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Odour characteristics of seafood flavour formulations produced with fish by-products incorporating EPA, DHA and fish oil.

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Abstract.

Thermal degradation of eicosapentaenoic (EPA) and docosahexanoic (DHA) acids was investigated. As a novelty, EPA, DHA or fish oil (FO) were incorporated as ω-fatty acid sources into model systems containing fish powder produced via Maillard reactions. Aroma composition of the resulting products was determined and complemented with sensory evaluation. Heating of the oils led to a fast decrease of both, EPA and DHA, and to the development of characteristic volatile compounds including hexanal, 2,4-heptadienal and 4-heptenal, the most abundant being (E,E)-2,4-heptadienal (132 ± 44 to 329 ± 122 μmol/g). EPA and DHA addition to the model systems increased the concentration of these characteristic volatile compounds. However, it did not have a considerable impact on the development of characteristic Maillard reaction products, such as pyrazines and some aldehydes. Finally, the results of the sensory evaluation illustrated that panellists would chose samples fortified with FO as the ones with a more pleasant aroma.

Chemical compounds studied in this article:

Eicosapentaenoic acid (EPA) (Pubchem CID 446284); docosahexaenoic acid (DHA) (Pubchem CID 445580); (*E*)-3-hexen-1-ol (Pubchem CID: 5281167); 1-octen-3-ol (Pubchem CID: 18827); (*E,E*)-2,4-heptadienal (Pubchem CID: 20307); (*Z*)-4-heptenal (Pubchem CID:71590); 1-hepten-4-ol (Pubchem CID: 19040).

Key words: Maillard Reaction, volatiles, 2,4-heptadienal, 4-heptenal, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA).

1. Introduction

Consumption of omega-3 polyunsaturated fatty acids (ω-3 PUFAs), such as eicosapentaenoic acid (EPA; C20:5) or docosahexaenoic acid (DHA; C22:6), has been reported to have beneficial effects on human health, such as lowering blood cholesterol and reduced risk of several diseases (Hammer & Schieberle, 2013). The fact that their biosynthetic pathways are slow encourages nutritionists to enrich the human diet with fatty fish containing high amounts of ω-3 PUFAs. Fresh fish however is not the only way of incorporating these fatty acids into the diet. There are in fact many other sources of fatty acids such as fish oils, pills or different foods enriched with specific fatty acids (Caprino, Moretti, Bellagamba, et al., 2008; Dawczynski, Schubert, & Jahreis, 2007; Hammer & Schieberle, 2013; Sanchez-Machado, Lopez-Cervantes, Lopez-Hernandez, & Paseiro-Losada, 2004).

Flavour is an important factor in determining the quality of processed fish and fish-derived products as well as overall consumer acceptance. Fresh fish characteristic aroma compounds, might be accumulated either *via* endogenous and/or exogenous processes. Sometimes volatile compounds can be absorbed from the water, in most cases, during fish respiration, and then stored under fish skin in the lipid layer; lipid-derived volatile compounds, such as aldehydes and ketones, are frequently generated by oxidative enzymatic reactions and autoxidation of lipids. Moreover, compounds such as pyrazines and furans also make important contributions to the flavour and aroma of fish products; although they have been found in raw fish, they are mainly derived from Maillard reaction, after frying or grilling (Giri, Osako, & Ohshima, 2010). Salmon or trout, for instance, have a strong pleasant characteristic smell after cooking attributed to aldehydes and ketones generated mainly by oxidative enzymatic reactions and autoxidation of lipids (Ganeko et al., 2008; Whitfield et al., 1982; Whitfield, Last, Shaw, & Tindale, 1988). It is well known that unsaturated fatty acids are prone to rapid oxidation, and their instability increases with the number of double bonds. The rapid formation of

hydroperoxides as primary oxidation products and their degradation into short-chain volatile compounds is most of the time considered as a disadvantage or challenge for the food industry (Ganeko et al., 2008). Nevertheless, formation of volatile compounds derived from fatty acid oxidation leading to specific aromas could also be seen as an opportunity to develop and enhance the sensory quality of food products containing fish or seafood.

Recently, there is a growing interest in using food waste materials as sources of ingredients for uses in foods. Some examples of this are the extraction of amino acids, peptides, collagen or fatty acids from fish waste (Guerard, Dufosse, Broise, & Binet, 2001), while fish powders or fish protein hydrolysates have been produced using fish wastes as starting material. Development of novel means of processing is required to convert the waste and by- products into forms that are safe, marketable and acceptable to consumers (Peinado, Koutisids & Ames, 2016).

The aim of this work was to study the effect of polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on fish by-products (fish hydrolysate powder) upon subsequent heating in the presence of glucose and/or fish oil. Volatile formation as well as sensory perception were evaluated.

2. Materials and Methods

2.1. Chemicals

Enzyme Flavopro 373 (Biocatalysts Ltd, Cardiff, UK), fish oil (Seven Seas Pure Cod Liver Oil High Strength with Omega-3 Plus Vitamins D & E – 300 mL) and fish powder (Croda International plc, Snaith, UK), as well as glucose and glycerol (Sigma-Aldrich, St Louis, MO) used to produce food model systems were food grade. Chemicals used for analytical determinations: petroleum ether 40:60, BF_3-methanol, hexane, sulfuric acid, hydrochloric acid, *iso*-octane, C7–C30 alkanes (1,000 µg/mL each component in hexane), as well as the

standards for volatiles and the standards for fatty acids, eicosapentanoic acid (EPA), docosahexanoic acid (DHA), tricosanoic acid and tricosanoate methyl esters were all analytical grade purchased from Sigma-Aldrich.

2.2. Oil thermal degradation and formation of aromas

2.2.1. Thermal degradation

The evolution of volatile compounds and fatty acids caused by thermal degradation of EPA and DHA was investigated. For this purpose, individual EPA and DHA (50 µg; 200 µL of a 0.25 mg/mL oil in hexane solution) were placed into glass reaction vials, left to evaporate the hexane and then heated to 110 °C in a thermostatic oil bath. At pre-determined times (0, 15, 30, 45, 60, 75, 90 min), specific samples were removed from the oil bath and cooled on ice before performing the correspondent volatile analysis. For fatty acids, the same procedure was followed but this time, 5 µg (200 µL of a 25 mg/mL oil in hexane solution) were used and, once removed from the oil bath, samples were derivatised to fatty acid methyl esters (FAMEs) for analysis by gas chromatography with flame ionisation detection (GC-FID).

2.2.2. Maillard reaction of food model systems

Aroma composition of food model systems produced *via* Maillard reaction containing fish powder hydrolysates as a source of amino acids, combined with an ω-fatty acids source was investigated. A full factorial design of 3 independent variables at 2 levels each was applied. The 3 independent variables studied were the different sources of ω-fatty acids (EPA, DHA or fish oil), and the 2 levels were the concentrations of them added to the food model system (1.5 or 3 g/100 g). For the control, hydrolysed fish powder (FPH) was heated under the same conditions but no external ω-fatty acids source was added.

Hydrolysed fish powder (FPH) was obtained following the procedure by Peinado et al., (2016). In short, commercially available enzyme Flavopro 373 (15 g/L) (Biocatalysts Ltd) was added to fish powder aqueous solution (100 g/L), hydrolysed overnight (15h) with

constant stirring and controlled conditions of temperature and pH (60 ºC at pH 6). The reaction was terminated by heating the mixture to 95 °C for 20 minutes to inactive the enzyme. The resulting slurry was centrifuged at 7,200 *g* for 20 minutes. The obtained supernatant, fish powder hydrolysate (FPH), was then used for subsequent reactions to generate aroma compounds *via* Maillard reactions. Aliquots of FPH (0.2 mL) were mixed and homogenised with glucose solution (0.05 mL (100 µmol/mL) in glass reaction vials and freeze-dried. Glycerol (200 µL) was added to each freeze-dried sample as carrier agent. Fish oil (1.5 or 3 g/100 g) was added to some of the samples according to the experimental design. Finally, samples in closed vials were homogenised and heated in a thermostatic oil bath at 110 ºC for 30 min to promote flavour formation, and then cooled on ice. All samples were prepared in triplicate and stored at –80 °C before volatile and sensory analyses.

2.2.3. Sensory evaluation

FPH-Maillard reaction model systems were evaluated using a triangle test (Kemp, Hort, & Hollowood, 2009; Meilgaard, Civille, & Carr, 2007). As the aim of the sensory evaluation was to investigate whether panellists would be able to differentiate between samples containing EPA or DHA, only one percentage of oil was selected (1.5 g/100 g). Testing was conducted using a non-trained panel of assessors aged 20–40 (n = 21; 13 female, 8 male). Three sets of the total possible combinations were presented to each assessor; each vial was anonymised and coded with random three-digit numbers. Assessors were asked to sniff the samples and select the different one. Moreover, an extra set containing the three coded samples was served to the panellists and they were asked which samples they preferred. The sensory testing took place as according to British Standard 5929 (1986).

2.3. Analyses

2.3.1. Chemical analyses. Composition of fish powder and fish oil.

The moisture, ash and extractable fat content of the fish powder were calculated according to the Association of Official Analytical Chemists (AOAC, 2000). Total protein was determined by the Kjeldahl method using a nitrogen conversion factor of 6.25. pH and colour analyses of the FPH-Maillard reaction systems were conducted after the addition of 2 mL of Milli-Q water. Spectrophotometric measurements were carried out at room temperature on an Ultrospec 2000 UV/Vis spectrophotometer (Pharmacia Biotech, Little Chalfont, UK) at 420 nm and pH measurements were obtained using an electronic pH meter (Mettler-Toledo, Greifensee, Switzerland).

Analysis of FAMEs was carried out by GC-FID with a mixture BF_3-methanol at 20 °C according to the IUPAC standard method (IUPAC, 1992) (Peinado et al., 2016; Peinado, Girón, Koutsidis & Ames, 2014; Yaich et al., 2011). The fatty acids composition was analysed after transesterification of extracted fat or fish oil (10 mg) to methyl esters (FAMEs) with a DANI Master GC equipped with an autosampler, a DANI FID detector (DANI Instruments S.p.A, Italy) and an Agilent DB-23 (60 m × 0.25 mm, 0.25 μm) capillary column (Agilent Technologies, Santa Clara, CA). The oven temperature ramp was programmed from 90 °C to 240 °C at 4 ºC/min and the injector and detector temperatures were set at 250 °C. The carrier gas was helium at 1.0 mL/min constant flow (split ratio 10:1). Data analysis, identification and quantification of FAMEs was accomplished by comparing the retention times and areas of the peaks with those of pure standards (Supelco® 37 Component FAME Mix; Sigma-Aldrich) analysed under the same conditions. The results were expressed as g of each fatty acid/100 g of the lipid fraction. GC-MS was carried out for identification and confirmation of the FAMEs on a Hewlett Packard 6890 GC with an autosampler coupled to a

159 5973N MSD instrument (Agilent Technologies) and an Agilent DB-23 (60 m × 0.25 mm,
160 0.25 μm) capillary column under the same program conditions.
161 The free amino acid content of the fish powder and hydrolysed fish powder solutions was
162 measured following the same method as Peinado et al. (2016) using the EZ-Faast amino acid
163 derivatization technique for GC-MS (Phenomenex, Torrance, CA). An aliquot of either
164 unhydrolysed fish powder solution or fish powder hydrolysate solution (200 μL) was
165 combined with distilled water (800 μL). An extract of that solution (500 μL) was combined
166 with hydrochloric acid (500 μL, 0.01 N, HCl), stirred at room temperature for 15 minutes and
167 left to settle for 45 minutes. An aliquot (100 μL) was then derivatised using the EZ-Faast
168 amino acid analysis kit. The derivatised amino acids were extracted into *iso*-octane (100 μL)
169 and analysed in electronic ionisation mode at 70 eV using a 6890 GC coupled to a 5973 MSD
170 instrument (Agilent, Palo Alto, CA). Derivatised amino acid solution (2 μL) was injected at
171 250 °C in split mode (10:1) onto a 10 m × 0.25 mm × 0.25 μm Zebron ZB-AAA capillary
172 column (film composition 50 % phenyl 50 % dimethyl polysiloxane; Phenomenex, Torrance,
173 CA). The oven temperature was 110 °C for 1 min, then increased at 30 °C/min to 320 °C, and
174 held at 320 °C for 2 min. The transfer line was held at 320 °C, and the carrier gas was helium
175 at a constant flow rate of 1.1 mL/min. The ion source was maintained at 320 °C
176 Standard mix stock solution (200 μM each) of 15 non-basic amino acids (Ala, Asp, Glu, Gly,
177 His, Ile, Leu, Lys, Met, Orn, Phe, Pro, Ser, Thr, Val) in hydrochloric acid (0.1 M) and 2 basic
178 amino acids (Asn and Gln) in water were prepared; different dilutions (10 to 150 μM) were
179 derivatised and calibration curves were plotted for each amino acid (effect of food matrix
180 composition was studied by spiking samples). Norvaline (100 μL, 0.2 mM) was used as the
181 internal standard.

182 *2.3.2. Volatiles analysis*

183 GC-MS analyses were performed using an Agilent 7890A gas chromatograph equipped with
184 a DB-WAX capillary column (60 m × 0.25mm × 0.25 μm) coupled to a BenchToF Time of
185 Flight Mass Spectrometer (Markes International Ltd, Llantrisant UK) and a CTC CombiPal
186 autosampler (CTC Analytics AG, Zwingen, Switzerland). Headspace solid-phase
187 microextraction (HS-SPME) was performed on aqueous extracts (200 μL) in 2 mL of
188 saturated NaCl solution. Samples were incubated at 40 ºC for 40 min followed by a 1-min
189 extraction using a polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fibre (Supelco,
190 Bellefonte, PA) and desorption at 260 ºC for 5 min. The oven temperature was 40 ºC (held
191 for 5 min), 40–200 ºC at 4 ºC/min, then to 250 ºC at 8 ºC/min, held for 5 min. Helium was
192 used as the carrier gas at a flow rate of 1 mL/min. The transfer line was held at 250 °C.
193 The mass spectrometer was operated at an ionisation voltage of 70 eV and an ion source
194 temperature of 250 °C. The volatile compounds were identified by comparing their mass
195 spectra with spectral data from the National Institute of Standards and Technology 2008
196 library as well as retention indices published in the literature (Ganeko et al., 2008; Giri,
197 Osako & Ohshima, 2010; pherobase. org). Relative retention indices were determined by
198 injection onto the column of a solution containing a series of *n*-alkanes (C7–C30, saturated
199 alkanes (1,000 μg/mL in hexane) Sigma-Aldrich) using the same temperature programmed
200 run as described above. Quantification of selected compounds was carried out using external
201 calibration curves.

202 ***2.4. Statistics***

203 Analysis of variance (ANOVA) was carried out on the quantitative data for each compound
204 identified in the GC-MS analysis of volatiles (thermal degradation and Maillard reaction) and
205 also on data for the fatty acids after a test for normality assessment. Principal component

analysis (SPSS Inc., Chicago, IL) was applied to differentiate the Maillard-FPHs based on their volatile compounds.

3. Results and discussion

3.1. Fish powder composition

Table 1 illustrates the chemical composition of fish powder. The obtained composition is similar to that reported by other fish powders when different fish were used as substrate to prepare the subsequent powders (Ghorbelet al., 2005). The most abundant fatty acids found in fish powder were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and myristic acid (C14:0). Similar results were observed from fish oil with the most abundant fatty acids being palmitic acid (C16:0), stearic acid (C18:0), myristic acid (C14:0) and oleic acid (C18:1). However, a much larger amount of eicosapentaenoic acid (C20:5) was observed in fish oil in comparison to the fish powder (Table 1). Linolenic acid C18:3 was not present in the fish powder while a considerable amount (3.8 ± 0.6) was found in the fish oil.

A total of 14 amino acids were identified and quantified in the samples. As expected, only a few free amino acids were present in the un-hydrolysed fish powder, including alanine, glycine, leucine, asparagine and glutamic acid, this being the most abundant. In comparison, FPH contained 14 free amino acids; leucine, glutamic acid, lysine and alanine increased their concentrations 2–5 fold (365–675 µg/g) while glycine, valine, isoleucine, threonine, serine, proline, asparagine, methionine, phenylalanine and remaining amino acids that had not been detected in the un-hydrolysed fish powder were found in small concentrations. Other authors such as Sathivel, et al., (2003) and Shahidi, Han, & Synowiecki, (1995) found similar results although values might differ as different enzymes and raw materials were utilised across studies.

3.2. Thermal degradation of EPA and DHA

In order to better understand the effect of EPA and DHA added to model reaction systems on flavour formation, kinetic studies of the pure compounds were conducted at 110 ºC. Although both EPA and DHA followed a similar pattern (Figures 1 and 2), with a sharp decline in their concentration, circa 70% between 0–15 minutes of heating, EPA seemed to follow a slower degradation process compared to DHA. Figures 1 and 2 illustrate the concentration (%) of 17 volatile compounds, associated with the thermal degradation of both fatty acids (Table 2).

During heating of both fatty acids (EPA and DHA), most aldehydes concentrations increased between 2 and 8-fold after 15 minutes of heating, remaining stable thereafter. This trend was coincident with the degradation of the corresponding fatty acid. In contrast, pentanal remained stable while other aldehydes showed a small decrease, remaining steady with time of heating. In both cases, the most abundant aldehyde was (*E,E*)-2,4-heptadienal, regardless of time of heating (132 ± 44 to 329 ± 122 µmol/g). It is known that aldehydes, significantly contribute to the overall aroma of cooked fish/seafood due to their low threshold values, and they derive from the degradation of fatty acids and triglycerides by autoxidation and subsequent formation of hydroperoxides (Ganeko et al., 2008; Hammer & Schieberle, 2013; Varlet, Prost & Serot, 2007). Heating increases the hydroperoxide formation by autoxidation of the unsaturated double bonds found in large amounts in polyunsaturated fatty acids (DHA and EPA). In our case, the sharp increase in some aldehydes such as (*E,E*)-2,4-heptadienal and (*Z*)-4-heptenal suggests that they were the main oxidation products from DHA and EPA thermal degradation. In fact, in a previous study, an increase in (*E,E*)-2,4-heptadienal (0.212 ± 0.015 to 2.0 ± 0.9 µg/mL) was reported when fish powder hydrolysates produced by different commercial enzymes were heated, that increase being higher in samples containing fish oil (Peinado et al., 2016). This is in agreement with several authors, such as Decker,

Elias, and McClements, (2010), and Parker, Elmore and Methven (2014), who reported 2,4-heptadienal as a degradation product of linolenic acid.

Regarding alcohols, the biggest differences were those reported by (*E*)-3-hexen-1-ol and 1-octen-3-ol, especially in DHA-containing samples. Branched-chain alcohols are formed by secondary decomposition of hydroperoxides of the *n*–3 and *n*–6 polyunsaturated fatty acids (Giri, Osako & Ohshima, 2010). Although, alcohols have relatively high odour threshold values, unsaturated alcohols such as 1-octen-3-ol, with usually lower threshold values, are expected to have a higher impact on the overall aroma (Kawai & Sakaguchi, 1996; Selli & Cayhan, 2009).

Finally, the acids found such as acetic, propanoic, butanoic, pentanoic and hexanoic with relatively low threshold values (Table 2), are generally fermentation products in several fish products, and they can derive from the breakdown of fatty acid chains but also from the Maillard reaction (Giri, Osako & Ohshima, 2010; Montel, Masson, & Talon, 1998).

3.3. Development of aromas in the Maillard Reaction model systems

The development of characteristic volatile compounds related to fish aroma has been previously studied in detail (Grigorakis, Taylor, & Alexis, 2003; Varlet, Prost & Serot, 2007; Wong, Abdul & Mohamed, 2008). To better understand the relationship between samples and their composition in terms of aroma compounds, a principal component analysis (PCA) was carried out (Fig. 3). The first two components explain 74.6% (PC1: 47.0% and PC2: 27.6%) of the total variance. In the plot, proximity between samples indicates similarities in terms of volatile release, while proximity between aromas indicates similarities in their concentrations and distribution amongst different samples. The first two principal components differentiate between samples without addition of fish oil, bottom-left side of the plot, samples containing fish oil, top-middle part, and samples containing either EPA or DHA, bottom-right side of the plot. EPA and DHA samples were richer in components associated with fish aroma, such as

279 1-penten-3-ol, (*E*)-3-hexen-1-ol, (*Z*)-4-heptenal, or (*E,E*)-2,4-heptadienal, as well as some of
280 the Maillard-derived pyrazines. Dimethyl pyrazines were associated with FPH without
281 addition of EPA and DHA, or FPH incorporating fish oil, while most of the aldehydes were
282 present in higher concentrations in samples containing fish oil.
283 A general increase in the concentration of aldehydes in the Maillard reaction of model
284 systems with added oils was observed (table 3). This increase could be mainly attributed to
285 lipid oxidation of the respective fatty acids. Linoleic acid, in particular, a fish oil component
286 and a precursor of hexanal (Varlet, Prost & Serot, 2007), was not detected in FPH while, as
287 expected, a large increase in hexanal was observed in samples containing fish oil (from 230
288 to 34,320 nmol/g). Aldehydes octanal and nonanal increased in samples containing FO (Table
289 3); they might possibly derive from the high amounts of oleic acid present in both FPH and
290 fish oil (Table 1) (Varlet et al., 2007). Specifically, (*Z*)-4-heptenal and (*E,E*)-2,4-heptadienal
291 concentration was greater in samples containing EPA and DHA compared to samples
292 containing fish oil. Varlet et al. (2007), reported that omega-3 PUFAs (such as EPA and
293 DHA) are the leading precursor for the development of oxidation products such as 4-heptenal
294 and 2,4-heptadienal, that derive mainly from the oxidation of linoleic and linolenic acid.
295 Aldehydes, specifically Strecker degradation derived, might impart nutty/malty nuances to
296 the product while heptanal, octanal or nonanal might impart a more characteristic fishy
297 flavour (Caprino et al., 2008; Giri, Osako & Ohshima, 2010; Selli & Cayhan, 2009).
298 Ketones together with aldehydes are the main products of lipid autoxidation of fatty acids or
299 the auto-oxidation of unsaturated fatty acids *via* hydroperoxides (Girard & Durance, 2000;
300 Giri et al., 2010). In addition, they can also be produced as a secondary products from the
301 Strecker reaction in Maillard reaction systems (Giri et al., 2010). In this study a ~2-fold
302 increase was observed in the concertation of 3-octen-2-one in those samples containing fish
303 oil, suggesting it as a product from the oxidation of fatty acids other than EPA or DHA. In

304 contrast, significantly large increases were observed in 2-furyl methyl ketone with EPA and
305 DHA addition, suggesting them as its precursors.
306 Similar to aldehydes and ketones, alcohols may be formed by secondary decomposition of
307 hydroperoxides of fatty acids (Giri et al., 2010). However, they have also been attributed to
308 enzymic peroxidation of *n*–3 and *n*–6 polyunsaturated fatty acids. In fact, an important
309 increase in 1-penten-3-ol was observed in samples containing EPA and DHA, while increases
310 in (E)-3-hexen-1-ol and 4-hepten-1-ol were also observed. It is important to highlight, that as
311 mentioned before, unsaturated alcohols, such as 1-penten-3-ol, might have a greater impact
312 on the overall aroma (Kawai & Sakaguchi, 1996; Selli & Cayhan, 2009).
313 Pyrazines are characteristic compounds derived from the Maillard reaction imparting
314 amongst other roasted and nutty flavours (Giri et al., 2010; Taylor & Mottram, 1990). Table 3
315 illustrates a slight increase in the concentration of pyrazines compared to the control (no oils
316 added), which implies that the addition of lipid oxidation products did not significantly
317 contribute. In some cases a significant decline was observed for some individual compounds,
318 such as the dimethyl pyrazines with EPA and DHA addition; while it remained stable when
319 fish oil was added (table 3).
320 ***3.4 Sensory evaluation***
321 Sensory differences in aroma perception between FPH-Maillard reaction model systems
322 containing fish oil (FPHs-Mr-FO), EPA (FPHs-Mr-EPA) or DHA (FPHs-Mr-DHA) were
323 assessed using triangle tests. A minimum correct number of responses ($p < 0.05$ for 21
324 assessors) was established as 12 (Meilgaard et al., 2007).
325 Panellists were only able to differentiate between FPHs-Mr-FO and FPHs-Mr-EPA (15
326 correct responses). In fact, there were significant differences ($p < 0.05$) between the pH of
327 FPHs-Mr-FO and FPHs-Mr-EPA (Table 3). The addition of EPA led to samples with a more
328 acidic pH when compared to samples with added DHA or fish oil, which was attributed to the

329 formation of lower levels of propanoic acid, butanoic acid and pentanoic acid (Figures 1 and
330 2).
331 In addition, sensory results established that FPHs-Mr-FO was the singularly most preferred
332 sample among panellists. The most probable cause of these findings may be, once again,
333 down to the less acidic content of that sample together with the volatile profile. Generally, the
334 aroma of the FO fortified samples included less of the aliphatic aldehydes, which are prone to
335 produce fishy or rancid type odours, such as (*Z*)-4-heptenal, hexanal and (*E,E*)-2,4-
336 heptadienal. Similarly, the concentration of 1-penten-3-ol was smaller compared to samples
337 with EPA and DHA addition, while the concentration of pyrazines, associated with nutty,
338 sweet, baked and roasted aromas, and generally considered more appealing (Ganeko et al.,
339 2008b; Giri et al., 2010; Varlet et al., 2007) was higher in the FPHs-Mr-FO.
340

341 **4. Conclusions**

342 Heating of unsaturated fatty acids, EPA and DHA, led to characteristic oxidation products
343 such as hexanal, (*Z*)-4-heptenal and (*E,E*)-2,4-heptadienal. Alcohol formation, however,
344 remained generally stable, with the exception of 1-penten-3-ol, which decreased with heating
345 time. The addition of fish oil or EPA and DHA to fish-powder-based Maillard reaction
346 systems had a considerable impact on the characteristic volatile compounds associated with
347 fish aroma. It enhanced the formation of seafood and cooked fish character impact volatiles
348 derived from lipid oxidation, such as hexanal, (*Z*)-4-heptenal, (*E,E*)-2,4-heptadienal and 1-
349 penten-3-ol. The addition of oil did not have a considerable impact on the majority of
350 characteristic compounds derived from the Maillard reaction, such as pyrazines and some
351 aldehydes. Furthermore, sensory evaluation illustrated that only FPHs-Maillard reaction
352 model systems containing FO and EPA could be differentiated, FO fortified samples being
353 the most preferred in comparison to EPA and DHA fortified samples.

354 Future work involving addition of the flavouring compounds to specific foods together with
355 nutritional and sensory evaluation is suggested to investigate the acceptability of seafood-
356 derived fish-like flavouring formulations.

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445 **Figure Caption**

446 **Figure 1**. DHA heating at 110 ºC for 90 minutes. Evolution of docosahexaenoic acid (DHA),

447 and volatiles relseased expressed as % concentration.

448

449 **Figure 2**. EPA heating at 110 ºC for 90 minutes. Evolution of eicosapentaenoic acid (EPA),

450 and volatiles released expressed as % concentration.

451

452 **Figure 3**. Biplot for the seven selected sample types (PC 1, 47.0%; PC 2, 27.6%).

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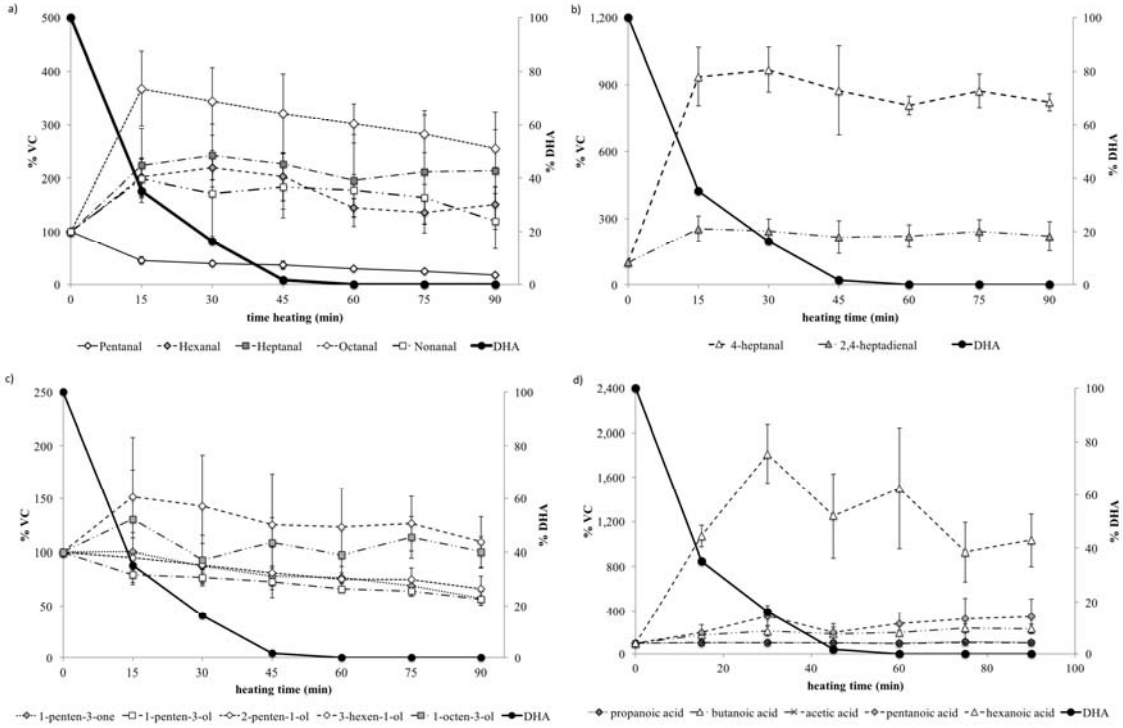

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460 Fig. 1

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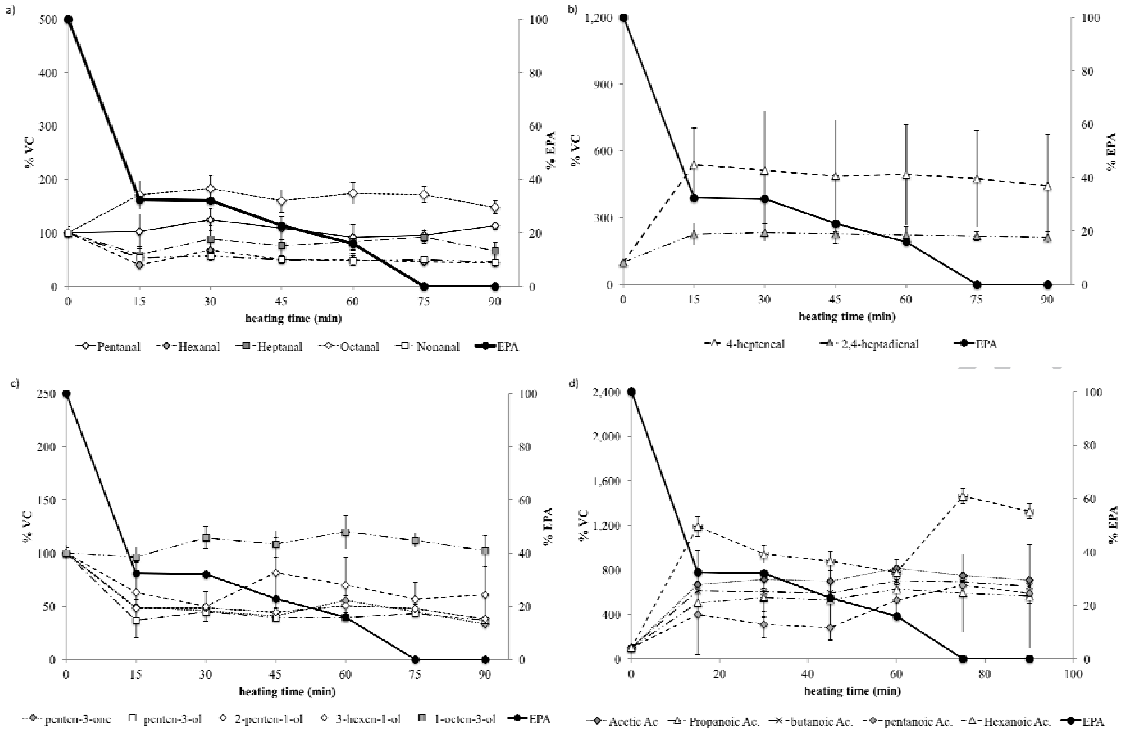

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466 Fig. 2

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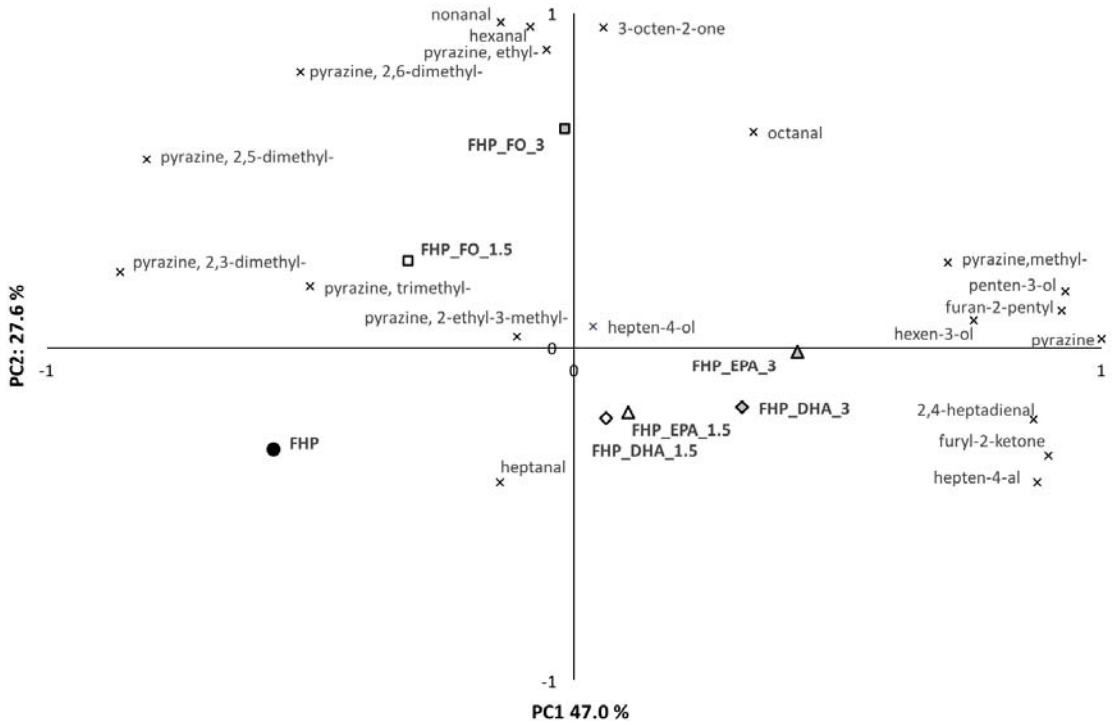

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471 Fig. 3

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Table 1: Description of commercial enzyme used for the fish powder hydrolysis. Characterization of fish powder (ash, moisture, fat, protein, carbohydrates (g/100 g)). Fatty acids composition of powder and fish oil (g/100 g total fat). Free amino acids composition of fish powder and fish powder hydrolysed. (n = 3).

enzyme characteristics*

enzyme	activity	optimum pH	Optimum T[a]
Flavopro 737	casein peptidase	5.5–7.5	45–55

fish powder composition (g/100 g)

x[w]	ash	protein	fat[a]	carbohydrates
4.67 ± 0.16	22.4 ± 0.3	60.3 ± 0.6	1.5 ± 0.4	11.1 ± 0.70

fat composition (g/100 g total fat)

	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	C20:5	C22:6
fish powder fat[a]	4.3 ± 0.5	47.0 ± 1.3	16.3 ± 0.5	15.3 ± 0.6	0.40 ± 0.03	-	0.67 ± 0.09	0.55 ± 0.02
fish oil	10.4 ± 0.3	29.1 ± 0.8	16.7 ± 1.8	8.2 ± 0.5	2.8 ± 0.3	3.8 ± 0.6	11.2 ± 0.7	6.5 ± 0.3

free amino acids composition (μg/g)

	ALA	GLY	VAL	LEU	ILE	THR	SER	PRO	ASP	MET	GLU	PHE	LYS	Other
fish powder	235±4	165±51		141±8					86±3		529±195			26±10
hydrolysed fish powder**	635±4	114±3	224±15	675±15	227±5	194±21	178±42	60±3	84±2	125±8	470±31	255±31	369±80	138±3

*Biocatalysts, Ltd.

**Production of fish powder hydrolysate (FPH): fish powder (100 g/L in water) + commercial enzymes (Flavopro), heated overnight (15 h) at constant stirring (pH 6, and 60 ºC, enzyme (10–20 g/L).

Table 2. Retention times, retention index, Identification, odour thresholds and odour descriptors of volatile compounds identify after EPA and DHA heating as well as in the fish powder hydrolisates after heating them with or without fish oil (n=3).

	RT	RI	Identification	Odour threshold[E]	odour description
Aldehydes					
hexanal[1,2]	13.5	1162	MS, RI Std	4.5–5[D]	fishy, grass[a,b,c]
heptanal[1,2]	17.5	1184	MS, RI Std	3[D]	citrus like[a], dry fish [b] green, fatty, solvent, smoky, rancid[c]
(*Z*)-4-heptenal[1,2]	19.7	1242	MS, RI Std	0.8–10[D]	boiled potato, creamy, sweet, biscuit-like, fishy[a,b,c,e]
octanal[1,2]	21.4	1290	MS, RI Std	0.7[D]	lemon, stew-like, boiled meat-like, rancid, soapy, citrus, green, flower, fruit, orange[a,b,c]
2-octenal[1,2]	26.4	1432	MS, RI	3[D]	aromatic, oxidised oil-like[a], green[c]
nonanal[1,2]	25.1	1357	MS, RI Std	1[D]	gravy, green, fruity, gas, chlorine, floral, waxy, sweet, melon, soapy, fatty, citrus fruit[a,b,c]
(*E,E*)-2,4-heptadienal[1,2]	27.5	1498	MS, RI Std	15–95[A]	deep fried, fatty, fishy[a,c],
Alcohols					
1-penten-3-ol[1,2]	16.4	1167	MS, RI Std	350–400 [A,D]	burnt, meaty[a,] paint-like chemical-like[b] grassy-green[c]
(*E*)-2-penten-1-ol[1,2]	22.4	1318	MS, RI Std	–	green, plastic[a]
(*E*)-3-hexen-1-ol[1,2]	24.7	1380	MS, RI Std	70 [D]	green[c]
1-octen-3-ol[1,2]	26.8	1444	MS, RI Std	1	fishy, grassy[a] garlic, mushroom, spicy, rubbery, carrots, herbal[c]
4-hepten-1-ol[1,2]	28.6	1468	MS, RI Std	–	fishy [c]
1-octanol[2]	30.3	1512	MS, RI Std	110–130 [D]	fatty, green[a]
Ketones					
1-penten-3-one[1,2]	11.3	993	MS, RI	1–1.3[D]	pungent, fish-like, rotten, fruity, plastic, leather[a,b,c]
3-octen-2-one[2]	25.6	1364	MS, RI Std	–	fatty, spicy[c]
Acids and esters					
acetic acid[1,2]	27.4	1426	MS, RI Std	30–150[D]	sour, vinegar, pungent[c]
propanoic acid [1,2]	30.1	1507	MS, RI Std	20000 [D]	pungent, rancid, soy, fruity, cheesy[c]
butanoic acid butyl ester[1,2]	32.7	1589	MS, RI Std	100[D]	fresh, sweet, fruity[c]
pentanoic acid[1,2]	35.9	1694	MS, RI Std	–	sweaty, pungent, sour, cheesy, beefy[c]
hexanoic acid[1,2]	38.9	1797	MS, RI Std	3000 [D]	sweaty, pungent, cheesy, goat-like, rancid[c]
Furans					
furan[2]	6.4	802	MS, RI Std	–	
2-pentyl furan[2]	19.2	1199	MS, RI Std	6 [A,D]	buttery, green bean-like[a,c]
Pyrazines					
pyrazine[2]	18.6	1183	MS, RI Std	—	pungent, sweet, corn, roasted hazelnut[a,c]
methylpyrazine[2]	20.6	1235	MS, RI Std	60–105,000[D]	nutty, roasty, cocoa, chocolate[c]
2,5-dimethyl pyrazine[2]	22.6	1289	MS, RI Std	800–1,800[D]	cocoa, roasted nut, roast beef, woody[c]
2,6-dimethyl pyrazine[2]	22.8	1295	MS, RI Std	200–9,000[D]	baked potato, nutty, fruity[c]

ethylpyrazine[2]	23.0	1302	MS, RI Std	6,000–22,000[D]	sweaty, nutty, peanut butter, musty[c]
2,3-dimethylpyrazine[2]	23.4	1313	MS, RI Std	2,500–35,000[D]	nutty, musty[c]
trimethylpyrazine[2]	25.3	1366	MS, RI Std	–	burnt, bread[a]

[A] Giri et al., 2010, [B] Ganeko et al., 2008, [C] pherobase.org, [D] http://www.leffingwell.com/odorthre.htm.
[E] Odour thresholds in water (μg/L).
[1] Volatiles identified in DHA and EPA heated samples; [2] Volatiles identified in fish powder hydrolysed samples.

Table 3. Volatile compounds associated with fish-like aroma identified in heated fish powder hydrolysate combined with EPA, DHA and FO (1.5 & 3 g/100g) expressed as nmol/g of FPH. The pH and absorbance readings of EPA, DHA and samples used for sensory evaluation (n = 3).

	Control	FPH+DHA		FPH + EPA		FPH + FO	
	FPH	**1.5 g/100 g**	**3 g/100 g**	**1.5 g/100g**	**3 g/100 g**	**1.5 g/100 g**	**3 g/100 g**
Aldehydes							
hexanal	230 ± 31 ^{a}	990 ± 6 ^{ab}	1,501 ± 523 ^{ab}	2,937 ± 1,046 ^{ab}	6,072 ± 2,638 ^{ab}	32,918 ± 10,638 ^{b}	34,320 ± 15,811 ^{c}
heptanal	11 ± 0.6 ^{a}	25 ± 2 ^{a}	39 ± 11 ^{a}	26 ± 4 ^{a}	73 ± 20 ^{a}	989 ± 67 ^{b}	446 ± 122 ^{c}
(*Z*)-4-heptenal	20 ± 10 ^{ab}	402 ± 33 ^{a}	703 ± 140 ^{c}	456 ± 69 ^{ab}	914± 187 ^{ab}	266 ± 4 ^{ab}	38 ± 1 ^{ab}
octanal	15 ± 5 ^{a}	18 ± 0.9 ^{ab}	25 ± 13 ^{ab}	19 ± 5 ^{a}	56 ± 34 ^{c}	172 ± 84 ^{ab}	39 ± 22 ^{ab}
nonanal	24 ± 1 ^{ab}	23 ± 3 ^{a}	26 ± 4 ^{b}	24 ± 0.3 ^{ab}	34 ± 6 ^{ab}	57 ± 5 ^{ab}	75 ± 7 ^{ab}
(*E,E*)-2,4-heptadienal	35 ± 0.1 ^{a}	884 ± 153 ^{b}	2,358 ± 567 ^{c}	909 ± 27 ^{b}	2,117 ± 164 ^{c}	236 ± 47 ^{a}	529 ± 49 ^{ab}
Ketones							
3-octen-2-one	4 ± 0.3 ^{a}	4 ± 0.1 ^{a}	4 ± 0.2 ^{a}	4 ± 0.2 ^{a}	5 ± 0.4 ^{a}	6 ± 0.1 ^{a}	8 ± 2 ^{b}
2-furyl methyl ketone	0 ^{a}	737 ± 18 ^{d}	960 ± 56 ^{e}	1,066 ± 17 ^{f}	1,355 ± 60 ^{g}	113 ± 0.08 ^{b}	265 ± 18 ^{c}
Alcohols							
1- penten-3-ol	1 ± 6 ^{a}	7,090 ± 276 ^{c}	8,397 ± 649 ^{d}	7,575 ± 212 ^{c}	9,048 ± 513 ^{e}	5,055 ± 225 ^{b}	6,670 ± 282 ^{c}
(E)-3- hexen-1-ol	12 ^{a}	14 ± 2 ^{b}	17 ± 3 ^{b}	13 ± 2 ^{ab}	15 ± 3 ^{b}	12 ± 2 ^{ab}	12 ± 0.5 ^{ab}
4-hepten-1-ol	15 ± 1 ^{ab}	12 ± 0.3 ^{ab}	18 ± 8 ^{b}	12 ± 0.3 ^{ab}	13 ± 0.4 ^{ab}	14 ± 0.2 ^{ab}	15 ± 0.5 ^{ab}
Pyrazines							
pyrazine	48 ± 7 ^{a}	186 ± 4 ^{bc}	397 ± 0.1 ^{e}	248 ± 129 ^{cd}	363 ± 54 ^{de}	108 ± 70 ^{ab}	238 ± 32 ^{c}
methylpyrazine	565 ± 101 ^{ab}	1,108 ± 48 ^{abc}	2,006 ± 432 ^{c}	1,185 ± 101 ^{abc}	1,544 ± 22 ^{bc}	1,524 ^{a}	1,909 ± 88 ^{c}
2,3-dimethylpyrazine	263 ± 9 ^{d}	39 ±1 ^{a}	42 ± 15 ^{a}	22 ± 1 ^{a}	26 ± 5 ^{a}	138 ± 20 ^{b}	169 ± 19 ^{c}
2,5-dimethylpyrazine	910 ± 26 ^{d}	309 ± 54 ^{c}	158 ± 40 ^{a}	273 ± 34 ^{b}	164 ± 47 ^{ab}	994 ± 30 ^{d}	991 ± 59 ^{d}
2,6-dimethylpyrazine	251 ± 9 ^{c}	154 ± 3 ^{b}	52 ± 13 ^{a}	118 ± 5 ^{b}	118 ± 5 ^{b}	581 ± 19 ^{d}	602 ± 68 ^{d}
trimethylpyrazine	329 ± 131 ^{c}	168 ± 11 ^{a}	45 ± 31 ^{a}	79 ± 8 ^{a}	127 ± 36 ^{a}	73 ± 19 ^{a}	284 ± 19 ^{bc}
2-ethyl-3-methylpyrazine	5.7 ± 1 ^{a}	6 ± 0.2 ^{a}	5 ± 0.2 ^{a}	5 ± 0.2 ^{a}	5 ± 0.3 ^{a}	5 ± 0.3 ^{a}	6 ± 0.6 ^{a}
pH	nd	5.222 ± 0.107 (ab)	nd	5.128 ± 0.076 (a)	nd	6.121 ± 0.108 (b)	nd
Abs^{420nm}	nd	1.388 ± 0.124 (a)	nd	1.336 ± 0.180 (a)	nd	1.154 ± 0.286 (a)	nd

Development of aroma: 1. Aliquots of FPHs (0.2 mL) mixed with a glucose solution (0.05 mL, 100 mM) and glycerol (200 µL); 2. Addition of oil (eicosapentanoic acid, docosahexanoic acid or fish oil, 1.5 or 3 g/100 g); 3. Samples heated at 110 °C for 30 minutes.
nd, not determined.
(a,b,c,d,e,f,g: homogenous groups obtained from the statistical analysis (ANOVA) for the different samples).

Highlights

- Thermal degradation of EPA and DHA was analysed for fatty acids and volatiles
- Aroma of fish powder systems with addition of ω-fatty acids was investigated.
- Hexanal, 2,4-heptadienal and 4-heptenal were found after heating EPA and DHA
- EPA and DHA added to model systems increased the fish aroma compounds
- Panellists only differentiated samples fortified with fish oil and EPA.