Anchovy mince (*Engraulis ringens*) enriched with polyphenol-rich grape pomace dietary fibre: *in vitro* polyphenols bioaccessibility, antioxidant and physico-chemical properties

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ABSTRACT

The aim of this study was to evaluate technological and antioxidant properties, including *in vitro* bioaccessibility of polyphenols, conferred on raw anchovy mince by the addition of polyphenol-rich grape pomace dietary fibre at different concentrations. For this purpose, headed and gutted anchovy was heat-flayed, deboned and mixed with 0%, 2%, 3%, 4% grape pomace dietary fibre. A significant increase ($P < 0.05$) in the concentration of polyphenols and associated antioxidant capacity was detected when grape pomace dietary fibre was incorporated in a proportion of at least 2% of the final mixture. *In vitro* digestion showed that the higher the grape pomace dietary fibre content, the higher was the proportion of polyphenols reaching the large intestine. Additionally, it was observed that the ABTS (2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) assay seems to be more suitable for evaluating antioxidant capacity in this kind of samples than FRAP (Ferric Reducing Antioxidant Power) assay. Technological properties such as mechanical and water holding, as well as sensory scores, indicated excellent qualities and acceptability of all samples. Hence, given the good acceptance of these samples, it should be feasible to make fish products based on mince anchovy as a means of increasing dietary intake of polyphenols with antioxidant capacity, especially considering the high concentration of polyphenols bioaccessible in the large intestine.

Keywords: valorization, anchovy mince, grape pomace, polyphenols, antioxidant capacity, bioaccessibility.

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1. Introduction

Anchovy (*Engraulis ringens*) is the most important resource in Peruvian waters and is mainly fished for use as raw material to make fish meal for fish farms or as feed for mammals for meat production. Storage, both fresh and frozen, is commonly difficult due to its high fat and haemoglobin contents, which facilitate oxidative processes (Tokur & Korkmaz, 2007). Human consumption of anchovy is very limited due to the strong taste; however it could be useful as a dietary component thanks to its great nutritive properties. Different synthetic antioxidants have been tried to preserve the anchovy mince from oxidation, but this approach presents several difficulties. First, its use is restricted in many countries and second, there is a tendency to minimize the addition of chemicals due to consumer concerns about safety, and therefore they tend to be substituted by natural substances (Frankel, 1999).

One strategy to improve the nutritional profile of fish products is the addition of dietary fibre, a food component with established health-related properties (ADA, 2008). Dietary fibres commonly used as ingredients in seafood products are usually very soluble and come mainly from algae such as carrageenan (Borderías, Montero, & Mati de Castro, 1996, Gómez-Guillen et al, 1996) and from seeds such as garrofin, guar, inulin and others (Pérez-Mateos, Hurtado, Montero, & Fernandez-Martín, 2001, Cardoso, Méndes, Pedro, & Nunes, 2008). Another kind of dietary fibre that is particularly suitable for addition to seafood products are so called antioxidant dietary fibres, a good example being dietary fibre derived from grape skin and seeds from wine processing (Sanchez-Alonso, Solas, & Borderías, 2007a).

In that connection wine processing yearly generates huge amounts of grape pomace, a material commonly discarded or used for low economic input activities. This by-product contains considerable amounts of dietary fibre and polyphenols, which makes it

64 suitable for a number of applications as an ingredient or dietary supplement (Zhu et al.,
65 2015). Moreover, the polyphenol profile of grape pomace is especially interesting in
66 that it falls into two categories with complementary health effects: the widely known
67 extractable polyphenols (EPP), in this case consisting mainly of flavonoids
68 (anthocyanins, flavonols, flavan-3-ols) and phenolic acids, and the less studied non-
69 extractable polyphenols (NEPP), classified into polymeric proanthocyanidins and high
70 molecular weight hydrolysable tannins. These have promising health-related properties
71 once released from the food matrix by colonic microbiota (Pérez-Jiménez, Díaz-Rubio,
72 & Saura-Calixto, 2013). Indeed, most phenolic compounds in grape pomace belong to
73 the class NEPP (Pérez-Jiménez, Arranz, Tabernero, Rubio, Serrano, Goñi, & Saura-
74 Calixto, 2008a). The capacity of flavonoids to inhibit lipid oxidation is well
75 documented in the literature, due to their ability to stop radical chain reactions (Frankel,
76 1999), as well as to their metal chelating properties (Bors, Michel, & Stettmaier, 2000).
77 The latter are very important in the development of rancidity in fish, especially fatty
78 species due to the high concentrations of fat and haemoglobin (Richards & Hultin,
79 2002; Sánchez-Alonso, Borderías, Larsson, & Underland, 2007b). Some studies have
80 been published addressing the antioxidant properties of grape polyphenols in fish
81 muscle such as horse mackerel and rainbow trout (Pazos, Gallardo, Torres, & Medina,
82 2005; Pazos, Alonso, Fernández-Bolaños, Torres, & Medina, 2006; Sánchez-Alonso et
83 al., 2007a, Sánchez-Alonso, Jimenez-Escrig, Saura-Calixto, & Borderías 2008; Gai,
84 Ortoffi, Giancotti, Medana, & Peiretti, 2015). The addition of antioxidant dietary fibre
85 also had a positive effect on technological properties of fish mince to which it was
86 added, improving water holding and cooking yield (Sánchez-Alonso, Jimenez-Escrig,
87 Saura-Calixto, & Borderías, 2006).

However, in most of these cases grape pomace was from skins and seeds prepared by freeze-drying, which is an expensive procedure on an industrial scale. Therefore, there is a need to explore other, more economical ways of grape pomace preparation without prejudicing the polyphenol profile of this material. Additionally, it is important to evaluate the effect of this kind of enrichment not only on oxidative stability of fish, but as a way to deliver bioaccessible polyphenols to the consumer.

The aim of this study was therefore to evaluate how the addition to minced anchovy muscle of different doses of dietary fibre from grape pomace, obtained by an inexpensive cool-drying system, affected the technological parameters, antioxidant capacity and sensory characteristics of the final products. The *in vitro* bioaccessibility of polyphenols contributed by dietary fibre in the enriched minced fish was also assessed.

2. Materials and Methods

2.1 Reagents

Pepsin (2000 FIP-U/g) was obtained from Merck (Darmstadt, Germany). Amyloglucosidase (14 IU/mg) was from Roche (Manheim, Germany). Pancreatin, α -amylase (17.5 IU/mg), ABTS (2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and gallic acid were all obtained from Sigma-Aldrich (St. Louis, MO, USA). 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) was from Fluka Chemicals (Madrid, Spain). Iron III-chlorure-6-hydrate were from Panreac (Castellar del Vallés, Barcelona, Spain).

2.2 Preparation of grape pomace dietary fibre

Grape (*Vitis vinifera* var. *Quebranta*) pomace, a by-product of *pisco* processing was collected in dark bags in Ica-province (Peru) during the harvest season (March) and was stored at 4°C. It was dried in a cool-air drying equipment (ASAHI Co Ltd. Mod. CV-20AN. Hiroshimasi, Japan) at 18-30°C for 36 h. Dried grape pomace was then milled in a centrifugal mill (Retsch ZM 200, Haan, Germany) to a particle size of 0.25 mm . Sugars were removed from dried and milled grape pomace with ethanol/water (80:20, v/v) at room temperature for 30 minutes, and the residue from this procedure was cool-dried anew.

The final product (grape pomace dietary fibre) contained 68.8% of dietary fibre (3.4% soluble dietary fibre and 65.4% insoluble dietary fibre) and 32.9 % of polyphenols (5.3% extractable polyphenols, 1.3 % hydrolysable polyphenols and 26.3% non-extractable proanthocyanidins, NEPA), as determined by standard procedures (Singleton, Orthofer, & Lamuela-Raventós, 1999; Pérez-Jiménez, Arranz, Tabernero, Díaz-Rubio, Serrano, Goñi, & Saura-Calixto, 2008b; Goñi, Diaz-Rubio, Pérez-Jiménez, & Saura-Calixto, 2009; Zurita et al., 2012). A fraction of hydrolysable polyphenols and NEPA is associated with dietary fibre

2.3. Preparation of anchovy mince enriched with grape pomace dietary fibre

Two hundred kg of anchovy (*Engraulis ringens*) caught at Pisco (Peru) were eviscerated, headed and kept in ice (3:1). Skin was removed at 85°C / 5 sec. A deboning machine with a 2.5 mm aperture diameter was used to remove the pin and skin (Bibun, model NF2DX, series 80). Fish mince proximal composition was determined by AOAC methodology (AOAC, 2005). The proximate composition was: protein 17.0±0.60%; fat 3.3±0.10%; ash 1.0±0.03 %; moisture 78.5±0.3 %.

The grape pomace dietary fibre (*Vitis vinífera* var. *quebranta*) (GPDF) was mixed with anchovy mince at different concentrations (0, 2, 3, 4%, all w/w) and frozen in blocks of 10 kg at -35°C wrapped in polyethylene bags. Samples were stored frozen at -25°C. [It was decided to start with a 2% of dietary fibre according with the aim of the study to produce a functional product with relevant amounts of grape pomace polyphenols as to exert antioxidant properties; previous studies reported that additions of 2-4% of dietary fibre resulted in a final product with acceptable sensorial properties \(Sánchez-Alonso et al., 2007b\).](#)

2.4. Evaluation of polyphenols and antioxidant capacity of grape pomace dietary fibre-enriched mince anchovy

For the determination of polyphenols and antioxidant capacity in grape pomace dietary fibre-enriched minced anchovy, an aqueous–organic extraction was carried out (Pérez-Jiménez et al., 2008b). Briefly, 0.5 g of the sample was placed in a capped centrifuge tube, 20 mL of acidic methanol/water/HCl (50:50, v/v; pH 2) was added and the tube was thoroughly shaken at room temperature for 1 h. The tube was centrifuged at 2500 g in a Thermo Heraeus Megafuge 11 (Thermo Fisher, Waltham, MA, USA) for 10 min and the supernatant was recovered. Twenty mL of acetone/water (70:30, v/v) was added to the residue, and shaking and centrifugation were repeated. The methanolic and acetonetic extracts were combined and they were used for the following determinations.

2.4.1. Determination of polyphenols

Polyphenols were determined by Folin-Ciocalteu assay (Singleton Orthofer, & Lamuela-Raventós, 1999). A test sample (0.5 mL) was mixed with 1 mL of Folin–Ciocalteu reagent and swirled. After 3 min, 10 mL of sodium carbonate solution (75 g/L) was added and mixed. Additional distilled water was mixed in thoroughly by

inverting the tubes several times. After 1 h, the absorbance at 750 nm was recorded. The results were expressed as mg/100 g gallic acid equivalents (GAE).

2.4.2. Determination of antioxidant capacity

For determining antioxidant capacity as free radical scavenging capacity, ABTS (2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid)) assay was used. After the addition of the sample or Trolox standard (0.1 mL) to the ABTS^{•+} solution (3.9 mL), absorbance readings were taken at 595 nm every 20 s for 6 min by a DU-640 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). The percentage inhibition of absorbance was plotted against time and the area under the curve (0-6 min) was calculated (Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999).

FRAP (Ferric Reducing Antioxidant Power) assay was used to determine the antioxidant capacity of the sample as reducing power. FRAP reagent, freshly prepared and warmed to 37 °C, was mixed (900 µL) with distilled water (90 µL) and the test sample, Trolox or appropriate blank reagent (30 µL). Readings at 595 nm in a Lambda 12 spectrophotometer after 30 min were selected to calculate the FRAP values (Benzie, & Strain, 1996; Pulido, Bravo, & Saura-Calixto, 2000).

In both assays, the results were expressed as µmol Trolox equivalents/100 g fw after interpolating in the calibration curves. No sample dilution was needed either for FRAP or ABTS assays.

2.5 Evaluation of technological properties of grape pomace dietary fibre-enriched mince anchovy

2.5.1 Water retention capacity.

2.5.1.1. Water holding capacity (WHC)

It was performed following Moreno, Carballo and Borderías (2008). Briefly, about 2 g (wet basis) of each sample were cut into small pieces and placed in a centrifuge tube ($\varnothing=10$ mm) using two filter paper (Whatman n°1 $\varnothing=90$ mm). The samples were centrifuged in a Jouan MR1812 centrifuge (Saint-Nazaire, France) for 10 min at 3000 x g at room temperature. WHC was expressed as percent of water retained per 100 g water in the sample prior centrifuging.

2.5.1.2. Cooking loss

It was determined as reported Moreno, Carballo and Borderías (2011) with slight modifications. The raw samples were placed weighed and vacuum wrapped. The bags with the samples inside were cooked in a bath at 90°C/30min. When samples reached room temperature the exudate was evacuated and the samples measured again. Water loss due to the cooking was expressed as percent of water retained per 100 g water in the sample prior cooking.

2.5.2 Mechanical properties

2.5.2.1. Shear strength

The analysis was carried out at room temperature (20-22 °C) and samples of 2*2 [cm](#) were penetrated up to breaking point simulating the chewing. It was performed using a mini-Kramer cell (HDP/MK05) attached to a 50 N cell connected to the crosshead on a TA-XT plus Texture Analyzer (Texture Technologies Corp., Scarsdale, NY, USA). From force-deformation curves derived at 0.2 mm.s⁻¹ crosshead speed, shear strength (N/g) was determined.

2.5.2.2. Texture Profile Analysis (TPA)

210 Texture Profile Analysis (TPA) was performed using a TA-XT plus Texture Analyzer
211 as described by Bourne (2002). Three cores (diameter 16 mm; height 16 mm) of
212 samples were axially compressed to 40% of their original height. Force-time
213 deformation curves were derived with a 50 N load cell applied at a crosshead speed of
214 0.8 mm s⁻¹. Attributes calculated were hardness (N); cohesiveness (dimensionless);
215 springiness (mm) and chewiness (N*mm).

216

217 2.5.3 Color

218 The color parameters L*, a*, and b* of the surface of the samples were determined
219 using a portable colorimeter (Minolta, CR-400 Konica-Minolta, Japan) (D65/2°), which
220 was standardized using a white calibration plate of CIELab scale. The L* value
221 indicates lightness (L* = 0 darkness, L* = 100 lightness); a* value indicates redness
222 (+60 = red, -60 = green); and b* value indicates yellowness (+60 = yellow, -60 = blue).
223 The determinations were carried out at least in quadruplicate.

224

225 2.6. *In vitro* evaluation of [the distribution of bioaccessible](#) polyphenols in grape pomace
226 dietary fibre-enriched mince anchovy

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227 In order to determine the [distribution \(in the small intestine and the large intestine\)](#) of
228 [bioaccessible](#) polyphenols, an *in vitro* procedure was used. This methodology was
229 initially designed to increase the physiological relevance of the official method to
230 determine dietary fibre content in foods (Saura-Calixto, García-Alonso, Goñi, & Bravo,
231 2000), a procedure where gastrointestinal digestion is emulated. The method was later
232 validated for evaluating the bioaccessibility of individual foods or even whole diets
233 (Saura-Calixto, Serrano, & Goñi, 2007; Tabernero, Serrano, & Saura-Calixto, 2007). A
234 general scheme of this process, only applied to samples with grape pomace dietary

Eliminado: bioaccessibility

237 fibre, is shown in Figure 1. Samples (300 mg), after being solved in phosphate buffer,
238 were incubated with pepsin (0.2 mL of a 300 mg/mL solution in 0.08 mol/L HCl–KCl
239 buffer, pH 1.5, 40 °C, 1 h), pancreatin (1 mL of a 5 mg/mL solution in 0.1 mol/L
240 phosphate buffer, pH 7.5, 37 °C, 6 h) and α -amylase (1 mL of a 120 mg/mL solution in
241 0.1 mol/L tris–maleate buffer, pH 6.9, 37 °C, 16 h). The samples were centrifuged (15
242 min, 3000 g) and the supernatant was separated from the residue.

243 The supernatant contained both polyphenols bioaccessible in the small intestine and
244 those associated with soluble dietary fibre. An aliquot of the supernatant was used to
245 determine polyphenols bioaccessible in the small intestine (Fraction A). While the rest
246 of the supernatant was incubated with 100 μ L of amyloglucosidase for 45 min at 60 °C
247 before being transferred to dialysis tubes (12,000–14,000 molecular weight cutoff,
248 Visking dialysis tubing; Medicell International Ltd., London, U.K.) and dialyzed against
249 water for 48 h at 25 °C to remove digestible compounds. Then, the retentate was
250 hydrolyzed with 1 M sulfuric acid at 100 °C for 90 min -the common procedure for the
251 hydrolysis of soluble dietary fibre- in order to release polyphenols associated with
252 dietary fibre (Fraction B).

253 The residue of the treatments with pepsin, pancreatin and amylase contained
254 polyphenols associated with insoluble dietary fibre. It was subjected to an extraction
255 with methanol/water and acetone/water, as described in 2.3.3. The derived supernatants
256 (Fraction C) correspond to low molecular weight polyphenols bioaccessible in the large
257 intestine. Independent residues of this extraction were subjected to two treatments: a)
258 methanol/H₂SO₄ at 85°C for 20 hours, in order to release hydrolysable polyphenols
259 from food matrix (Fraction D); b) butanol/HCl at 100°C for 60 minutes, in order to
260 release NEPA (Fraction E).

Polyphenol content was determined by Folin assay- see details in 2.3.3- in Fractions A-D, while specific spectrophotometry procedure for NEPA (Zurita et al., 2012) was applied in Fraction E.

In summary, only Fraction A corresponds to polyphenols bioaccessible in the small intestine, while Fractions B-E correspond to the different classes of polyphenols that would become bioaccessible in the large intestine in case they were released from the dietary fibre matrix by the action of the colonic microbiota- ~~Bioaccessible poyphenols~~ in each fraction was expressed as percentage of total polyphenols measured in the five fractions; the results were not associated with those measured directly in the samples, since it has been reported that polyphenols determinations after an aqueous-organic treatment are not directly comparable with those performed after an enzymatic procedure (Pérez-Jiménez, & Saura-Calixto, 2005; Koehnlein, Koehnlein, Correa, Nishida, Correa, Bracht, & Peralta, 2016).

2.7. Sensory Analysis

A Threshold Sensory analysis was performed with 10 trained panellists (middle-aged men and women). The analysis was performed between 2 and 4 hours after breakfast in a room at 20°C. Parameters such as dietary fibre lumpiness, strange taste, unpleasant flavour and general acceptance were evaluated after grilling pieces of 1 cm thick. Dietary fibre lumpiness, strange taste and unpleasant flavour detected in the samples were evaluated on a linear scale from 0 (nothing) to 10 (a lot). The general acceptance of the samples was measured over a hedonic scale ranging from 0 (do not like) to 10 (like a lot).

2.8 Statistics

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Three parallel extractions were carried out on each sample. The determinations were performed in duplicate for each extract and are reported on a fresh matter basis. Physicochemical properties analysis, were performed in triplicate. The results of all the determinations are expressed as the mean value $\pm$ s.d. Levene's test and the Kolmogorov-Smirnov test were applied to assess variance equality and normal distribution, respectively. One-way analysis of variance, followed by Tukey's post-hoc significance test, was used when the assumptions of normality and equal variance were met. Otherwise, non-parametric tests (Kruskal–Wallis and Mann–Whitney U rank-sum) were used to assess significance. Differences were considered to be statistically significant for $P < 0.05$. The SPSS IBM22 for Windows was used throughout.

3. RESULTS AND DISCUSSION

3.1 Evaluation of polyphenols and antioxidant capacity in grape pomace dietary fibre-enriched anchovy mince

Grape pomace dietary fibre is known to have not only high dietary fibre content, but also a significant proportion of polyphenols, and is therefore a good antioxidant (Saura-Calixto, 1998). For this reason, polyphenols and antioxidant capacity were measured in the products obtained, as shown in Table 1. The control sample contained 200 mg GAE/100 g fw, although it did not contain polyphenols. This is because, as we know, several compounds occurring at significant concentrations in fish, such as amino acids, may interfere with the Folin-Ciocalteu assay used to evaluate polyphenol content (Prior, Wu & Schaich, 2005). Nevertheless, there was a significant increase ($P < 0.05$) in the concentration of these compounds when at least 2% of grape pomace dietary fibre was added to the final mixture. As regards details of these compounds, Gai et al. (2015)

reported that (+)-catechin and (-)-epicatechin were the major polyphenols detected in minced rainbow trout muscle enriched with grape pomace. Indeed, *in vivo* studies on the bioavailability of a grape pomace derived product have identified (epi)catechin as the most abundant metabolites (Touriño, Pérez-Jiménez, Mateos-Martín, Fuguet, Vinardell, Cascante & Torres, 2011; Mateos-Martín, Pérez-Jimenez, Fuget, & Torres, 2012). Regarding antioxidant capacity, in both FRAP and ABTS assays a basal value was obtained for the control sample, but significant increases were detected when grape pomace dietary fibre was added to minced anchovy. In the case of the ABTS assay, a clear upward trend was observed with increasing percentages of grape pomace dietary fibre. In the FRAP assay, although there was also a significant increase from 2% of grape pomace dietary fibre upwards, the overall tendency was not so marked as in ABTS. Based on these results, the ABTS assay seems to be more suitable than the FRAP assay for evaluating antioxidant capacity in this kind of samples. This may be due to the different reaction mechanism involved in each assay; the radical scavenging ability measured by ABTS may be more specific of phenolic compounds than the reducing capacity assessed by FRAP assay, which could be altered by other non-phenolic substances present in this kind of complex matrixes. Thus, cool-dried grape pomace dietary fibre retained a polyphenol content and antioxidant capacity that originated significant increases in these parameters when added to anchovy mince, as previously reported for similar products prepared from freeze-dried grape pomace (Sánchez-Alonso et al., 2007a, b.; Sanchez-Alonso et al., 2008). Cool-drying is in fact a procedure that the food industry could adopt at a lower cost than other processes such as freeze-drying.

3.2 Physicochemical properties of anchovy mince enriched with grape pomace antioxidant dietary fibre.

3.2.1. Water holding capacity

Water holding capacity (WHC) was determined as such on the raw samples, and on the cooked samples as cooking loss.

WHC tended to increase in line with the increase in dietary fibre concentration, and statistical differences were observed in the samples with 4% GPDF. Dietary fibre from fruits and greens has been reported to possess a considerable affinity for water, thus enhancing WHC (Grigelmo-Miguel, & Martin-Belloso, 1999). Moreover, similar results to these were reported by Sánchez-Alonso et al. (2007a) and Sánchez-Alonso and Borderías (2008) in minced fish muscle enriched with dietary fibre from white and red grapes (*Vitis vinifera*).

On the other hand, when samples were cooked (90 °C/20 min), cooking loss was very low (Fig. 2). Results indicated that around three quarters of the water naturally present in the samples was retained during heating. This could be due to both the water-holding effect of dietary fibre (Grigelmo-Miguel & Martin-Belloso, 1999) and the ability of myofibrillar proteins to link water molecules after heating, through increased interactions among proteins-water-ingredients (Moreno, Carballo, & Borderías, 2013).

3.2.2 Mechanical properties

3.2.2.1 Shear strength

Shear strength was determined in raw and cooked samples (Fig. 3). In both groups of samples GPDF addition increased the shear strength, particularly with 4% of GPDF added. This could be due to a proportional reduction of moisture content in samples as a consequence of the increase in dietary fibre addition. The shear strength of cooked

samples in particular was greater than in raw samples, which may be related to the higher friction involved in extruding the sample by Ottawa cell (Sánchez-Alonso, Careche, & Borderías, 2010). As reported in the cooking loss section, after heating most of the water is retained in the mince matrix, meaning that the effect on shear strength is related to both, i.e. stabilization of the protein network by the interactions produced by heating, and a reduction in water release.

3.2.2.2 Texture Profile Analysis (TPA)

The parameters measured by Texture Profile analysis (Fig. 4) indicated that GPDF proportions higher than 2% significantly affected hardness, springiness and chewiness of raw and cooked samples, particularly chewiness. The values of hardness and chewiness of raw samples were considerably lower than in cooked samples, although in both groups these parameters increased significantly when 3% and 4% of dietary fibre was added. The increase in hardness and chewiness in cooked samples is due to a lack of moisture as a consequence of the increase in dietary fibre and the protein aggregation caused by heating (Stone and Stanley, 1992). Springiness was also higher in cooked samples than in raw ones, which could be influenced by improved stabilization of the anchovy mince protein network as a result of protein aggregation during heating.

It is interesting that the addition of up to 4% of dietary fibre did not modify sample cohesiveness in either raw or cooked minces. Some authors have reported a reduction in cohesiveness when adding GPDF dietary fibre obtained through various different processes (Sánchez-Alonso, & Borderías, 2008). The difference in cohesiveness between both studies could originate from the fact that the red grape cultivars did not have the same specific composition and thus could modify anchovy mince matrix cohesiveness differently.

388 In general, it is fair to say that the addition of dietary fibre to the anchovy mince matrix
389 modifies texture parameters to a limited extent.

390

391 3.2.3 Colour

392 The effect of GPDF addition on colour parameters of anchovy mince is shown in Table
393 2. Anchovy mince lightness (L*) was significantly reduced when the percentage of
394 GPDF was increased to 3%, which is due to the brownish colour of the added dietary
395 fibre. On the other hand, there was no change in redness (a*) or yellowness (b*) due to
396 the dietary fibre, as might have been expected given the dark colour of the anchovy
397 mince. This is a positive finding in that there was no change in the original colour of the
398 anchovy mince even with 4% of added GPDF.

399

400 3.3 Evaluation of the distribution of bioaccessible polyphenols in grape pomace dietary
401 fibre-enriched anchovy mince

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402 Previous studies on the addition of polyphenols and dietary fibre to seafood products
403 have mostly focused on the effects on technological properties and on lipid oxidation
404 (Sánchez-Alonso et al., 2006, Sánchez-Alonso et al., 2007a, b, Sánchez-Alonso et
405 al., 2008; Ribeiro, Cardoso, Silva, Serrano, Ramos, Santos, & Mendes, 2013; Gai et al.,
406 2015). However, the nutritional significance of such addition should also be assessed.

407 Therefore, this study included an *in vitro* evaluation of the distribution (in the small
408 intestine and the large intestine) of polyphenols in grape pomace dietary fibre-enriched
409 minced anchovy, being therefore potentially available for absorption after intake.

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410 Although all *in vitro* approaches are only an emulation of *in vivo* conditions, the
411 methodology used here has been previously used to determine this distribution (Saura-
412 Calixto et al., 2007; Tabernero et al., 2007). Taking into account the complexity of

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426 grape pomace, with more than one hundred individual phenolic compounds (Tourinho et
 427 al., 2008), this first exploratory study was focused on the determination of total
 428 polyphenols in order to determine whether the addition of [different amounts of GPDF](#)
 429 affected their [distribution throughout the digestive tube](#). Despite the known limitations
 430 of the Folin-Ciocalteu assay, it should be remarked that this approach was previously
 431 used, for instance for the evaluation of polyphenols bioaccessibility in a whole diet
 432 (Saura-Calixto et al., 2007). [Moreover, it has been reported that when *in vitro* results of](#)
 433 [Folin-Ciocalteu method in digestion assays are compared with those from *in vivo*](#)
 434 [studies, the bioavailability values appear to be in the same range \(Bohn, Carriere, Day,](#)
 435 [Deglaire, Egger, Freitas, Golding, LeFeunteun et al., *in press*\).](#)
 436 [Results for bioaccessibility distribution](#) are shown in Table 3. The specific aim [of this](#)
 437 [determination](#) was to evaluate whether further addition of dietary fibre also caused
 438 modifications in the relative bioaccessibility of polyphenols. [In this way, the results](#)
 439 [obtained should be considered mostly for comparative purposes between treatments. It](#)
 440 was observed that the higher the grape pomace dietary fibre content, the higher was the
 441 proportion of bioaccessible polyphenols in the large intestine. [These were](#) measured as
 442 the sum of the different fractions of polyphenols which may appear associated with
 443 soluble or insoluble dietary fibre [and that, *in vivo*, should be released from this matrix](#)
 444 [by the action of the colonic microbiota](#). Thus, the fraction of polyphenols bioaccessible
 445 in the small intestine was 53.98% in the product with 2% of grape pomace dietary fibre,
 446 40.02% in the product with 3% and 29.65% in the product with 4%. Interestingly, since
 447 [most](#) polyphenols [reaching the large intestine](#) belonged to the class of NEPA they could
 448 be measured by a much more specific method than Folin assay (Zurita et al., 2012).
 449 These results would imply that larger amounts of dietary fibre increase polyphenols

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Eliminado: They are expressed as percentages, since the absolute values were evident considering the amounts of the different polyphenol fractions present in each product.

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Eliminado: —

Eliminado: in this fraction

retention, a process that may take place through different mechanisms (Renard, Watrelot, & Le Bourvellec, 2017).

There is increasing evidence of the relevance of the metabolites produced from microbial fermentation for the health effects of polyphenols (Williamson & Clifford,

2010). Therefore, it is very promising that in all the concentrations tested, at least a 40% of polyphenols would reach the large intestine. This is due to the high proportion in grape of NEPP, with specific health-related properties (Pérez-Jiménez et al., 2013). It

should be remarked that these compounds, in order to be transformed into actual bioavailable polyphenols would need to be released from their association with dietary

fibre. In this way, a previous study on grape seed and peel that combined the *in vitro* digestion procedure used here with colonic fermentation showed that intestinal bacteria were able to transform 95-97% of polyphenols originally present in the sample (Goñi, Martín, & Saura-Calixto, 2005).

These results need to be confirmed by *in vivo* bioavailability studies. Nevertheless, should they be confirmed, taken together with the fact that significant proportions of dietary fibre are being incorporated in the anchovy matrix, these products would have considerable nutritional potential since the health effects of ω -3 PUFAs present in anchovy would be combined with those of dietary fibre and polyphenols. Indeed, recent reports have found beneficial interactions between ω -3 PUFAs and grape polyphenols in terms of various metabolic syndrome markers (Ramos-Romero, et al., 2016).

3.4. Sensory analysis

The sensory panel performed a threshold test on different parameters of the anchovy mince (scores shown in Fig. 5). These tracked the colour parameters determined

Eliminado: polyphenol

Eliminado: most polyphenols added to minced anchovy as grape pomace dietary fibre seemed to be able to reach the colon and could potentially be fermented.

Eliminado: Indeed,

Eliminado: These polyphenols can spread through the digestive tube after intake because most grape pomace polyphenols belong to the class of non-extractable polyphenols—in particular non-extractable proanthocyanidins—with specific health-related properties (Pérez-Jiménez et al., 2013). ¶

Eliminado: bioaccessibility of polyphenols incorporated in minced anchovy

analytically (Table 2); panellists noticed no differences in the visual appearance of the anchovy mince, and the scores of all samples were quite similar.

Despite the increase in added GPDF, no significant strange tastes or unpleasant flavours were detected in the samples. This also agrees with the fact that the acceptability scores of all samples were much higher (Fig. 5). It is significant that the control sample without GPDF achieved the lowest general acceptability. This could be due to the strong fishy flavour of anchovy, which is attenuated by the addition of dietary fibre (Sanchez-Alonso et al., 2010). The lumpiness of samples was clearly related to the amount of dietary fibre added to the mince, especially at a 4% GPDF concentration. Increased lumpiness, mainly in sample with 4% dietary fibre, did not affect the general acceptability of the product.

All these results are interesting from a sensory point of view given that up to 4% GPDF could be added without detriment to general acceptance.

CONCLUSIONS

Cool-drying was used to obtain a dietary fibre and polyphenol-rich material from grape pomace, which was successfully added in concentrations of up to 4% to minced anchovy, in different proportions, inducing little change in techno-functional properties or sensory acceptability.

In the mince enriched with GPDF, there were significant increases in polyphenol contents and antioxidant capacity at final concentrations of grape pomace dietary fibre from 2% upwards. Moreover, in vitro digestion showed that the higher the grape pomace dietary fibre content, the higher was the proportion of polyphenols reaching the large intestine.

Eliminado: *in vitro* bioaccessibility evaluation of GDAF-enriched fish mince showed a significant concentration of bioaccessible polyphenols in the large intestine.

534 The approach followed in this study could assist the valorization of local Peruvian
535 varieties of grape and anchovy, through an inexpensive procedure easily applicable at
536 industrial level. The possible implications of this product for human health remain to be
537 elucidated.

538

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Table 1. Polyphenols content and antioxidant capacity (by FRAP and ABTS assays) in grape pomace dietary fibre-enriched minced anchovy.

<u>Grape pomace dietary fibre content (%)</u>	<u>Polyphenols (mg GAE/100 g fw)</u>	<u>ABTS (μmol Trolox/100 g fw)</u>	<u>FRAP (μmol Trolox/100 g fw)</u>
<u>0</u>	<u>200.50 + 5.30^a</u>	<u>1235.00 + 233.35^a</u>	<u>10.90 + 0.98^a</u>
<u>2</u>	<u>224.58 + 18.72^b</u>	<u>4024.00 + 303.85^b</u>	<u>49.70 + 4.78^b</u>
<u>3</u>	<u>254.64 + 14.79^c</u>	<u>10,995.00 + 806.10^c</u>	<u>419.21 + 58.50^c</u>
<u>4</u>	<u>232.09 + 3.86^b</u>	<u>14,021.67 + 1,128.55^c</u>	<u>426.77 + 81.86^c</u>

GAE, gallic acid equivalents; FRAP, Ferric Reducing Antioxidant Power; ABTS, 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid); fw, fresh weight. Different superscript letters in the same column indicate significant differences ($P < 0.05$).

Table 2. Effect of the addition of different percentages of GPDF over the color parameters of anchovy mince.

<u>Grape pomace dietary</u> <u>fibre content (%)</u>	<u>L*</u>	<u>a*</u>	<u>b*</u>
<u>0</u>	<u>$36,01 \pm 0,76^a$</u>	<u>$9,81 \pm 0,39^a$</u>	<u>$-0,90 \pm 0,36^a$</u>
<u>2</u>	<u>$34,18 \pm 0,81^{ab}$</u>	<u>$10,41 \pm 0,65^a$</u>	<u>$-1,79 \pm 0,56^a$</u>
<u>3</u>	<u>$33,14 \pm 0,59^b$</u>	<u>$10,81 \pm 0,29^a$</u>	<u>$-2,04 \pm 0,57^a$</u>
<u>4</u>	<u>$33,02 \pm 0,78^b$</u>	<u>$10,45 \pm 0,30^a$</u>	<u>$-2,79 \pm 0,37^a$</u>

Different letters in the same column indicate statistical differences (p<0.05).

Table 3. Distribution (%) of bioaccessible polyphenols in grape pomace dietary fibre-enriched minced anchovy.

Grape pomace dietary fibre content (%)	Bioaccessible in the small intestine	Bioaccessible in the large intestine ¹			
	Fraction A	Fraction B	Fraction C	Fraction D	Fraction E
2	53.98 + 5.87 ^a	5.81 + 0.97 ^a	0.60 + 0.14 ^a	8.83 + 1.05 ^a	30.78 + 0.19 ^a
3	40.02 + 0.15 ^b	11.00 + 1.64 ^b	1.91 + 0.28 ^b	13.51 + 0.96 ^b	33.56 + 3.10 ^a
4	29.65 + 2.91 ^c	6.56 + 0.69 ^a	1.78 + 0.19 ^b	14.10 + 1.07 ^b	47.91 + 0.77 ^b

Fraction A, polyphenols non associated with dietary fibre; Fraction B, polyphenols associated with soluble dietary fibre; Fraction C, low molecular weight polyphenols associated with insoluble dietary fibre; Fraction D, hydrolysable polyphenols associated with insoluble dietary fibre; Fraction E, non-extractable proanthocyanidins associated with insoluble dietary fibre. Different superscript letters in the same column indicate significant differences ($P < 0.05$).

¹ In order to become bioaccessible, these polyphenols need to be released from the dietary fibre matrix by the action of colonic microbiota.

FIGURE LEGENDS

Figure 1. Scheme of the *in vitro* procedure carried out in grape pomace dietary fibre-enriched minced anchovy for the determination of the distribution of bioaccessible polyphenols.

Eliminado: bioaccessibility

Figure 2. Effect of the addition of different percentages of GPDF over water binding capacity (WHC) and Cooking loss of anchovy mince. Different letters indicate statistical differences ($p < 0.05$).

Figure 3. Effect of the addition of different percentages of GPDF over Shear strength (N/g) of anchovy mince. Different letters in the raw or heated indicate statistical differences ($p < 0.05$).

Figure 4. Effect of the addition of different percentages of GPDF over texture profile analysis parameters of raw (a) and heated (b) anchovy mince. Different letters in the same parameter with different amount of GPDF indicate statistical differences ($p < 0.05$).

Figure 5. Effect of the addition of different percentages of GPDF to anchovy mince on sensory parameters.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.