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*Effects of novel modified atmosphere packaging on lipid quality and stability of sardine (*Sardina pilchardus*) fillets*

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which has been published in final form at [doi:10.1111/ijfs.14747](https://doi.org/10.1111/ijfs.14747)

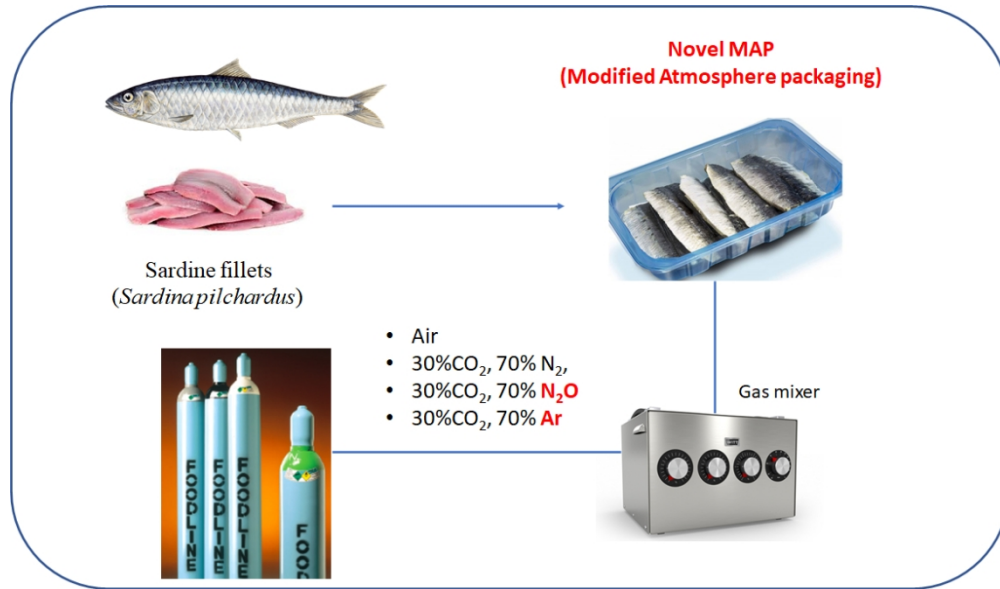
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Effects of novel modified atmosphere packaging on lipid quality and stability of sardine (*Sardina pilchardus*) fillets

Journal:	<i>International Journal of Food Science and Technology</i>
Manuscript ID	IJFST-2020-29507.R2
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	n/a
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Keywords:	Sardine, Modified Atmosphere Packaging (MAP), argon, nitrous oxide, Lipid Oxidation

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Graphical abstract

309x181mm (96 x 96 DPI)

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3 1 **Effects of novel modified atmosphere packaging on lipid quality and stability of**
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6 2 **sardine (*Sardina pilchardus*) fillets**

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8 3 **Running title: Novel modified atmosphere packaging for fish**
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35
36 15 **Abstract**
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38 16 Modified atmosphere packaging (MAP) is an efficient method to increase shelf-life of fishery
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40 17 products by inhibiting bacterial growth and oxidative reactions. Beside the traditional gases used for
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42 18 MAP, novel gases such as argon (Ar) and nitrous oxide (N₂O) were approved for food use in the
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44 19 European Union. The present research investigates the effect of MAP with unconventional gas
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46 20 mixtures, that previously positively affected microbial shelf-life, on colour, lipid oxidation and
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48 21 sensorial characteristics of sardine fillets during storage. Four atmosphere conditions were tested: Air
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50 22 (20.8% O₂/79.2% N₂), N₂ (30% CO₂/70% N₂), N₂O (30% CO₂/70% N₂O) and Ar (30% CO₂/70%
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52 23 Ar). Samples were stored for 12 days at 3 °C. Results showed that the removal of oxygen significantly
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54 24 inhibited the oxidation process; however, most of the investigated parameters related to fat oxidation
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3 25 did not show any improvement, except for a slight decrease in lipid hydrolysis and improvement in
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5 26 sensory properties in the packaging containing Ar.
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12 29 **Keywords:**

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14 30 Sardine; modified atmosphere packaging; argon; nitrous oxide; oxidation
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19 32 **1. Introduction**

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21 33 Seafood products play an important role in a nutritionally balanced diet, however, fresh products are
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23 34 highly perishable due to endogenous enzymes and metabolic activities of microorganisms (Sivertsvik
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25 35 et al. 2002). One of the major deteriorative process that occurs during the storage of fish and which
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27 36 has a major influence on quality is lipid oxidation. Lipid oxidation may affect both odour and flavour
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29 37 of fish and, in severe cases, its nutritional value (Pacetti et al. 2015). Fish and shellfish are excellent
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31 38 sources of polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA, 20:5v3) and
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33 39 docosahexaenoic acid (DHA, 22:6v3). PUFAs are known to be markedly susceptible to peroxidation
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35 40 and to be readily incorporated into the mechanism of lipid peroxidation to yield free radicals and lipid
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37 41 peroxy radicals. Therefore, their protection against oxidation by use of novel packaging technologies
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39 42 as well as storage conditions is essential.
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44 43 Modified atmosphere packaging (MAP) can extend the shelf-life of most fishery products by
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46 44 inhibiting bacterial growth and oxidative reactions. The achievable extension of shelf-life depends on
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48 45 species, fat content, initial microbial population, gas mixture, the ratio of gas volume to product
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50 46 volume, and storage temperature (Sivertsvik et al. 2002). Traditional MAP uses different
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52 47 concentrations of O₂, CO₂ and N₂, generally removing or reducing oxygen and increasing the
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54 48 concentration of CO₂ (Erkan et al. 2006), while N₂ is used as filler gas. In fish products, the aim is to
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56 49 inhibit lipid oxidation and microbial growth.
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3 50 However, since Directive 95/2/CE and further amendments the EU has also approved argon (Ar) and
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5 51 nitrous oxide (N₂O) for food use. They are generally used to replace N₂, an inert gas, in packaging,
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8 52 hence as fillers. However, both Ar and N₂O were observed to increase the sensitivity of different
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10 53 microorganisms to anti-microbial compounds (Qadir and Hashinaga 2001). Moreover, some reports
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12 54 indicate that Ar is able to interfere with receptor sites of enzymatic oxygen (Spencer and Humphreys
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14 55 2003; Rocculi et al. 2005). Choubert et al. (1988) and Randell et al. (1977) found contrasting results
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17 56 regarding the increase of shelf-life due to the use of Ar in rainbow trout and herring fillets packaging.
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19 57 On the other side, positive effects of N₂O have been reported to mainly for fruit packaging, on account
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21 58 of its ability to partially inhibit respiration and senescence (Rocculi et al. 2005).
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23
24 59 Sardine (*Sardina pilchardus*) is an important Mediterranean commercial fish species; its high fat
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26 60 content makes it very susceptible to oxidation. In a previous study of our group, we investigated the
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28 61 effect of Ar and N₂O in MAP of sardine fillets on microbiological quality and freshness indicators
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30 62 during storage. Results indicated that Ar allowed to inhibit bacterial spoilage and freshness decay,
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33 63 increasing the shelf-life of the product (Pinheiro et al. 2019).
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35 64 The present research is focused on the evaluation of the effect of MAP with unconventional gas
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37 65 mixtures (Ar and N₂O) on the colour and different lipid oxidation indicators of sardine fillets during
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40 66 refrigerated storage.
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45 68 **2. Material and methods**

46 69 **2.1 Fish Samples**

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49 70 Sardines (*Sardina pilchardus*), fished in the Adriatic Sea (Cesenatico, Italy), were mechanically
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51 71 filleted (8.5 ± 0.5 cm average length and 10 ± 1 g average weight). A total of 35 kg were packed in
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54 72 different MAP conditions.
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56 73 57 58 74 **2.2 Modified atmosphere packaging (MAP)**

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3 75 250 g of fillets were placed in polypropylene trays and sealed with a PET/PP high barrier film
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5 76 (permeability to O₂ <123.2 cm³/m² day bar and water vapor transmission rate, WVTR < 4.8 g/m² day)
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8 77 with a volume ratio product:gas of 1:1. A gas quaternary mixer mod. KM100-4 (Witt-100
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10 78 Gasetechnik, Witten, Germany) combined to a gas-flushing welding machine mod. Multiple 315
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12 79 (Orved Srl, Venezia, Italy) was used to obtain different combinations of selected gases: Air (20.8%
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14 80 O₂ + 79.2% N₂), N₂ (30% CO₂ + 70% N₂), N₂O (30% CO₂ + 70% N₂O) and Ar (30% CO₂ + 70%
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16
17 81 Ar). For each atmosphere, 24 packages were prepared and stored at 3 ± 1°C for 12 days. For the
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19 82 analytical determinations, 4 trays for each MAP condition were collected after 0, 1, 2, 5, 6, 8, 12 days
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21 83 of storage. Sampling times were chosen to be representative of the shelf life of the fillets according
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24 84 to previous experiments.
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28 86 **2.3 Physico-chemical analyses**

30 87 pH values were assessed in samples homogenized with distilled water (1:2 sample:water ratio) with
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33 88 a pH meter (Crison, Barcellona).
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35 89 Colour parameters lightness (L*), redness (a*) and yellowness (b*) were measured with a
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38 90 spectrophotometer mod. ColorFlex™ (Hunterlab, Reston, Virginia). The tristimulus L*, a*, b*
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40 91 measurement mode (CIE, 1976) was used. The hue angle (H⁰) was calculated as follow:
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$$42 \quad 92 \quad H = \arctan \frac{b^*}{a^*}.$$

45 93 For each sample and storage time, the average of at least 15 measurements was calculated.
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50 95 **2.4 Lipid Oxidation indexes**

52 96 **2.4.1 Total Lipid extraction**

54 97 Lipids were extracted with a method previously described by Bligh and Dyer (1959) according to the
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57 98 modification of Vernocchi et al. (2007).
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3 99 Peroxide value (PV), conjugated diene (CD) and trienes (CT) measurements were used for the
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5 100 determination of primary lipid oxidation. Thiobarbituric acid reactive substances (TBARS) was used
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8 101 to determine the secondary oxidative products and lipid hydrolysis was measured by free fatty acids
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10 102 (FFA) contents. Moreover, fatty acid composition was determined by gas chromatography (GC).

11 12 103 13 14 15 104 **2.4.2 Peroxide value (PV)**

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17 105 The value of PV was determined by the ferrothiocyanate method (Chapman and McKay 1949).
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19 106 Results were expressed as millimoles of O₂ per kg of lipid.

20 21 107 22 23 24 108 **2.4.3 Conjugated diene (CD) and triene (CT) hydroperoxides**

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26 109 CD and CT were measured according to the method of Abdalla and Roozen (1999). Results were
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28 110 expressed in terms of absorbance (232 nm and 268 nm for dienes and trienes respectively) relative to
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30 111 50 mg of fat. The measurement was performed in duplicate for each extract.

31 32 33 112 34 35 113 **2.4.4 Thiobarbituric acid reactive substances (TBARS)**

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37 114 The 2-thiobarbituric acid-reactive substances (TBARS) were measured according to the method
38
39 115 described by Bao and Ertbjerg (2015). Results were expressed as mg of malondialdehyde (MA) per
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41 116 kg of fillet, calculated using a standard curve of 1,1,3,3-tetraethoxypropane.

42 43 44 117 45 46 47 118 **2.4.5 Free fatty acid (FFA)**

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49 119 FFA content was determined using the method of Lowry and Tinsley (1976) modified according to
50
51 120 Bernárdez et al. (2005). The absorbance was read at 710 nm and the amount of FFA determined,
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53 121 using a standard curve prepared from oleic acid. Results were expressed as g oleic acid/100 g lipids.

54 55 56 122 57 58 123 **2.4.6 Fatty acid composition**

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60 124 *Lipid purification and methyl ester synthesis*

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3 125 The lipid fractions were obtained using an aminopropyl bonded sorbent columns (SPE-NH₂)
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5 126 ISOLUTE (Biotage, Milan, Italy). Columns were equilibrated with 9 mL of hexene and loaded with
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8 127 20 mg of total lipid extract. FFAs fractions were recovered using 2% formic ethyl conveyed in diethyl
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10 128 ether. After purification samples were dried at room temperature under nitrogen flux and the methyl
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12 129 ester synthesis was carried out using diazomethane. Fatty acids methyl esters were dried under
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15 130 nitrogen flux, resuspended into hexane and stored at -40°C until the GC-MS analysis.

17 131 *GC–Mass Spectrometry analysis and fatty acid identification*

19 132 GC–MS analysis were carried out on an Agilent 6890 gas chromatograph (Agilent Technologies,
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22 133 Palo Alto, CA) coupled to an Agilent 5970 mass selective detector operating in electron impact mode
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24 134 (ionization voltage, 70 eV). A Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm
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26 135 i.d., 1.2 µm df) was used (Chrompack, Middelburg, The Netherlands). The temperature program was
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28 136 50 °C for 2 min, then programmed at 1 °C min⁻¹ to 65 °C and finally at 5 °C min⁻¹ to 220 °C, which
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31 137 was maintained for 22 min. Injector, interface, and ion source temperatures were 250, 250, and 230
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33 138 °C, respectively. Injections were performed in triplicate with a split ratio of 1:30 and helium (1 mL
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35 139 min⁻¹) as the carrier gas.

37 140 FFAs methyl esters samples were prepared by adding 100 ppm of undecanoic acid methyl ester as
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40 141 internal standard. Injections were performed in triplicate. FFAs were identified using the NIST
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42 142 library. The amount of each FFA (mg/kg) were determined by the comparison of each peak area with
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45 143 the peak area of the internal standard (C11:0). The results are means of three independent
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47 144 experiments.

51 146 **2.5 Sensory evaluation**

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54 147 Sensory evaluation was carried out according to the quality index method (QIM) described in detail
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56 148 by Stamatis and Arkoudelos (2007) for sardine fillets. The attributes examined were: (1) the
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58 149 development of slime on the surface of the fillet; (2) muscle incision and firmness; (3) odour; and (4)
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60 150 overall appearance of the fish fillet. Each assessment was carried out by a minimum of six trained

panellists with a long-term training and experience in fish evaluation. Four categories were ranked: highest quality or **excellent** (E), good quality (A), fair quality (B), and unacceptable quality (C).

2.6 Statistical analysis

Significant differences (p-level <0.05) between means at different storage times were explored by the analysis of variance (ANOVA with post-hoc Tukey HSD); Kruskal-Wallis test was used if significant differences emerged by the Levene's test.

3. Results and Discussion

3.1 Physico-chemical parameters

The initial pH values of sardine samples was 6.24 and, after 12 days of storage, a slight increase up to 6.52 was observed for all samples. Similar results were previously reported for sardine fillets (Erkan et al. 2006). However, the observed changes were not statistically significant (data not reported).

Changes in the L*, a* and H° colour parameters of sardine fillets packed in air and MAP during storage are shown in **Table 1**. During storage, L* (lightness) showed a slight decrease in all samples, but at the end, sardine fillets packaged in N₂O showed a significantly lower L* value compared to the N₂ and air samples (p < 0.05). On the contrary, a*(redness) values increased in all samples but after the fifth day until the end of storage, in fillets packed in N₂ and Ar, it was significantly higher compared to samples in air.

The hue angle (H°) decreased during storage showing differences among the tested atmospheres. From day 5 until day 8, hue values in Air samples were significantly higher than the MAP samples. However, at the end of the storage, no significant differences were observed among the different MAP samples (p > 0.05).

As shown by Thippareddi and Phebus (2002), the concentration of oxygen in the package determines the oxidative state of the myoglobin in fresh meats (**Fig. 1**). From a macroscopic point of view, this

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3 177 phenomenon is evident considering the images of sardine fillets acquired in conditions of
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5 178 standardized illumination reported in **Fig. 2**. After 12 days of storage, while sample packed in air (b)
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8 179 evidenced a yellow/brown colour, the N₂, N₂O and Ar samples (c, d, e) evidenced an evident purple-
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10 180 red colour component, even more evident also respect to the fresh sample (a). The yellow-brown tint
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12 181 and the inhomogeneous distribution of colour in the surface of the sample packed in air is indicating
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15 182 an advanced state of oxidation. On the contrary, **the absence of oxygen or an oxygen concentration**
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17 183 **very close to 0%** promotes deoxymyoglobin (DMb) development, that is probably the reason for the
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19 184 increase of the redness observed in fillets packed in N₂, N₂O and Ar.
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23 24 186 **3.2 Lipid Oxidation indexes**

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26 187 Primary lipid oxidation was evaluated by PV. Evolution in the PV during storage is shown in **Fig.**
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28 188 **3A**. At the beginning, the value was rather low (1.56 meq O₂/kg fat), indicating a good oxidative
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31 189 quality of the raw material. In the sample packed in air, the increase in PV began just on day one,
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33 190 following the typical trend of peroxide formation. At the end of the 12 days, PV was significantly
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35 191 greater (8.34 meq O₂/kg fat) in Air samples compared to the MAP samples, in which PV remained
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38 192 almost constant during storage with values that never exceeded 4 meq O₂/kg fat.

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40 193 The low peroxide values in the MAP samples are probably due to the low concentration of this gas
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42 194 in the headspace of the packages. According to the classification reported by Pinheiro et al. (2019),
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44 195 samples in air could be considered 'spoiled' (PV=8-10) at the end of the storage, while the other
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47 196 samples were still 'good' (PV=2-5).

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49 197 **Fig. 3B** and **3C**, shown the absorbance at 232 nm and 268 nm of the conjugable oxidation products,
50
51 198 dienes and trienes respectively. For all samples, no difference in CD and CT of sardine fillets was
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54 199 observed within the first 8 days of storage ($p > 0.05$). Similarly, Chaijan et al. (2006) reported that in
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56 200 the sardine muscle (*Sardinella gibbosa*), no difference in CD was observed within the first 12 days
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58 201 of iced storage.
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3 202 After day 8, the CD and CT values in the Air samples increased significantly ($p < 0.05$) compared to
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5 203 the MAP samples that instead remained almost constant. At the end of storage, the CD and CT values
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8 204 were highest in the Air samples compared with the MAP samples.

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10 205 CD and CT are oxidation indexes for commonly used vegetable oils and for olive oils EU regulation
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12 206 (EC 796/2002) sets specific threshold values. However, for different oils and fats UV absorbance
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14
15 207 values should be evaluated individually due to their own chemical and physical characteristics. The
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17 208 CD and CT values in all MAP samples remained very low and did not increase with the storage time,
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19 209 indicating a protective effect of MAP on lipid oxidation.

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22 210 According to Pinheiro et al. (2019), fish quality can be considered good until TBARS levels are below
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24 211 5 mg malonaldehyde/kg, while the threshold for consumption is 8 mg malonaldehyde/kg.

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26 212 TBARS values measured during storage of packed sardine fillets are reported in **Table 2**. Fresh
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28 213 samples showed values of about 0.33 mg MA/kg, similarly to values reported by other authors
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31 214 (Méndez et al. 2017), that increased during storage in all samples. However, for MAP samples values
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33 215 were far lower compared to sample packed in Air and were maintained low until the end of the
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35 216 storage. These results are in agreement with previous researches (Caglak et al. 2014; Messina et al.
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38 217 2015). After 12 days, a value of 3.39 mg MA/kg was recorded for the Air sample, while in MAP
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40 218 samples the higher value was 1.09 mg MA/kg. Both values are lower than the reported critical values.
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42 219 Moreover, values are also lower compared to the ones reported by Erkan et al. (2006) for sardine
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44 220 fillets after 5 days of storage in two different MAP conditions. This difference may be explained by
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47 221 the fact that in the mentioned research the atmosphere composition included 5% of O₂ that probably
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49 222 allowed a faster lipid oxidation.

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51 223 While Choubert et al. (2008) observed an improvement using Ar instead of N₂, that was attributed to
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53 224 its higher density that allows it to remove oxygen better than nitrogen, inhibiting the initiation and
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56 225 progression of oxidation, in the present research, although the three different tested MAP (containing
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58 226 N₂, N₂O and Ar) were effective in minimizing TBARS development, no significant difference
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60 227 ($p < 0.05$) was observed among traditional and innovative gases.

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3 228 Hydrolysis of ester bonds in lipids by enzymatic action or heating in the presence of water liberates
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5 229 FFAs (Bernárdez et al. 2005). In general, hydrolysis, induced by lipases and phospholipases, produces
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8 230 FFAs that can undergo further oxidation and production of low-molecular weight compounds
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10 231 responsible for the rancid off-flavour of fish products (Chaijan et al. 2006). Furthermore, FFAs and
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12 232 their oxidation products could impact muscle texture and functionality due to their ability to interact
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15 233 with myofibrillar proteins and to promote protein aggregation (Pacheco-Aguilar et al. 2000).
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17 234 The evolution of lipid hydrolysis is presented in **Fig. 3D**. The initial fresh fillets value (0.09 ± 0.01 g
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19 235 oleic acid/100g lipids) was similar to that obtained by Chaijan et al. (2006) for another sardine specie
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21 236 (*Sardinella gibbosa*). FFAs values for all samples increased slightly with storage time. In Ar samples,
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24 237 the higher value was observed at the day 8 (0.73 ± 0.22 g /100g lipids) followed by a decrease on day
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26 238 12. At the end of the storage period, the samples packed on Ar gas mixture was lower than all samples.
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28 239 Chaijan et al. (2006) reported a high lipid hydrolysis (> 6 g FFA/100g lipid) in iced sardines at the
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31 240 end of the storage (15 days), probably because the **authors** used whole fish in which hydrolytic
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33 241 enzymes can derive also from internal organs. In this study, the highest FFAs value (0.96 ± 0.44 g
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35 242 /100g lipids) was found on N₂ samples on day 12 and was similar to the values reported by Fagan et
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38 243 al. (2004) for mackerel and salmon fillets packed in MAP (60% N₂/40% CO₂) combined with freeze-
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40 244 chilling. However, no significant differences were found among Air, N₂ and N₂O samples throughout
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42 245 the storage period.
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44
45 246 In order to understand the effect of the packaging atmosphere on the qualitative release of FFAs, gas-
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47 247 chromatographic analyses were performed, results are reported in **Table 3**. The data showed that the
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49 248 patterns of fat hydrolysis were different according to the atmosphere considered. The raw material
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51 249 was characterized by the release of mainly saturated FFAs such as C16:0, C18:0 and, **to a minor**
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54 250 **extent**, C14:1, respectively 92 and 8% of the total. After 8 days of storage, all samples were
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56 251 characterized by an increase of the lipolysis although slightly higher in the Ar samples with a major
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58 252 release of saturated and monosaturated fatty acids such including stearic (C18:0) and oleic (C18:1)
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60 253 ones. On the other hand, the sardine fillets packed in Ar, after 8 days of storage, presented high total

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3 254 amount of polyunsaturated FFAs, related in particular to an increase content of 22:6 (n-3). However,
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5 255 this sample was characterized by the absence of C18:2n6c and C20:4n6, present both in the Air and
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8 256 N₂ samples. The highest amount of PUFA, after 8 days, was detected in Air samples, characterized
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10 257 also by the highest amount of TBARS (Table 2). The Air sample was characterized by the presence
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12 258 of C18:2n6c, C20:4n6, C20:5n3, 22:6 (n-3). Similar data were found by Chaijan et al. (2006) who
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15 259 found that the FFAs released in sardines were prone to oxidation as shown also by the marked increase
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17 260 in TBARS.

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19 261 After 12 days of storage at 3 °C, the release of saturated and polyunsaturated fatty acids increased in
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21
22 262 all samples with the exception of the Ar sample that showed a slight decrease.

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24 263 Regarding the monounsaturated fatty acids, for each considered sample, the total amounts found after
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26 264 12 days was lower with respect the samples analysed at 8 d of storage.

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28 265 Considering the relative quantities (%) of the three fractions on the total amount of FFA for each
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31 266 sample, it is possible to notice how in the Air sample, the release of PUFA was higher compared to
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33 267 the other samples at both considered sampling times, probably causing the higher oxidation level as
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35 268 shown by the previously considered indexes.

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37 269 According to Choubert et al. (2008), the positive effect of replacing N₂ with Ar is related to its
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40 270 physical properties, particularly to its higher density, **that allow for better inhibition of lipid oxidation.**
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42 271 However, the authors did not specifically investigate lipid hydrolysis.

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44 272 The accumulation of FFAs in fish muscle is mainly due to the enzymatic activity of lipase and
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47 273 phospholipase found in muscle, in digestive organs when present, but also deriving from
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49 274 microorganisms (Chaijan et al. 2006). **In previous research**, we showed that Ar was able to reduce
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51 275 microbial growth in sardine fillets compared to N₂, however, the effect on enzymatic activity was not
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54 276 investigated. It could be possible that the observed differences in FFAs release are related to an effect
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56 277 **of** enzymatic reactions in the fish tissue, however, specific research should be carried out to clarify
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58 278 this aspect.

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280 3.3 Sensory evaluation

281 **Samples** packed in air reached the unacceptable condition (C) at the 5th day of storage (data reported
in the supplementary material), while for samples packed in MAP the sensorial shelf-life was
significantly improved. N₂ and N₂O samples were considered unacceptable at the 8th day, while
sample packed in Ar maintained the “fair” score (B) until the end of the storage. These results are in
agreement with previous data on microbial spoilage (Pinheiro et al., 2019), confirming that the use
of Ar allowed to increase shelf life of sardine fillets because of improved microbiological status and
sensorial characteristics.

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289 4. Conclusions

290 As extensively known, the use of modified atmosphere in fish packaging with the removal of oxygen
has confirmed to significantly inhibit the oxidation process during refrigerated storage.

291 Although previous studies have shown that Ar allowed to increase microbial shelf-life of sardine
fillets most of the investigated parameters related to fat oxidation did not show any improvement
during storage, except for a slight decrease in lipid hydrolysis in the packaging containing Ar.
However, sensorial analysis showed that Ar sample was considered acceptable until the end of the
storage.

292 Considering that the two investigated novel gases are at present more expensive compared to nitrogen,
before a possible utilization at industrial level, a careful consideration of benefits versus costs should
be carried out. Moreover, further studies are in due course in our lab in order to consider the
reversibility of the visual quality improvement promoted by MAP after packaging opening, and in
simulated preparation and consumption operations.

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303 Conflict of interest

304 The authors declare no conflict of interest.

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3 306 **Data availability statement**
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5 307 Research data are not shared.
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10 309 **Acknowledgments**
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13 310 The authors acknowledge the financial support of the project “PRIZEFISH - Piloting of eco-
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15 311 innovative fishery supply-chains to market added-value Adriatic fish products” funded by the Call
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17 312 2014 - 2020 Interreg V-A Italy - Croatia CBC Programme - Priority Axis: Blue innovation. Subsidy
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19 313 Contract N° 10043583.
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24 315 **Ethical guidelines**
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27 316 Ethics approval was not required for this research
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31 318 **References**
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47 424 This work evaluated different parameters related to fish oxidation in sardine fillets during
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49 425 storage. It was very important to discuss results observed in this research.

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51 426 Choubert, G., Brisbarre, F., Parfouru, D., & Baccaunaud, M. (2008). Argon modified atmosphere
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54 427 packaging for fillets of rainbow trout (*Oncorhynchus mykiss*) fed astaxanthin or canthaxanthin.
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58 429 This work is one of the very few researches that evaluated the sue of argon in th packaging of
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60 430 fish fillets. The comparison with our results was very important

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434 This research is focused on use of MAP for sardine fillets, which is a similar topic of the
435 present study. The development of a sensorial analysis method for sardine fillets was based
436 on this research that was also used for the discussion of results

437 Pinheiro, A. C. D. A. S., Urbinati, E., Tappi, S., Picone, G., Patrignani, F., Lanciotti, R., ... & Rocculi,
438 P. (2019). The impact of gas mixtures of Argon and Nitrous oxide (N₂O) on quality parameters of
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440 268-275.

441 This work was carried out by our own research group and evaluated the use of novel gases for
442 MAP of sardine fillets determining the effect on microbial shelf life. Results were exploited
443 and used for explaining some of the results of the present study.

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3 444 **Figure captions**
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5 445 Fig. 1. Relationship of oxygen concentration on myoglobin chemical state (modified from
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8 446 Thiipareddi and Phebus 2002).

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10 447 Fig. 2. Images of fresh sample (a), and of samples air (b), N₂ (c), N₂O (d) and Ar (e) after 12 days of
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12 448 storage at 3 ± 1°C
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14 449 Fig. 3. Peroxide values (A), Conjugated Dienes (B) and Conjugated Trienes (C) and Free Fatty acids
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17 450 (D) content measured in sardine fillets in MAP during storage at 3 °C. Different letters indicate
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19 451 significant differences among samples at the same storage time (p<0.05).
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For Peer Review

Table 1. Changes in the colour parameters of sardine fillets under different MAP conditions at 3°C.

Samples	Storage time (days)						
	0	1	2	5	6	8	12
L*							
Air	46.56±1.5	45.52±0.5 ^a	43.71±2.8 ^a	43.68±2.6 ^a	45.07±2.7 ^a	43.94±2.6 ^a	45.55±2.4 ^a
N ₂	-	45.47±1.8 ^a	43.98±3.1 ^a	43.15±1.74 ^a	43.43±2.2 ^a	42.03±2.6 ^a	44.33±1.6 ^a
N ₂ O	-	42.85±1.3 ^b	43.63±2.9 ^a	41.91±1.95 ^a	41.25±2.5 ^a	41.95±2.8 ^a	42.66±2.7 ^b
Argon	-	43.78±1.2 ^{ab}	45.30±2.0 ^a	42.61±1.92 ^a	43.50±2.1 ^a	42.66±2.4 ^a	43.70±1.6 ^{ab}
a*							
Air	1.22±0.9	1.96±2.1 ^a	3.07±1.8 ^a	2.35±1.1 ^b	4.41±1.8 ^a	4.36±2.7 ^b	3.89±2.5 ^b
N ₂	-	0.99±1.5 ^a	3.52±1.6 ^a	5.05±2.4 ^a	5.71±1.3 ^a	6.53±2.3 ^a	5.94±2.6 ^a
N ₂ O	-	1.45±1.0 ^a	4.36±2.2 ^a	4.40±2.8 ^a	5.89±1.6 ^a	6.64±1.4 ^a	4.96±2.9 ^{ab}
Argon	-	2.05±1.4 ^a	2.94±2.3 ^a	4.55±2.6 ^a	5.89±1.3 ^a	6.24±1.5 ^a	6.26±2.6 ^a
H⁰							
Air	80.21±11.2	79.02±19.7 ^a	70.29±13.4 ^a	75.68±12.3 ^a	62.91±11.2 ^a	65.88±13.8 ^a	54.45±13.3 ^b
N ₂	-	83.89±11.6 ^a	66.59±13.6 ^a	59.84±11.6 ^b	54.12±5.0 ^b	51.48±9.1 ^b	56.20±11.1 ^a
N ₂ O	-	77.95±8.4 ^a	63.91±10.7 ^a	62.99±14.6 ^b	52.68±6.1 ^b	50.54±5.6 ^b	54.19±14.2 ^a
Argon	-	74.25±10.4 ^a	71.22±14.4 ^a	62.37±13.2 ^b	54.18±5.2 ^b	52.56±6.4 ^b	56.73±10.9 ^a

Different letters in the same column indicate significant differences (P < 0.05).

Table 2. Changes in the TBARS values (mg Malondialdehyde/kg) of sardine fillets under different MAP conditions at 4 °C.

Samples	Storage time (days)						
	0	1	2	5	6	8	12
Air	0.33±0.0	1.34±0.1 ^a	0.58 ±0.2 ^a	1.21 ±0.5 ^a	2.62 ±1.1 ^a	2.00 ±0.7 ^a	3.39 ±0.9 ^a
N ₂	-	0.61±0.4 ^b	0.61 ±0.3 ^a	0.62 ±0.2 ^a	0.55 ±0.1 ^b	1.11 ±0.3 ^a	1.19 ±0.1 ^b
N ₂ O	-	0.59±0.1 ^b	0.58 ±0.3 ^a	0.73 ±0.1 ^a	0.63 ±0.1 ^b	1.61 ±0.7 ^a	1.01 ±0.1 ^b
Argon	-	0.44±0.2 ^b	0.45 ±0.0 ^a	1.08 ±0.3 ^a	0.71 ±0.0 ^b	1.34 ±0.1 ^a	1.09 ±0.4 ^b

Different letters in the same column indicate significant differences ($P < 0.05$).

Table 3. Release of FFAs (mg/kg) in *Sardina pilchardus* fillets during storage at 3°C in relation to the packaging atmosphere.

	Raw material	Air - 8d	Air - 14d	N ₂ - 8 d	N ₂ - 14d	N ₂ O - 8d	N ₂ O - 14d	Ar - 8d	Ar - 14d
C13:0	-*	-	4.28±0.34	-	-	6.52±2.39	-	-	-
C14:0	-	3.24±0.13	3.25±0.48	3.47±0.05	3.53±0.40	3.96±0.52	4.49±0.78	1.56±2.21	4.10±0.40
C16:0	37.25 ±5.92	26.94±2.62	36.23±1.23	36.49±1.90	30.90±2.38	35.58±5.33	49.26±18.76	38.78±5.63	38.16±1.35
C17:0 iso	-	9.99±4.13	-	-	-	-	-	-	-
C18:0	29.81 ± 2.25	8.74±0.18	12.09±1.2	13.88±0.72	9.98±0.88	9.07±1.25	10.79±1.10	16.39±3.25	12.37±1.14
C22:0	-	-	-	-	30.15±2.63	-	-	-	-
Σ saturated	67.06	48.91	55.85	53.84	74.56	55.13	64.54	56.73	54.63
% saturated	91.93	49.70	51.77	52.73	60.84	51.28	53.37	50.39	51.45
C14:1n9	5.89 ±1.67	-	-	-	-	-	-	3.63±0.87	-
C16:1n9	-	5.00±0.26	4.63±0.1	4.97±0.57	5.45±1.30	5.56±0.25	9.33±1.74	4.94±1.00	6.54±0.17
C18:1n9c	-	3.07±0.82	-	3.14±1.00	2.55±0.37	2.55±0.05	-	5.14±0.10	2.80±0.76
C18:1n9t	-	2.02±0.01	-	2.23±0.39	-	2.53±0.37	-	-	2.89±0.42
Σ monounsaturated	5.89	10.09	4.63	10.34	8.00	10.64	9.33	13.71	12.23
% monounsaturated	8.07	10.25	4.29	10.13	6.53	9.90	7.72	12.18	11.52
C18:2n6c	-	1.82±0.16	3.62±1.10	1.34±0.60	2.64±0.22	1.39±0.64	6.55±0.89	-	-
C18:3n3	-	-	2.59±0.2	2.26±0.40	2.12±0.37	-	2.86±0.98	-	-
C20:4n6	-	1.70±0.16	-	1.27±0.13	-	1.39±0.64	-	-	-
C20:5n3	-	14.05±1.63	13.68±2.14	12.53±0.90	13.91±4.33	14.71±0.04	20.50±9.00	13.67±1.40	16.10±1.59
22:6 (n-3)	-	21.85±7.77	27.51±5.61	20.53±1.75	21.43±5.53	24.24±2.84	17.14±2.56	28.47±0.48	23.22±7.90
Σ polyunsaturated		39.42	47.4	37.93	40.1	41.73	47.05	42.14	39.32
% polyunsaturated	0.00	40.05	43.94	37.15	32.72	38.82	38.91	37.43	37.03
Total	72.95	98.42	107.88	102.11	122.56	107.5	120.92	112.58	106.18

* not detected

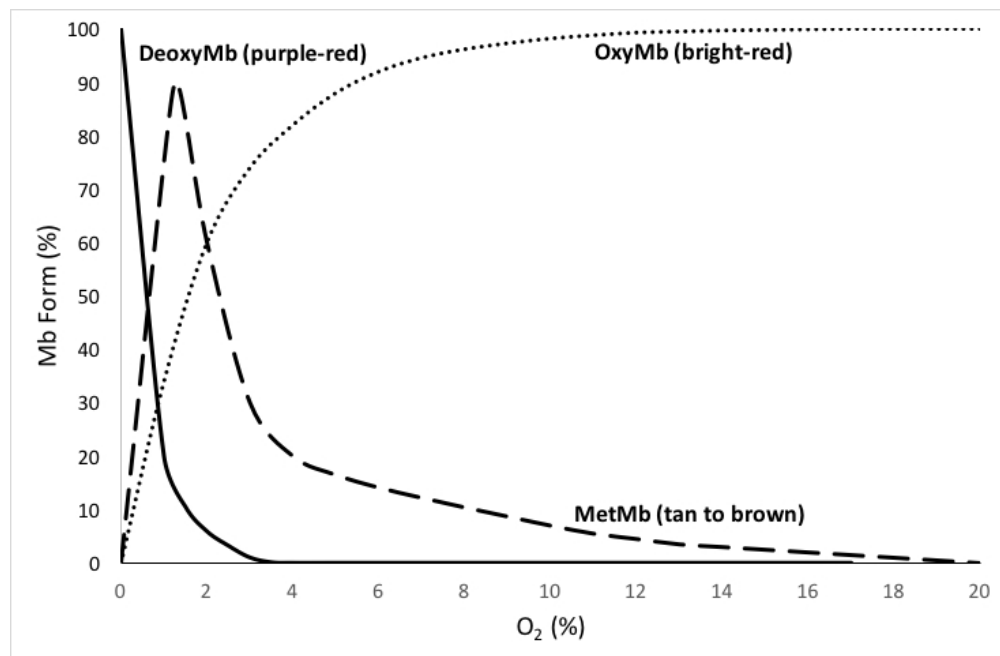


Fig. 1. Relationship of oxygen concentration on myoglobin chemical state (modified by Thippareddi and Phebus 2002).

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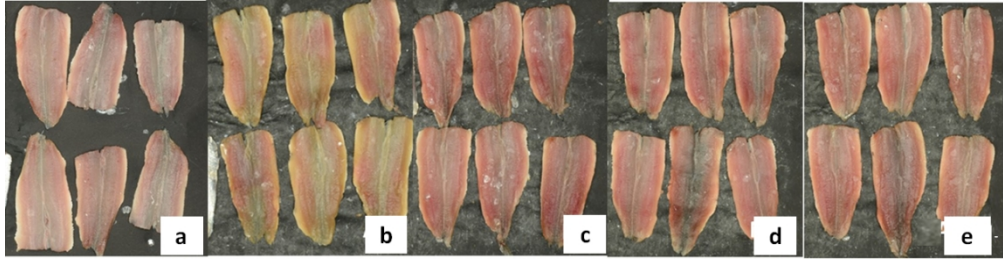


Fig. 2. Images of fresh sample (a), and of samples air (b), N₂ (c), N₂O (d) and Ar (e) after 12 days of storage at 3 ± 1°C

334x87mm (96 x 96 DPI)

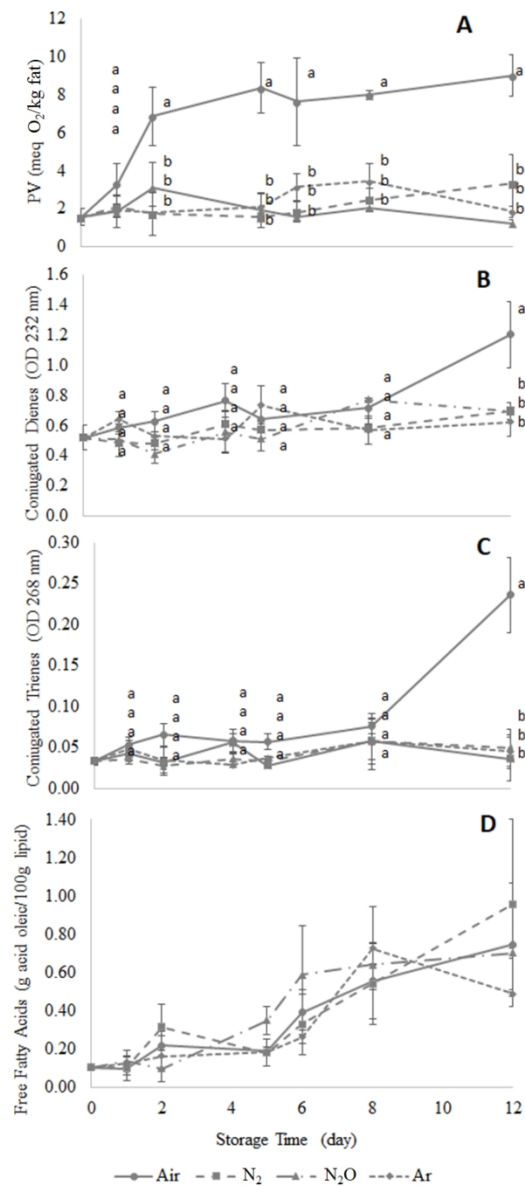


Fig. 3. Peroxide values (A), Conjugated Dienes (B) and Conjugated Trienes (C) and Free Fatty acids (D) content measured in sardine fillets in MAP during storage at 3 °C. Different letters indicate significant differences among samples at the same storage time (p < 0.05).

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Table S1. Sensory acceptance* during sardine storage under different MAP conditions at 3 °C.

Sample	Storage time (days)					
	1	2	5	6	8	12
Air	E	B	C	C	C	C
N2	E	A	A	B	C	C
N2O	E	A	A	B	C	C
Ar	E	A	A	B	B	B

* Freshness categories: E (excellent), A (good), B (fair) and C (unacceptable). Raw fish was category E.

For Peer Review