.74 ± 0.18
iso-C15:0	0.01 ± 0.00	0.01 ± 0.00	0.05 ± 0.06	0.02 ± 0.01	0.01 ± 0.00	0.03 ± 0.03	0.03 ± 0.02
anteiso-C15:0	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
C14:1	0.15 ± 0.01	0.13 ± 0.00	0.14 ± 0.00	0.15 ± 0.01	0.14 ± 0.01	0.13 ± 0.04	0.15 ± 0.01
C15:0	0.82 ± 0.01	0.79 ± 0.01	0.81 ± 0.02	0.88 ± 0.06	0.82 ± 0.08	0.81 ± 0.23	0.87 ± 0.04
C15:1 Δ10	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
C16:0	20.58 ± 0.46	20.20 ± 1.31	20.62 ± 0.45	20.60 ± 0.33	20.36 ± 0.66	21.78 ± 0.64	22.05 ± 0.54
iso-C17:0	0.74 ± 0.01	0.73 ± 0.02	0.73 ± 0.03	0.80 ± 0.02	0.68 ± 0.01	0.81 ± 0.15	0.83 ± 0.01
anteiso-C17:0	0.27 ± 0.01	0.26 ± 0.02	0.28 ± 0.01	0.26 ± 0.01	0.23 ± 0.02	0.28 ± 0.01	0.28 ± 0.01
C16:1 Δ9	6.82 ± 0.21	6.70 ± 0.74	7.10 ± 0.71	6.22 ± 0.68	9.40 ± 1.07	6.22 ± 0.98	6.62 ± 1.00
C17:0	0.97 ± 0.03	0.95 ± 0.04	0.95 ± 0.02	1.01 ± 0.05	0.91 ± 0.02	0.99 ± 0.15	1.05 ± 0.08
C17:1 Δ10	0.47 ± 0.01	0.47 ± 0.07	0.40 ± 0.03	0.42 ± 0.04	0.39 ± 0.05	0.40 ± 0.01	0.44 ± 0.01
C18:0	3.94 ± 0.30	3.97 ± 0.23	3.90 ± 0.07	4.00 ± 0.03	3.84 ± 0.16	4.16 ± 0.62	4.37 ± 0.16
C18:1 Δ9	7.36 ± 1.07	6.04 ± 1.67	7.02 ± 0.06	6.33 ± 0.06	4.91 ± 0.71	7.40 ± 1.92	6.14 ± 0.42
C18:1 Δ11	2.30 ± 0.01	2.26 ± 0.18	2.41 ± 0.01	2.31 ± 0.23	2.45 ± 0.13	2.24 ± 0.08	2.39 ± 0.19
C18:2 Δ9,12	1.90 ± 0.06	1.98 ± 0.23	1.77 ± 0.01	1.83 ± 0.01	1.83 ± 0.01	1.77 ± 0.33	1.88 ± 0.08
C18:3 Δ6,9,12	0.18 ± 0.01	0.18 ± 0.03	0.17 ± 0.01	0.16 ± 0.04	0.23 ± 0.02	0.16 ± 0.02	0.17 ± 0.02
C20:0	1.65 ± 0.04	1.57 ± 0.59	1.46 ± 0.08	1.25 ± 0.25	1.68 ± 0.88	1.23 ± 0.04	1.29 ± 0.07
C18:3 Δ9,12,15	1.48 ± 0.11	1.75 ± 0.06	1.64 ± 0.16	1.69 ± 0.01	1.23 ± 0.28	1.57 ± 0.51	1.80 ± 0.16
C20:1 Δ11	0.90 ± 0.06	0.69 ± 0.16	0.95 ± 0.11	0.71 ± 0.10	0.54 ± 0.06	1.03 ± 0.50	0.71 ± 0.14
C21:0	0.05 ± 0.00^{ab}	0.05 ± 0.01^{b}	0.06 ± 0.01^{ab}	0.08 ± 0.01^{a}	0.05 ± 0.01^{b}	0.05 ± 0.01^{ab}	0.05 ± 0.00^{ab}
C20:2 Δ11,14	0.34 ± 0.01^{ab}	0.37 ± 0.01^{ab}	0.35 ± 0.02^{ab}	0.39 ± 0.01^{a}	0.31 ± 0.00^{b}	0.35 ± 0.02^{ab}	0.34 ± 0.01^{ab}
C20:3 Δ8,11,14	0.11 ± 0.01^{ab}	0.11 ± 0.01^{ab}	0.11 ± 0.01^{ab}	0.11 ± 0.01^{ab}	0.14 ± 0.00^{a}	0.10 ± 0.01^{b}	0.10 ± 0.01^{b}
C22:0	0.90 ± 0.03^{ab}	0.91 ± 0.05^{ab}	0.92 ± 0.03^{ab}	1.02 ± 0.03^{a}	0.99 ± 0.06^{ab}	0.84 ± 0.05^{b}	0.95 ± 0.01^{ab}
C20:4 Δ5,8,11,14	0.14 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.12 ± 0.01	0.15 ± 0.04	0.14 ± 0.02
C22:1 Δ13	0.11 ± 0.01	0.08 ± 0.01	0.13 ± 0.01	0.11 ± 0.03	0.08 ± 0.01	0.12 ± 0.05	0.09 ± 0.01
C22:1 Δ11	1.06 ± 0.05	0.98 ± 0.01	0.99 ± 0.08	0.97 ± 0.01	0.86 ± 0.06	0.89 ± 0.09	1.01 ± 0.01
C20:5 Δ5,8,11,14,17	10.34 ± 0.21	10.69 ± 0.66	10.23 ± 0.08	10.04 ± 0.03	11.01 ± 0.20	10.16 ± 0.21	10.06 ± 0.03
C23:0	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
C22:2 Δ13,16	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.01
C24:0	0.60 ± 0.01	0.61 ± 0.05	0.63 ± 0.01	0.65 ± 0.01	0.65 ± 0.01	0.58 ± 0.01	0.61 ± 0.01
C24:1 Δ15	1.47 ± 0.02	1.37 ± 0.05	1.42 ± 0.11	1.53 ± 0.07	1.35 ± 0.08	1.52 ± 0.04	1.42 ± 0.05
C22:5 Δ7,10,13,16,19	1.37 ± 0.09	1.37 ± 0.38	1.54 ± 0.12	1.34 ± 0.17	1.45 ± 0.29	1.20 ± 0.05	1.23 ± 0.06
C22:6 Δ4,7,10,13,16,19	25.64 ± 1.00	27.16 ± 0.81	25.17 ± 1.58	27.10 ± 1.31	24.52 ± 0.33	25.37 ± 0.35	24.94 ± 2.08
SFA	37.87 ± 0.69^{ab}	37.56 ± 0.30^{b}	38.34 ± 1.24^{ab}	38.45 ± 0.26^{ab}	39.03 ± 0.37^{ab}	39.24 ± 0.81^{ab}	40.39 ± 0.43^{a}
MUFA	20.62 ± 1.37	18.71 ± 1.29	20.55 ± 0.48	18.75 ± 1.20	20.13 ± 0.08	19.93 ± 1.46	18.96 ± 1.80
PUFA	41.51 ± 0.69	43.74 ± 1.62	41.12 ± 1.75	42.83 ± 1.46	40.84 ± 0.42	40.84 ± 0.64	40.67 ± 2.24
n-3 PUFA	38.82 ± 0.78	40.97 ± 1.79	38.57 ± 1.77	40.16 ± 1.51	38.20 ± 0.44	38.30 ± 0.32	38.03 ± 2.20

[a] Means (n = 3) followed by a different letter are significantly different at an alpha level of 0.05 according to an HSD test.
[b] Cm:n Δx, m = number of carbon atoms; n, number of double bonds; x, position of double bonds; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; *n*-3 PUFA = Σ(C18:3 Δ9,12,15 + C20:5 Δ5,8,11,14,17 + C22:5 Δ7,10,13,16,19 + C22:6 Δ4,7,10,13,16,19).

both volatile (short-chain alcohols, aldehydes and ketones) and non-volatile (cholesterol oxidation products, COPs) substances were performed to provide an overall assessment of the oxidative state of the sardine lipids.

2. Material and methods

2.1. Sampling and plasma treatments

Fresh sardines were caught in the Adriatic Sea and processed (headed, gutted and filleted) by a local trader (Ecopesce S.r.l., Cesenatico, FC, Italy). PAW was prepared by exposing 500 ml of sterile distilled water (SDW) for one minute to a pulsed corona discharge driven by a high voltage generator (AlmaPulse, AlmaPlasma s.r.l., Bologna, Italy) with a peak voltage of 18 kV and a pulse repetition frequency of 5 kHz (Laurita et al., 2021). Plasma is generated above the water surface in the air gap (5 mm) between the tip of the stainless-steel working electrode and the liquid surface. The reaction chamber was not hermetically sealed, so that the plasma was generated in the ambient air in a static state. PAW parameters (pH, electrical conductivity, hydrogen peroxide, nitrites, ozone) were measured according to methods described in Laurita et al. (2021) to verify compliance with the specifications given by the plasma source developers. Different dipping times in freshly prepared PAW (10, 20 and 30 min) were tested for the degree of oxidation of fish lipids, while the solid to liquid ratio was kept constant (1:3 w/v). The water used to prepare PAW also served as a control dipping medium. The treatments (three replicates each) were carried out in a benchtop oscillating stirrer set at 90 rpm. As a further control, analyses of the lipids extracted from the raw material (fresh sardine fillets) were carried out.

2.2. Total lipid extraction

Total lipids were extracted from the PAW-treated samples and controls using the "Bligh and Dyer" extraction procedure (Zhao et al., 2021). Briefly, each sample (30 ± 2 g) was homogenised with 25 ml chloroform and 50 ml methanol in an Ultra-Turrax® T10 disperser (IKA-Werke GmbH & Co. KG, Staufen, Germany). The slurry was filtered through a glass Gooch funnel and the cake was homogenised again with 25 ml chloroform. Both filtrates were collected in a separating funnel and finally a biphasic system was generated by adding 30 ml aqueous potassium chloride 0.88 % w/v. The lower phase, consisting of chloroform with small amounts of water and methanol, was collected and the solvent was removed at 40 °C in a BÜCHI Rotavapor® Model R-124 (BÜCHI Labortechnik AG, Flawil, Switzerland). The lipid extracts were stored at −20 °C until they were analysed.

Table 2
MS spectral data and chromatographic behaviour (relative retention time (RRT) to 5α-cholestane on a DB5-type capillary column) used for peak identification in the COPs-containing fraction of sardine lipids.

Peak #	RRT	Pure analytical standard	$[M]^+$ [a]	Major significant ions[a] (base peak in bold)	Name (IUPAC)	Synonym	Abbreviation
1	1.18		456 (4)	441 $[M-15]^+$ (2); 366 $[M-90]^+$ (7); 351 $[M-90-15]^+$ (4); 327 $[M-Aring]^+$ (7); 255 $[M-90-SC]^+$ (15); 129 $[Aring]^+$ (58); 111 $[SC]^+$ (70)	Cholesta-5,22-dien-3β-ol	22-Dehydroxycholesterol	
2	1.19	Y	n.d.	**456 $[M-90]^+$ (100)**; 441 $[M-90-15]^+$ (4); 129 (23)	Cholest-5-ene-3β,7α-diol	7α-Hydroxycholesterol	7α-OH
3	1.20		456 (9)	441 $[M-15]^+$ (2); 366 $[M-90]^+$ (13); 351 $[M-90-15]^+$ (16); 327 $[M-Aring]^+$ (14); 255 $[M-90-SC]^+$ (20); 129 $[Aring]^+$ (75); 111 $[SC]^+$ (80)	Cholesta-5,24-dien-3-ol	Desmosterol	
4	1.27	Y	458 (20)	443 $[M-15]^+$ (7); 368 $[M-90]^+$ (45); 353 $[M-90-15]^+$ (25); 329 $[M-Aring]^+$ (60); 255 $[M-90-SC]^+$ (12); 129 **$[Aring]^+$ (100)**	Cholest-5-en-3β-ol	Cholesterol	
5	1.27		546 (2)	531 $[M-15]^+$ (5); 456 $[M-90]^+$ (37); 441 $[M-90-15]^+$ (30); 403 $[M-15-15-SC]^+$ (64); 194 (37)	Cholest-4-ene-3β,6β-diol	6β-Hydroxycholesterol	6β-OH
6	1.29		456 (35)	441 $[M-15]^+$ (5); 366 $[M-90]^+$ (2)	Cholesta-4,6-dien-3β-ol	4,6-Cholestadienol	
7	1.30	Y	n.d.	456 $[M-90]^+$ (2); 366 $[M-90-90]^+$ (35); 353 (75); 145 (92)		19-Hydroxycholesterol	19-OH (IS)[b]
8	1.34		546 (2)	531 $[M-15]^+$ (2); 456 $[M-90]^+$ (6); 441 $[M-90-15]^+$ (8); 403 $[M-15-15-SC]^+$ (25); 194 (17)	Cholest-4-ene-3β,6α-diol	6α-Hydroxycholesterol	6α-OH
9	1.35	Y	n.d.	**456 $[M-90]^+$ (100)**; 441 $[M-90-15]^+$ (4); 129 (23)	Cholest-5-ene-3β,7β-diol	7β-Hydroxycholesterol	7β-OH
10	1.37		470 (2)	455 $[M-15]^+$ (3); 380 $[M-90]^+$ (8); 341 $[M-Aring]^+$ (11); 129 **$[Aring]^+$ (100)**	Ergosta-5,22-dien-3β-ol	Brassicasterol	
11	1.38		472 (4)	457 $[M-15]^+$ (4); 382 $[M-90]^+$ (12); 367 $[M-90-15]^+$ (9); 343 $[M-Aring]^+$ (22); 129 $[Aring]^+$ (85)	Ergost-5-en-3β-ol	Campesterol	
12	1.39		546 (1)	531 $[M-15]^+$ (1); 456 $[M-90]^+$ (9); 441 $[M-15-90]^+$ (4); 417 (6) $[M-Aring]^+$; 366 (14) $[M-90-90]^+$; 327 $[M-90-Aring]^+$ (8); 147 (48); 129 $[Aring]^+$ (25); **73 (100)**	Cholest-5-ene-3β,4β-diol	4β-Hydroxycholesterol	4β-OH
13	1.41	Y	474 (7)	459 $[M-15]^+$ (3); 445 $[M-29]^+$ (4); 384 $[M-90]^+$ (19)	5β,6β-Epoxycholestan-3β-ol	β-Epoxycholesterol	5,6β-epoxy
14	1.43	Y	474 (3)	459 $[M-15]^+$ (2); 445 $[M-29]^+$ (4); 384 $[M-90]^+$ (9)	5α,6α-Epoxycholestan-3β-ol	α-Epoxycholesterol	5,6α-epoxy
15	1.45		n.d.	456 $[M-90]^+$ (55); 441 $[M-15-90]^+$ (18); 366 $[M-90-90]^+$ (5); 194 (35); 179 (56); 147 (71); **73 (100)**	Cholest-6-ene-3,5-diol	5-Hydroxycholesterol	5-OH
16	1.46		546 (1)	531 $[M-15]^+$ (1); 456 $[M-90]^+$ (8); 441 $[M-15-90]^+$ (4); 417 (6) $[M-Aring]^+$; 366 (19) $[M-90-90]^+$; 327 $[M-90-Aring]^+$ (12); 147 (50); 129 $[Aring]^+$ (27); **73 (100)**	Cholest-5-ene-3β,4α-diol	4α-Hydroxycholesterol	4α-OH
17	1.53	Y	n.d.	546 $[M-90]^+$ (5); 531 $[M-90-15]^+$ (5); 517 $[M-90-29]^+$ (4); 456 $[M-90-90]^+$ (25); 441 $[M-90-90-15]^+$ (9); 403 $[M-90-15-15-SC]^+$ (43); 321 (25); 129 $[Aring]^+$ (40); **73 (100)**	Cholestane-3β,5α,6β-triol	Cholestanetriol	3,5,6-triol
18	1.56		n.d.	546 $[M-90]^+$ (4); 531 $[M-90-15]^+$ (2); 456 $[M-90-90]^+$ (47); 441 $[M-90-90-15]^+$ (8); 403 $[M-90-15-15-SC]^+$ (31); **73 (100)**	Cholestane-3β,5,6-triol	Cholestanetriol	triol
19	1.67	Y	472 (42)	457 $[M-15]^+$ (5); 382 $[M-90]^+$ (18); 367 $[M-90-15]^+$ (52); 129 $[Aring]^+$ (95); **73 (100)**	Cholest-5-en-3β-ol-7-one	7-Ketocholesterol	7-keto

[a] Relative abundance in parenthesis.
[b] Internal standard.
n.d., not detectable; Aring, A ring fragment characteristic of Δ^5 sterols (*m/z* 129); SC, side chain fragment (C_8H_{17}).

2.3. Determination of lipid oxidation indexes

The total amount of primary lipid oxidation products (hydroperoxides) was measured by iodometric titration (Zhao et al., 2021). The peroxide value (PV) was expressed as meq active oxygen/kg lipid extract. Conjugated polyenoic structures in fish lipids were quantified by measuring the absorbance of oil solutions in *iso*-octane (2,2,4-trimethylpentane) for spectroscopy (Supelco, Bellefonte, PA, USA) against the solvent at 232, 270 and 315 nm, which correspond to the maximum characteristic absorbance of conjugated diene, triene and tetraene systems, respectively. The concentration of the fish oil solutions was chosen so that the absorbance values were in the range of 0.2–0.8 AU (Absorbance Units). The results were expressed as specific absorbance K_λ, defined as the absorbance of a 1 % w/v sample solution measured in a 1 cm cuvette at the specific wavelength, according to the formula:

$$K_\lambda = \frac{AU_\lambda}{C \times s}$$

where AU_λ was the absorbance reading at the wavelength λ, C was the concentration (g/100 ml) of the oil solution, and s was the length (cm) of the optical path.

2.4. Analysis of total fatty acids

The fatty acid (FA) composition of the crude lipid extracts was determined by gas chromatography (GC) using a Trace 1300 instrument (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a TG-Polar capillary column 60 m × 0.25 mm i.d., 0.20 μm film thickness (Thermo Fisher Scientific, Waltham, MA, USA) and a flame ionisation detector, in the operating conditions described in Haddad, Mozzon, Strabbioli and Frega (2010). A standard mixture of 37 fatty acid methyl esters (FAMEs) provided by Supelco, (Bellefonte, PA, USA) was used for

Table 3
Content of cholesterol and COPs (mean[a] ± SD, n = 3) of crude lipids extracted from sardine fillets soaked in PAW and distilled water. P10-30 are PAW-dipped fillets for 10 to 30 min; C10-30 are water-dipped fillets for 10 to 30 min. RAW are the fresh, untreated fillets.

Analyte[b]	RAW	C10	C20	C30	P10	P20	P30
Cholesterol (mg/100 g fat)	695.6 ± 10.3	693.6 ± 44.7	625.0 ± 114.8	671.7 ± 58.0	679.3 ± 26.1	627.2 ± 71.4	709.0 ± 45.6
Cholesterol (mg/100 g FM)	29.9 ± 0.4	29.9 ± 1.9	26.9 ± 5.0	28.9 ± 2.5	29.3 ± 1.2	27.0 ± 3.1	30.5 ± 2.0
7α-OH (μg/g fat)	17.4 ± 2.6	25.9 ± 10.5	30.0 ± 4.8	26.8 ± 12.5	19.4 ± 0.2	21.9 ± 2.4	17.1 ± 1.8
6β-OH (μg/g fat)	5.2 ± 1.6	7.4 ± 1.0	7.8 ± 0.0	8.0 ± 3.7	2.9 ± 1.8	5.7 ± 3.4	6.6 ± 3.3
6α-OH (μg/g fat)	2.8 ± 0.1	3.2 ± 0.5	3.7 ± 0.5	3.3 ± 0.7	3.3 ± 0.3	3.7 ± 0.5	2.3 ± 0.1
7β-OH (μg/g fat)	35.0 ± 3.2	37.7 ± 14.5	43.9 ± 7.0	45.1 ± 13.5	57.9 ± 19.0	51.8 ± 9.2	41.4 ± 9.1
4β-OH (μg/g fat)	1.3 ± 0.1	1.4 ± 0.5	2.1 ± 0.7	1.3 ± 0.1	2.7 ± 2.4	2.3 ± 0.8	2.0 ± 0.2
5,6β-epoxy (μg/g fat)	9.3 ± 1.8	15.3 ± 2.2	20.9 ± 6.1	13.5 ± 5.4	19.9 ± 3.6	21.0 ± 0.4	19.4 ± 1.5
5,6α-epoxy (μg/g fat)	4.5 ± 1.0	7.4 ± 4.4	12.2 ± 5.5	5.4 ± 1.1	11.3 ± 5.4	13.0 ± 1.3	10.1 ± 0.4
5-OH (μg/g fat)	0.8 ± 0.1	1.6 ± 0.2	1.9 ± 0.1	1.4 ± 0.8	2.7 ± 0.1	1.9 ± 1.1	1.4 ± 0.5
4α-OH (μg/g fat)	1.1 ± 0.0	1.8 ± 0.1	1.6 ± 0.4	1.6 ± 0.6	1.1 ± 0.3	1.2 ± 0.0	1.8 ± 0.3
3,5,6-triol (μg/g fat)	2.2 ± 1.2	3.2 ± 1.3	4.6 ± 1.5	2.7 ± 0.5	3.9 ± 1.3	4.6 ± 0.7	3.4 ± 2.7
Triol (μg/g fat)	1.0 ± 0.2	1.0 ± 1.0	1.0 ± 0.0	1.3 ± 0.5	0.5 ± 0.2	1.1 ± 0.8	2.2 ± 0.8
7-keto (μg/g fat)	34.8 ± 18.2	32.7 ± 5.9	41.5 ± 8.2	37.4 ± 4.2	43.0 ± 15.9	38.3 ± 8.3	47.9 ± 1.5
Total COPs (μg/g fat)	115.4 ± 13.5	138.5 ± 12.8	171.1 ± 34.0	147.9 ± 43.4	168.7 ± 7.7	166.4 ± 10.0	155.8 ± 18.7
Total COPs (μg/g FM)	4.96 ± 0.58	5.96 ± 0.55	7.36 ± 1.46	6.36 ± 1.87	7.25 ± 0.33	7.16 ± 0.43	6.70 ± 0.80

[a] No significant differences were found between the treatments at an alpha level of 0.05 according to an HSD test.
[b] COPs abbreviations as in Table S2. FM, fresh matter.

peak identification. The total FA compositions (weight percent of total FA) were calculated by the peak area normalisation method.

The FAMEs for GC analysis were previously prepared by acid-catalysed transesterification of crude fats. Approximately 200 mg of the lipid extract was weighed into a screw-capped test tube (5 ml volume) and 2 ml of methanolic HCl 3 M (Supelco, Bellefonte, PA, USA) was added. The test tube was tightly closed with a cap with PTFE seal and heated in an oven at 70 °C for two hours. After cooling, 1 ml of water was added and two extractions were performed with 1 ml of *n*-hexane each; the layers were gently shaken and then allowed to settle. The hexane layers were collected in a clean tube and dried by adding the appropriate amount of anhydrous sodium sulphate.

2.5. Analysis of total unsaponifiable and COPs

5α-Cholestane (1 ml of a solution of 1000 mg/l in hexane) and 19-hydroxycholesterol (25 μl of a solution of 500 mg/l in hexane/isopropanol 3:2 v/v) were added to a screw-capped Erlenmeyer flask as internal standards for the quantification of total cholesterol and COPs, respectively. After evaporation of the solvent under a stream of nitrogen, about 250 mg of the crude lipids were accurately weighed into the flask and allowed to react overnight with 10 ml of methanolic potassium hydroxide 1 mol/l at room temperature and protected from light. The saponified mixture was transferred to a separating funnel with the aid of 10 ml water. Three extractions were carried out with 10 ml diethyl ether each and the organic layers were collected in a clean separating funnel. The combined extracts were washed three times with 10 ml water each, filtered over anhydrous sodium sulphate and evaporated to dryness in a rotary evaporator at 35 °C (BÜCHI Labortechnik AG, Flawil, Switzerland). The total unsaponifiable matter was finally dissolved in 1 ml *n*-hexane and divided into a 100 μl fraction, for direct cholesterol determination, and a 900 μl fraction, for COPs recovery, according to the procedure described in Foligni et al. (2022). All solvents, reagents and pure analytical standards were provided by Merck Life Science S.r.l. (Milan, Italy), except for 19-hydroxycholesterol, which was supplied by Vinci-Biochem S.r.l. (Florence, Italy). Total unsaponifiables and COPs were analysed using a Trace 1300 gas chromatograph equipped with a flame ionisation detector (FID) and coupled to an ISQ 7000 single quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). A Dual Detector Microfluidics kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to split the injected sample 1:1 between the mass spectrometer (qualitative analysis) and the FID (quantitative analysis). Details on the conditions of use were described in Foligni et al. (2022).

2.6. Analysis of volatile components

Volatile components were collected from the static headspace of crude lipids by solid phase microextraction (SPME), using a Supelco (Bellefonte, PA, USA) "Grey fibre" (divinylbenzene /carboxen/ polydimethylsiloxane), and analysed by GC–MS on a Varian 3900 gas chromatograph coupled to a Saturn 2100 T ion trap mass detector (Varian Analytical Instruments, Walnut Creek, CA) and equipped with a DB-5 type capillary column. Peak identification was performed by: (i) comparison of electronic impact fragmentation patterns (EI; 70 eV) and linear retention indices (RIs) with data published in the NIST 2020 Mass Spectral Library (National Institute of Standards and Technology); (ii) analysis and interpretation of chemical ionisation (CI; reagent gas: methanol) mass fragmentation patterns; (iii) comparison with MS data and RIs from analytical standards available in our laboratories (Mozzon, Foligni, & Mannozzi, 2020).

2.7. Data analysis

Analytical data were analysed using JMP® Version 10 (SAS Institute Inc., Cary, NC, USA). The one-way ANOVA and the Tukey-Kramer test for honest significant differences (HSD) were used to compare the experimental variables between the samples treated with PAW and the control samples. The significance level was set at $p < 0.05$.

3. Results and discussion

Although a variety of plasma generation devices and reaction chambers have been designed and developed for the production of PAW, most food-related applications are batch systems in which a fixed volume of water is subjected to plasma discharge. In our experiments, we used pulsed corona discharges generated in the air above the water surface. Atmospheric air was used extensively as a working gas to make the scale-up of the process feasible and sustainable. Perinban et al. (2019) pointed out that corona discharges are one of the most studied plasma sources for food applications despite their unstable discharges and electrode corrosion. The reactive species were transferred to the plasma-liquid interface and the bulk liquid region by a diffusion process enhanced by forced convection through intense liquid mixing (a magnetic stirrer was set at 500 rpm). The physicochemical properties of the water changed significantly during the plasma treatment: the pH dropped from 6.12 ± 0.3 to 3.64 ± 0.8, while the enrichment of RONS was accompanied by an increase in conductivity from 8.87 ± 0.59 to 76.1 ± 0.7 μS/cm, according to the PAW parameters provided by the developers

Table 4
Volatile compounds detected in the headspace of sardine lipids using SPME-GC/MS.

Peak #	RI[a]	CI[b] parent and base ions	Compound name	Identification method[c]
1	704	97 $[M + 1]^+$	furan, 2-ethyl-	Tentative
2	747	85 $[M + 1]^+$	3-penten-2-one, (E)-	Tentative
3	763	85 $[M + 1]^+$	2-pentenal, (E)-	Confirmed
4	780	69 $[M + 1\text{-}H_2O]^+$	2-penten-1-ol, (Z)-	Tentative
5	799	111 $[M + 1]^+$, 69	1,4-octadiene	Partial
6	807	101 $[M + 1]^+$, 83 $[M + 1\text{-}H_2O]^+$	hexanal	Confirmed
7	822	111 $[M + 1]^+$, 69	2,4-octadiene	Partial
8	829	111 $[M + 1]^+$, 69	3,5-octadiene	Partial
9	861	99 $[M + 1]^+$, 81 $[M + 1\text{-}H_2O]^+$	2-hexenal, (E)-	Confirmed
10	870	107 $[M + 1]^+$	ethylbenzene	Confirmed
11	878	107 $[M + 1]^+$	*m/p*-xylene	Confirmed
12	899	107 $[M + 1]^+$	*o*-xylene	Confirmed
13	902	95 $[M + 1\text{-}H_2O]$	4-heptenal, (Z)-	Tentative
14	994	57	1-decene	Tentative
15	999	111 $[M + 1]^+$, 93 $[M + 1\text{-}H_2O]^+$	2,4-heptadienal, (E,Z)-	Tentative
16	1002	57	*n*-decane	Confirmed
17	1004	129 $[M + 1]^+$, 111 $[M + 1\text{-}H_2O]^+$	*n*-octanal	Confirmed
18	1016	111 $[M + 1]^+$, 93 $[M + 1\text{-}H_2O]^+$	2,4-heptadienal, (E,E)-	Tentative
19	1063	57	decane, 5-methyl-	Tentative
20	1065	57	decane, 4-methyl-	Tentative
21	1069	57	decane, 2-methyl-	Tentative
22	1075	125 $[M + 1]^+$	3,5-octadien-2-one, (E,E)-	Tentative
23	1101	57	*n*-undecane	Confirmed
24	1106	143 $[M + 1]^+$, 125 $[M + 1\text{-}H_2O]^+$	*n*-nonanal	Confirmed
25	1188	57, 71	x-dodecene	Partial
26	1195	57	1-dodecene	Tentative
27	1201	57	*n*-dodecane	Confirmed
28	1207	57, 71	2-dodecene, (E)-	Tentative
29	1217	57, 71	2-dodecene, (Z)-	Tentative
30	1283	57	dodecane, 3-methyl-	Tentative
31	1293	57	1-tridecene	Tentative
32	1301	57	*n*-tridecane	Confirmed
33	1393	71	1-tetradecene	Tentative
34	1400	57	*n*-tetradecane	Confirmed
35	1501	57	*n*-pentadecane	Confirmed
36	1594	71	1-hexadecene	Tentative
37	1600	57	*n*-hexadecane	Confirmed
38	1681	71	x-heptadecene	Partial
39	1685	71	x-heptadecene	Partial
40	1695	57	1-heptadecene	Tentative
41	1702	57	*n*-heptadecane	Confirmed

[a] Kovats Retention Index experimentally determined on a DB-5 type column.
[b] CI = chemical ionization experiment (methanol was the reagent gas).
[c] Confirmed = identification supported by co-injection of pure analytical standards; Tentative = identification based on the MS and RI matching (NIST 2020 MS Library, Rev. A) and consistent with CI fragmentation pattern; Partial = MS and RI matching were not enough to completely define the chemical structure.

of the device (Supplementary Materials, Table S1) (Laurita et al., 2021).

The type of water is a critical parameter: sterile distilled, deionised, osmotised and tap water have been used to prepare PAW, but the biocidal efficacy of PAW seems to be strongly dependent on low pH values, which in turn are associated with low hardness. To avoid the introduction of other variables (physico-chemical parameters of the water), we used SDW, which is free of minerals, organic matter and microorganisms and has shown optimal efficacy in deactivating microorganisms in several studies (Zhao, Patange, Sun, & Tiwari, 2020). Be that as it may, the properties of PAW are influenced by many critical parameters that directly affect the type and concentration of RONS produced: plasma source, energy supply (power, voltage, frequency), feeding gas and flow rate, pressure (atmospheric, vacuum chamber), water volume, plasma-liquid interface contact type (in water, above water, aerosol, bubble), activation time. The application mode of PAW introduces further variables (immersion time, stirring speed, temperature), which makes it extremely difficult to compare the experimental data and clarify the relationships between the reactive species and the chemistry of lipid oxidation.

3.1. Lipid oxidation indices

Peroxide value and UV absorbance were used as measures of the primary lipid oxidation products in the sardine fillets examined (Fig. 1.). PVs were in the range of 4.6–7.9 meq O_2/kg lipids, which were comparable to hydroperoxide levels previously reported in PAW-processed mackerel fillets (Zhao et al., 2021). To quantify lipid hydroperoxides, several authors (Chaijan et al., 2021, 2022; Li et al., 2019; Singh & Benjakul, 2020) used the spectrophotometric method based on the oxidation of ferrous to ferric ions and subsequent complexation of these ions by thiocyanate, which makes comparison with literature data difficult. Our experimental data showed no clear effect of PAW on the induction of fish lipid oxidation: Soaking time seemed to have more influence on PV than on the immersion medium (PAW vs. distilled water), although statistics did not fully support the differences. The agitation during solid/liquid contact could promote the uptake of oxygen from the surrounding atmosphere, thus favouring the oxidation of polyunsaturated fatty acids (EPA, DHA). The comparison with the untreated samples (RAW) supports this hypothesis. No significant changes in PV between controls and treated samples were also reported by Zhao et al. (2021) in mackerel fillets dipped in PAW and in dried Alaska pollock shreds (Choi, Puligundla, & Mok, 2016) and gwamegi (semi-dried Pacific saury) (Puligundla, Choi, & Mok, 2018) directly exposed to a corona discharge plasma jet.

In the early phase of oxidation, the formation of lipid hydroperoxides is accompanied by a shift of the double bonds in methylene-interrupted polyenes: conjugated dienoic, trienoic and tetraenoic systems with characteristic absorption in the UV range of the electromagnetic spectrum are formed. Contact of sardine fillets with PAW resulted in higher specific absorptions at 232, 270 and 315 nm than controls immersed in distilled water, especially at the longer soaking time (30 min), although the level of significance did not fully support the differences. Albertos et al. (2017) also reported an increase in UV absorbance in the conjugated triene region (268 nm) in mackerel fillets exposed to direct contact with plasma generated in the packaging atmosphere by a DBD source. Secondary oxidation products are also capable of significantly absorbing UV photons, in particular α,β-unsaturated carbonyl compounds (enones, enals) at 232 nm, bifunctional oxidation products (ethylenic diketones) and oxo-dienes at 270 nm. The results of the analyses of the volatile components (section 3.4.) showed a contribution of the secondary volatile oxidation products to the specific absorption measured in the crude lipids of PAW-treated sardines, especially in the region of the trienes.

3.2. Fatty acid composition

The total FA composition of tissue lipids of seafood treated with cold plasma has been studied by several authors as an indirect marker for assessing the degree of lipid oxidation. Decreases in MUFA and/or PUFA were observed in slices of Asian sea bass and in pre-packaged Atlantic mackerel fillets exposed to direct plasma treatment by a DBD source (Albertos et al., 2017; Singh & Benjakul, 2020), but no data are currently available on the effects of PAW on the FA composition of seafood lipids.

The FA compositions of the lipids of the controls (untreated) and the PAW-treated sardines are summarised in Table 1. The average lipid content of the samples tested was 4.3 ± 0.8 g/100 g. Docosahexaenoic acid (DHA, C22:6 Δ4,7,10,13,16,19) was the most abundant (24.52–27.10 % of total FAs), followed by palmitic acid (C16:0;

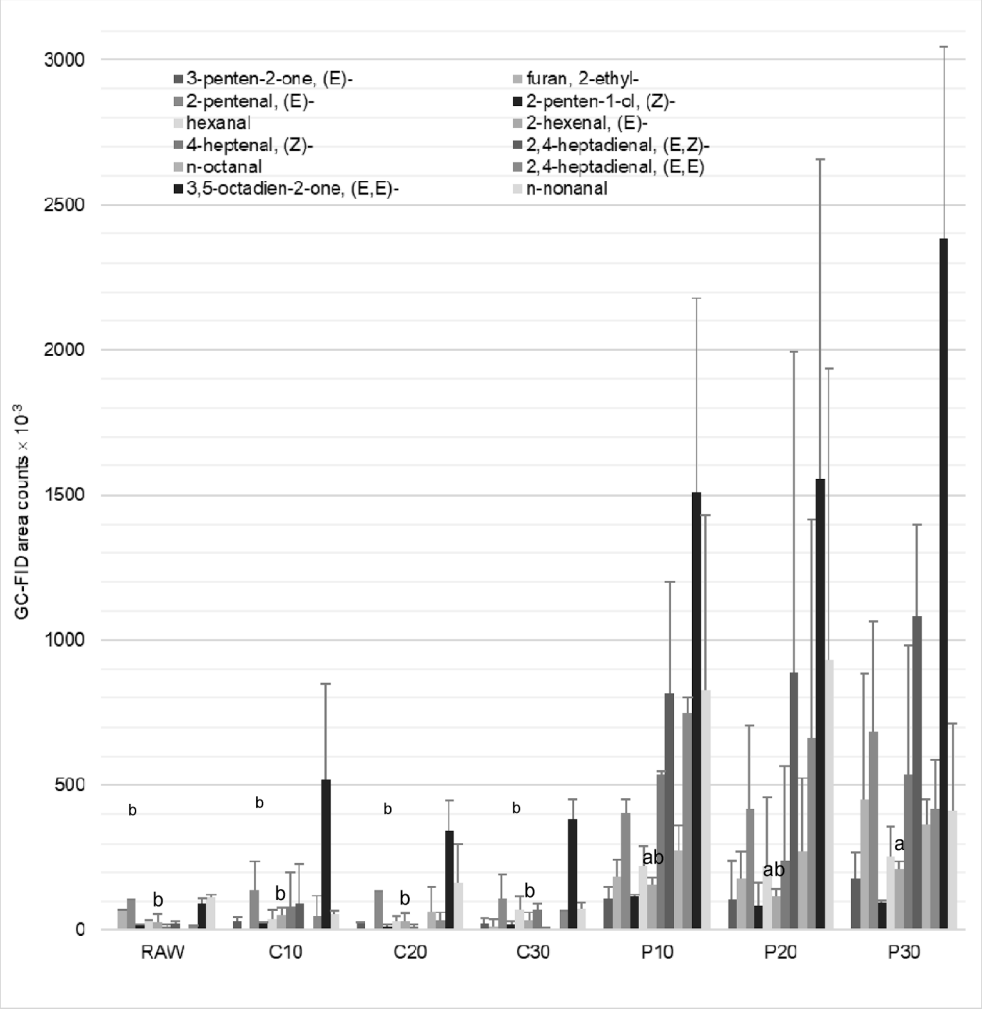

Fig. 2. Levels of volatile oxidation markers of (mean ± SD, n = 3) in sardine lipids extracted from PAW-processed fillets (P samples) and controls immersed in distilled water (C samples). 10–30 are the dipping times (min). Different lower case letters indicate a significant difference at the 5 % level.

20.20–22.05 %) and eicosapentaenoic acid (EPA, C20:5 Δ5,8,11,14,17; 10.04–10.69 %). PUFAs, the most sensitive lipids to oxidative degradation, accounted for a total of 40.67–43.74 % of total FAs. Despite the results summarised above (section 3.1.), no significant differences were observed between the controls and the PAW-soaked fillets. Although there was evidence of PAW-induced FA oxidation (increase in UV absorption and secondary volatile oxidation products), the degree of oxidation was not sufficient to cause a reduction in nutritionally valuable EPA and DHA. Also, in sushi products (nigiri and hosomaki) (Kulawik et al., 2018) and mackerel fillets (Pérez-Andrés et al., 2020a), no significant differences in the content of each FA were found between the treated (DBD plasma source) and the untreated samples.

3.3. Unsaponifiable matter composition

An enlargement of the portion of interest of the GC–MS trace of the polar fraction obtained from the unsaponifiable matter of the sardine lipids can be found in the supplementary materials (Fig. S1).

The availability of pure analytical standards made the identification of the common COPs straightforward: this was true for the peaks at RRT (relative retention time to 5α-cholestane) 1.19, 1.35 (7-OH epimers), 1.41, 1.43 (5,6-epoxy isomers), 1.53 (cholestane triol), and 1.67 (7-keto). Neither the common 25-hydroxycholesterol (pure standard was available) nor other side-chain hydroxy derivatives of cholesterol were detected. The presence of less common COPs was determined by careful interpretation of mass fragmentation patterns and comparison with MS and chromatographic retention data published in the literature (Table 2). The low abundance of these compounds made their identification challenging, as the quality of their mass spectra was sensitive to background noise and chromatographic separation efficiency. The solid phase extraction (SPE) step to enrich the polar fraction of the unsaponifiables also proved crucial. Indeed, significant amounts of cholesterol could be collected in the COPs-containing fraction, making the chromatographic separation of the oxidation derivative at RRT 1.27 critical (Supplementary Materials, Fig. S2). The peaks at RRTs 1.27 and 1.34 were attributed to stereoisomers of cholest-4-ene-3,6-diol. The presence of fragments resulting from the loss of the methyl group and/or the trimethylsilyl hydroxy group (TMSOH) at *m/z* 531 ($[M-15]^+$), 456 ($[M-90]^+$) and 441 ($[M-90-15]^+$) confirmed the molecular ion at *m/z* 546 corresponding to the monohydroxy derivatives of cholesterol. According to literature data (Grandgirard, Martine, Joffre, Juaneda, & Berdeaux, 2004), the ion at *m/z* 403 is considered characteristic of the 4-ene-6-hydroxy structure. The fragmentation pattern involves the loss of two methyl groups and the side chain (C_8H_{17}), confirming the localisation of the two hydroxy groups in the ring system. The RRT of peak #5 agreed with the values reported by Grandgirard et al. (2004) for 6β-hydroxycholesterol; therefore, the peak at RRT 1.34 was tentatively assigned to 6α-hydroxycholesterol. The prominent ion at *m/z* 147 (whose origin is not yet known) has been reported as a marker for cholestene-3,4-diols (Breuer, 1995), while fragments at *m/z* 129 (A-ring

fragment), 417 [M−Aring]$^+$, and 327 [M−90−Aring]$^+$ characterise the A-ring cleavage of TMS derivatives of Δ^5-steroids. Based on these fragmentation patterns and the chromatographic behaviour reported by Grandgirard et al. (2004), the peaks at RRT 1.39 and 1.46 were therefore assigned to 4β- and 4α-hydroxycholesterol, respectively. The mass spectrum of the component at RRT 1.45 was consistent with the monohydroxy derivative of cholesten-3-ol, which was thought to be 6-cholesten-3,5-diol formed, together with 4-cholesten-3,6-diols (peaks at RRT 1.27 and 1.34), by a concerted ene addition of singlet oxygen to the Δ^5 unsaturation of cholesterol. As early as 1988, Bachowski, Thomas and Girotti (1988) observed that 5α-hydroperoxide is the major oxidation product of cholesterol in photooxidised cell membranes. The peak at RRT 1.56 showed a similar fragmentation pattern to pure cholestane-3β,5α,6β-triol and was tentatively attributed to a cholestane-triol stereoisomer.

Some other substances not due to oxidation derivatives of native sterols were detected in the COPs fraction of unsaponifiables. The fragmentation patterns of the peaks at RRT 1.18 and 1.20 were consistent with the isomers of dehydrocholesterol (cholest-5,*x*-dien-3-ol). Fragments due to loss of the methyl group and/or TMSOH at *m/z* 441, 366 and 351 confirmed a MW, as a TMS derivative, of 456 Da, while the intense fragment at *m/z* 129 and the complementary ion at *m/z* 327 [M−129]$^+$ were characteristic of Δ5-sterols. The fragments at *m/z* 111 and 255, which were attributed to the side chain (SC) and the ion [M−90−SC]+, respectively, indicated the presence of another double bond in the SC. According to the literature (Gerst, Ruan, Pang, Wilson, & Schroepfer Jr., 1997), they were tentatively attributed to 22-dehydrocholesterol (cholesta-5,22-dien-3β-ol) and desmosterol (cholesta-5,24-dien-3β-ol), respectively, which had previously been identified in zooplankton of Adriatic Sea (Serrazanetti, Conte, Pagnucco, Bergami, & Milani, 1992). The mass spectrum of the peak at RRT 1.29 showed only one intense fragment ion at *m/z* 456 and much less abundant fragments at *m/z* 441 and 366. The fragmentation pattern and chromatographic behaviour were consistent with 4,6-cholestadienol (Gerst et al., 1997). A base peak at 57 *m/z* together with a series of heavier fragment ions at *m/z* C_nH_{2n+1} and a series of less intense peaks at C_nH_{2n-1}, both decreasing exponentially, characterised the lower mass range (50–150 Da) of the spectrum, indicating co-elution of a long-chain linear alkane. Two Δ^5-phytosterols were also detected in the COPs containing fraction: brassicasterol, with RRT 1.37 and campesterol, with RRT 1.38. As noted by Cardenia, Rodriguez-Estrada, Baldacci, and Lercker (2013), the sterol composition of sardines feeding on both zooplankton and phytoplankton consisted predominantly of C27 (e.g. cholesterol) and C28 (e.g. campesterol, brassicasterol) sterols. The same authors reported the presence of dehydrocholesterol in sardine lipids.

The total cholesterol content of the fish fillets ranged from 543.9 to 741.2 mg/100 fat (corresponding to 23.4–31.9 mg/100 g fresh mass) (Table 3), which was consistent with previously reported data on *S. pilchardus* caught in the Mediterranean Sea (Cardenia et al., 2013). No significant differences in total cholesterol content were observed between controls and treated samples, nor in the levels of other oxidation-sensitive substances identified in the unsaponifiable matter of sardine lipids (Mozzon, Pacetti, Frega, & Lucci, 2015): squalene (17.7–35.6 mg/100 g fat; 0.8–1.5 mg/100 g fresh mass) and α-tocopherol (2.1–5.6 mg/100 g fat; 0.1–0.2 mg/100 g fresh mass). Total COPs ranged from 115.4 to 171.1 μg/g fish fat, corresponding to 4.96–7.36 μg/g fresh mass and 19.5–29.0 μg/g dry mass (average water content of fillets was 74.6 ± 1.1 g/100 g). These values were in agreement with the COPs values reported by Barreira et al. (2023) (39.53 ± 2.14 μg/g dry weight), de Carvalho et al. (2021) (11.5 ± 0.1 μg/g dry weight) and Saldanha, Benassi, and Bragagnolo (2008) (19.4 ± 0.4 μg/g dry matter) in raw sardines, but were lower than the levels observed by Ferreira et al. (2017) (61.2 ± 2.8 μg/g dry matter) and higher than the levels observed by Cardenia et al. (2013) (62–371 μg/100 g fresh matter). The calculated percentage of cholesterol oxidation ranged from 1.66 to 2.74, which was much higher than the values reported by Cardenia et al. (2013) (0.1–0.9 %) for sardine fillets stored at 4 °C for 4 h under light exposure and in the dark. The experimental data showed no significant effect of PAW treatment on cholesterol oxidation. It is noteworthy that storage appears to be a much more critical factor than processing in controlling the formation of COPs. Indeed, a 6-fold increase in total COPs of sardine fillets was observed by Cardenia et al. (2013) after 4 h of exposure to light at 4 °C and by Saldanha et al. (2008) after 120 days of storage at −18 °C. To the authors' knowledge, only Pérez-Andrés et al. (2020b) reported the ability of a cold plasma source (DBD) to degrade pure cholesterol in a model system, but no data on the degradation product were reported. The reaction mechanisms involved in the formation of COPs have been largely elucidated (Iuliano, 2011). Therefore, the qualitative-quantitative profile of COPs could provide useful information on the preferred mechanism of oxidation, thus helping to clarify the relationships between causes (plasma reactive species) and effects (COPs). In particular, 7-hydroxy and 7-keto derivatives are usually expected to be the major COPs. Our data confirmed this general behaviour, thus showing a major contribution of the free radical-mediated oxidation pathway driven by a chain reaction mechanism. The dominance of the β-epimer among the 7-hydroxy compounds has been frequently reported, and it was confirmed by our experimental data (ratio 7β-OH/7α-OH = 1.5–3.0). The very low amount of 4-OH-5-ene isomers confirmed a large asymmetry in the behaviour of the two allylic positions (C4 and C7) with respect to Δ^5-unsaturation. Epoxidation also contributed significantly to cholesterol degradation. Both free radical and non-radical mediated pathways can be involved in the formation of 5,6-epoxides, depending on the lipid species (FA hydroperoxides and peroxyl radicals) and non-lipid species (hydrogen peroxide, peroxynitrite and ozone) that can trigger the oxidative cascade. The 5,6β-epoxy/5,6α-epoxy ratio was in the range of 1.8–2.4, lower than the values reported by Iuliano (2011) (3–11). The structures of 4-en-6-OH and 6-en-5-OH were consistent with photosensitised, non-radical mediated oxidation of cholesterol by singlet oxygen, which can react directly with the Δ^5 double bond through a concerted ene addition involving the shift of the double bond to an allylic position.

3.4. Volatiles composition

As briefly summarised in the introduction, many papers report global indices (TBARs) for quantifying secondary oxidation products. However, we have focused on the secondary volatile lipid oxidation products as they are more relevant markers for understanding the onset and evolution of lipid oxidation and could strongly influence sensory properties at a very early stage of the oxidation process.

Approximately 40 volatiles were identified or tentatively identified in the headspace of sardine oils sampled with a divinylbenzene/carboxen/polydimethylsiloxane SPME fibre. A summary of the mass spectral data and chromatographic retentions used for peak identification is reported in Table 4. A GC–MS chromatogram of the static headspace of sample P30 can be found in the Supplementary Material (Fig. S3). Long-chain alkanes and alkenes dominated the fish lipid headspace, but key markers of lipid peroxidation were also detected, namely saturated (hexanal, n-octanal, n-nonanal) and unsaturated (2-pentenal, 2-hexenal, 4-heptenal, 2,4-heptadienal) aldehydes, ketones (3-penten-2-one, 3,5-octadien-2-one), alcohols (2-penten-1-ol) and cyclic compounds (2-ethylfuran). Conjugated trienes (2,4-heptadienal, octadienone) were the most abundant and were formed from n-3 FAs (which accounted for 40.67–43.74 % of the total FAs, see Section 3.2) by β-cleavage of hydroperoxide isomers. Cyclic (furans) and C5 volatiles (pentenal, pentenol, pentenone) also originated almost exclusively from the terminal end of n-3 PUFA. A mechanism for the formation of 2-alkylfurans from the corresponding (E)-2-alkenals was proposed by Adams et al. (cited by Gómez-Cortés, Sacks, and Brenna, 2015). The origin of the straight-chain alkanals, hexanal, nonanal and octanal, could be traced to the cleavage of 13-OOH of linoleic acid, 10-OOH and 11-OOH of oleic acid, respectively (Xu, Yu, Li, Chen, & Wang, 2017). An average increase of 5-,

7-, and 8-fold in the total amount of secondary volatile oxidation products was observed after soaking sardine fillets in PAW for 10, 20, and 30 min, respectively, compared to the corresponding controls immersed in distilled water (Fig. 2.). When volatiles are used to measure the extent of lipid oxidation of foods, hexanal is often chosen as a typical marker. However, in fish oils, due to their FA composition which is very rich in PUFAs, other products such as (E,E)-2,4-heptadienal and 4-heptenal have been characterised as very strong odorants that contribute to the unpleasant fishy aftertaste and should be considered as more effective markers of lipid oxidation. Although the PAW treatments increased the amount of secondary volatile oxidation products of sardine lipids, the effect of the treatment could not be fully demonstrated statistically due to the very large variance between replicates. In addition, no fishy odour was detected in the fillets by the authors immediately after treatment.

4. Conclusions

The oxidative state of saponifiable (FAs) and unsaponifiable (cholesterol) lipids was assessed in sardine fillets soaked in PAW and compared with controls soaked in demineralised water. Significant undesirable changes occurred in the lipid fraction of the sardine fillets under the conditions we used to produce PAW. Volatiles derived from the terminal end of n-3 PUFA (2,4-heptadienal isomers, 3,5-octadien-2-one, 2-pentenal, 2-penten-1-ol, 3-penten-2-one, 2-ethylfuran) increased in the PAW processed samples, as did the non-volatile conjugated polyenoic structures. However, due to the high variance of the replicates, the statistics could not fully confirm the effect of the PAW treatment. The degree of oxidation of the PUFAs was not sufficient to cause a significant reduction in the nutritionally valuable EPA and DHA.

Oxidative degradation of tissue cholesterol yielded twelve polar derivatives (seven monohydroxy, two dihydroxy, two epoxy, one keto). Although oxidative pressure exerted by PAW did not increase COPs in a statistically robust manner, the levels detected in sardine fillets (115.4–171.1 μg/g fish fat, corresponding to 4.96–7.36 μg/g fresh weight) could pose a risk to human health. As reported by Cardenia et al. (2013), despite the documented wide range of adverse biological effects, a toxicity threshold for COPs has not yet been established and the threshold of toxicological concern (TTC) for unclassified compounds (0.15 μg/person/day) has been proposed as a reference. Therefore, it would be highly desirable that processing and storage do not further increase the COPs content in cholesterol-containing foods.

The large number of variables affecting the qualitative and quantitative composition of the RONS mixture make comparison of the experimental data and clarification of the relationships between the reactive species and the chemistry of lipid oxidation a major challenge. Further studies are needed to clarify the actual mechanisms of generation of reactive species and the mechanisms of their interactions with sensitive food components, to increase the knowledge of critical parameters and safety aspects of the technology and to support the scale-up of PAW applications in industrial food processing.

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CRediT authorship contribution statement

Massimo Mozzon: . **Roberta Foligni:** Conceptualization, Formal analysis, Investigation, Supervision, Visualization, Writing – original draft. **Cinzia Mannozzi:** Investigation, Writing – review & editing. **Filippo Galdenzi:** Investigation, Visualization. **Romolo Laurita:** . **Silvia Tappi:** Investigation, Project administration, Resources, Writing – review & editing. **Marco Dalla Rosa:** Funding acquisition, 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

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113823.

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