New species for EU aquaculture

Deliverable Report

Deliverable No:	D28.5		**Delivery Month:**	55
Deliverable Title	Report on results of quality evaluation study on basic quality characteristics of the developed products.			
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Objective: The objective of this Deliverable he report will refer: to the total proximate composition of the products (protein, lipid moisture, inorganic content and carbohydrates), the energy contents of the selected products and the quantitative nutritional value in aspects of fatty acids.

Description: All products developed in D.28.4 were evaluated for their physicochemical properties and their chemical quality.

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1. Introduction

Processing is known to cause alterations both in the nutritional value and sensory profiles of seafood (Sampels, 2015). Thus, it is important to evaluate the quality of seafood products that have undergone processing, since the benefits of consuming those can be altered. Specifically, it can drastically change the nutritional value of the seafood, particularly when referring to highly processed products that involve processes, which can lead to chemical- or heat-induced changes and added materials (i.e. oil or animal fats) (Sampels, 2015). Within these frames, we have examined the nutritional values of all processed products and

assessed both changes that proximate composition and fatty acids undergone due to processing / product formulation and their general physicochemical quality in order to make comparisons among them.

This is done in order to give potential fish farmers and other potential chain partners insights in the nutritional values of the generated products. This is furthermore dictated by the need of the presence of nutritional information on the respective food packages. Besides, this information can be used for further optimization of the products' generation processes (e.g. potential changes of the formulation to improve the nutritional value).

2. Materials and Methods

Basic analysis of nutritional values, namely the proximate composition and the fatty acid composition, has taken place for both the unprocessed products, i.e. the fillets of the species used for processing and the processed products obtained through deliverable D28.4.

Prior to analyses samples were homogenized down to ground level. However in the case of fillet in olive oil some degree heterogeneity was unavoidable. All analyses have been conducted in five samples per product (n=5) to ensure representative values and eliminate possible effect of sample heterogeneities. The proximate and fatty acid composition of unprocessed raw tissues (fish fillets) was also conducted in triplicate (n=3). The pH was determined using a conventional pH-meter on 10 g of ground sample in 100 mL of distilled water. The water activity (aw) was determined in the homogenate by means of a water activity meter (AquaLab).

The proximate composition (protein, fat, moisture and ash) of physical prototypes was determined as described in the AOAC Official Methods (AOAC, 2005). Briefly, moisture was determined gravimetrically after drying the samples in an oven at 103 °C ± 2 °C. Ash content was measured gravimetrically after drying and incinerating the samples in a muffle furnace at 550 °C to obtain white ashes. The protein content was determined based on the Dumas combustion method (N content x 6.25). The fat content was measured after acid hydrolysis with HCl in a boiling bath. The residue was filtrated and dried and subsequently the lipid content was extracted with ether by means of a Soxhlet extractor.

The fatty acids were extracted and esterified by a direct trans-esterification process in methanol-benzene (4:1) solution with acetyl chloride (Lepage & Roy, 1986). Esters were then analysed in a gas chromatograph – flame ionization detector (GC-FID) (Varian 3300, Walnut Creek, CA, USA) equipped with a flexible fused silica Megabore column (30 m x 0.32 mm x 1 μm) with a bonded stationary phase of CP-WAX. Helium (purity 99.999%) was used as the carrier gas with a flow rate of 5 mL/min. Fatty acid identification was performed according to Fountoulaki, Alexis, Nengas, and Venou (2003). Carbohydrates were calculated by difference whereas the sugar content was determined after samples clarification with Carrez reagents. Identification and quantification was performed by means of High Performance Liquid Chromatograph equipped with a refractive index detector. The salt content (Na content x 2.5) was determined by means of an Atomic Absorption Spectroscopy in sample extracts obtained after mineralization with nitric acid and hydrogen peroxide in a microwave digester. The gross energy content was determined in the freeze-dried samples, by total burning of samples by the means of an IKA C4000 (IKA Analysentechnik, Heitersheim, Germany) adiabatic calorimeter.

Cholesterol, phytosterols, and squalene were determined in aliquots of the Folch extract (containing 20-30 mg of lipids) by GC/FID after hot saponification followed by methylation with BF_3/MeOH and silylation with BSTFA. For preparing the trimethyl-silyl ether (TMS) derivatives of cholesterol, appropriate amounts of the hexane extracts were transferred to autosampler vials and evaporated to dryness under nitrogen, 0.25 mL of BSTFA containing 1% v/v trimethylchlorosilane was added, and the vials were sealed and heated for 20 min at 70 °C. An Agilent HP series GC 6890 (Avondale, PA) equipped with a flame ionization detector, split-splitless injector, and HP 6890 autosampler was employed. One microliter of each sample and standards were injected in the gas chromatograph at a split ratio of 20:1. Separation of squalene and sterols was achieved on a SGE (Melbourne, Australia) BPX50 capillary column (30 m long, 0.25 mm internal diameter)

coated with a 0.25 μm thick film of 50% PH phenyl-methylpolysiloxane. The identification and quantification of squalene, cholesterol, β-sitosterol, and 5Δ-avenasterol were performed by using standard solutions and by constructing the respective standard curves using 5-R-cholestane as the internal standard. The peak corresponding to Δ5-avenasterol was recognized as the first major peak eluting after β-sitosterol in chromatograms obtained from virgin sesame oil, which has been reported to be relatively rich in the specific phytosterol and was quantified according to β-sitosterol reference curve.

Proximate composition and fatty acid results are presented as means and the coefficient of variation (CV) corresponded to the variation among the different samplings performed. Statistical analysis was performed separately for all products, including the comparison of products compositions with those of the respective raw fillets. Determination of significant differences ($P<0.05$) was achieved by 1-way ANOVA using the *lsmeans* package (Length, 2016), in R version 3.4.2 (R, 2016). Post hoc analysis of the results was achieved by the Tukey test.

3. Results and Discussion

Interspecies variations in the proximate composition of the fish raw material (the unprocessed fillets of the four studied species) used for obtaining the processing are included in **Table 1**. The results indicated that greater amberjack exhibited the most distinctive proximate composition profile amongst the other species, which was connected to the significantly higher fat content, when compared to those. The reverse moisture /fat relationship (meaning increase of fat with respective decrease of moisture content) as well as the generally stable protein has been also demonstrated within these results.

Table 1: Mean values and coefficient of variation (CV) of fillet proximate composition parameters of wreckfish, greater amberjack, grey mullet, meagre and pikeperch (n=5). Different letters in the same row indicate statistically significant differences ($P <0.05$) between the mean values of each species.

Proximate composition (%)	Greater amberjack		Grey mullet		Meagre		Pikeperch	
	Mean	CV	Mean	CV	Mean	CV	Mean	CV
Moisture	69.46b	0.04	76.53a	0.01	77.17a	0.04	76.58a	0.01
Protein	22.21a	0.07	21.37ab	0.03	20.65ab	0.09	21.80a	0.03
Fat	6.28a	0.64	0.58b	0.44	0.52b	0.36	0.06b	0.30
Ash	1.44a	0.01	1.27ab	0.01	1.35ab	0.07	1.30ab	0.01

Moreover, to complete the knowledge on the nutritional value of the raw fish tissues, the fatty acid composition of the four examined species' fillets are also included (**Table 2**). Meagre and greater amberjack exhibited an expected fatty acid quality with regards to the rearing origin (reared specimens). The higher ratio of PUFA and n-3 fatty acids of grey mullet, when compared to meagre and greater amberjack, was expected, since the specimens examined received natural feeding from the environment (not commercial feeds), whereas pikeperch specimens originated from fresh water intensive farming (France) and were fed a commercial extruded feed, their fatty acid composition resembled wild specimens, exhibiting the highest proportions of PUFA and n-3 fatty acids as well as the highest n-3/n-6 ratio.

Table 2: Mean values and coefficient of variation (CV) of the fillet main fatty acid groups (% of total fatty acids) of greater amberjack, grey mullet, meagre and pikeperch (n=3). Post-hoc analysis by Tukey test in R software. Different letters in the same row indicate statistically significant differences (P <0.05) between the mean values of each species.

Fatty acid groups	Greater amberjack		Grey mullet		Meagre		Pikeperch	
	Mean	CV	Mean	CV	Mean	CV	Mean	CV
SFA	22.7d	15	33.8ab	8	27cd	1	33.2a	5
MUFA	45.6a	2	15.9c	4	28.7b	11	13.8c	15
PUFA	31.7d	9	47.9b	4	44.4bc	7	53.1a	3
n-9	38.7a	3	8.16d	14	22.5b	12	9.89d	17
n-6	17.4b	3	12.2c	19	26.4a	5	6.03d	8
n-3	13.6c	17	35b	3	17.2c	11	47.0a	5
18:2n-6	15.5b	2	2.15d	75	20.6a	4	4.00c	13
ARA	0.70c	6	8.28a	14	1.35c	13	2.03c	12
EPA	3.41d	6	12.8a	9	3.01d	2	7.78b	7
DHA	8.06d	36	17.6c	8	12.7c	15	38.6a	5
n-3/n-6	0.78c	16	2.94c	19	0.65c	7	7.86a	12

The basic physicochemical characteristics of the products, namely the pH and water activity (aw) appear in **Table 3**. With the exceptions of salad that exhibit an acidic pH, all other products are lightly acidic to neutral. The high water activity observed in burgers and fish pate together with their almost neutral pH makes these two products particularly prone to bacterial activity and therefore proper cold-chain preservation is essential since their complexity due to high processing degree makes difficult the detection of advanced spoilage unlike the other products where spoilage is more apparent (steak, salad) or they are less prone (fillets in olive oil).

Table 3. Physicochemical characteristics of the six generated products.

	Steak[1]	Pate[2]	Salad[3]	Burger[3]	Smoked fillets[4]	Fillets in Olive oil[4]
pH	5.90	6.69	4.2	6.45	5.90	5.90
aw	-	0.988	0.988	0.992	-	-

[1]Greater amberjack, [2] Pikeperch, [3] Meagre, [4] Grey mullet,

The proximate composition of the six generated products appears in **Table 4**. The key fatty acids and the fatty acid groups characterizing the products are presented in **Table 5**. The proximate and fatty acid composition of all products underwent changes from that of their respective raw fish fillets (changes presented in **Table 6**). The increase of fat percentage in the processed products can be justified due to the addition of fatty raw materials, during product formulation. Those were olive oil in the meagre salad and grey mullet fillets in oil, and Emmental cheese in meagre burger. Furthermore, it can also be attributed to salting or thermally-induced decrease of moisture, which applied for the fillets in olive oil and smoked fillets or cooking of burgers. The only product where increase in moisture was observed is the fish salad, due to the addition of salad leaves that contain high water. Similar composition alterations have been observed in numerous smoked and brined/marinated products (Lipato & Kapute, 2017; Ljubojević et al., 2016).

Protein contents changes depended on the formulation of products (**Table 6**). In products where other raw materials are included (salad, fish burgers, fillets in olive oil), protein was decreased. The decrease of protein in marinated fish, similar as the fillets in olive oil experienced, has been also confirmed in other studies (Yeannes & Casales, 2008). Opposite to the latter, other studies referring to marinated products found increase of protein; however, these changes and their differentiations can be associated with pre-treatment the products underwent, i.e. salting that causes dehydration (Mattioli et al., 2017; Sampels, 2015). The smoked fillet, on the other hand, was the only product that experienced an increase in its protein content, and this can be attributed to water loss due to the heat treatment it underwent, i.e. hot smoking (Ljubojević et al., 2016; Mattioli et al., 2017). It seems, nevertheless, that effects of hot smoking in proximate composition can be variable depending on smoking temperature, duration of prior storage of fillets, and pre-treatment like brining (Romotowska et al., 2016). Another characteristic proximate composition change is the increase in the inorganic content (ash) of both fish burgers and smoked fillets, which besides thermal removal of moisture, can be attributed due to the addition of salt as part of seasoning or salt-drying procedure and this has been confirmed in other marinated and hot-smoked species (Cieślik et al., 2017).

Table 4. Proximate composition and energy contents Mean (±CV) of the 6 generated products (n=5). Different letters in the same row indicate statistically significant differences (P <0.05) between the mean values of each species.

Proximate composition (/100g)	Steak[1]		Pate[2]		Salad[3]		Burger[3]		Smoked fillets[4]		Fillets in Olive oil[4]	
	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
Moisture	72.0b[5]	2	66.0c	1	79.8a	1	71.8b	<1	63.3c	4	57.3d	11
Fat	3.67c	38	14.9b	3	2.93c	8	4.82c	3	2.87c	24	21.85a	16
Protein	22.4a	4	17.6cd	3	13.0d	8	18.8bcd	1	27.4a	8	19.4bc	23
inorganic c	1.48d	9	1.58cd	2	0.8e	5	2.47b	2	4.82a	8	1.99bc	20
total CHS	0	0	0.02b	0	8.4	0	0.09	0	0	0	0	0
sugars	0	0	0	0	6.0	0	0	0	0	0	0	0
fibers	0	0	0	0	1.9	0	0	0	0	0	0	0
salt (mg)	0.29	0	824	8	295	0	503.8	12	-*	-	0.16	0
Energy (kcal)	116b	7.6	66.0d	1.1	83.3cd	5.8	119.3b	1	184.2ab	4.3	287.8a	11

[1]Greater amberjack, [2] Pikeperch, [3] Meagre, [4] Grey mullet, [5] * missing value

The fatty acid profile of the products was altered mainly due to the addition of specific exogenous fat sources, which were added to the initial fillets. Specifically, in fish burger, the SFA proportion increased drastically, due to the addition of Emmental cheese that is characterized by high SFA content (Domagała, Pluta-Kubica, & Pustkowiak, 2013). The increase in saturated fatty acids has generally observed in the fish burgers made from different fish species, and the degree of the increase depends on the formulation of the burger (Branciari et al., 2017). Although the burgers have been proposed as a mechanism for inducing EPA and DHA intake for modern consumers and especially children, this drawback of SFA increase has to be taken into account (Branciari et al., 2017). The big increase in n-9 and therefore in total MUFA for fish in olive oil is expected due to the olive oil fatty acid profile, rich in 18:1n9 (De Leonardis, 2014). The fact that no respective changes were found for fish salad may be attributed to the small proportion of added olive oil in this product. In smoked fillets, the source of change in fatty acids was less profound; the respective changes included reduction of EPA and ARA percentages, but also an increase in DHA and total n-3 PUFA. This can attributed to hot smoking, since grilling has also been shown to increase essential fatty acids through the loss of moisture (Costa et al., 2013). Other studies involving smoking of fish have shown variable changes in fatty acid composition, but may depend on the type of smoking that was applied during product formulation (Ljubojević et al., 2016; Mattioli et al., 2017; Strobel, Jahreis, & Kuhnt, 2012).

Table 5: Mean values and coefficient of variation (CV) of the processed products main fatty acid groups (% of total fatty acids) of fish steak, fish pate, fish salad, fish burger, smoked fish fillets and fillets in olive oil (n=5). Post-hoc analysis by Tukey test in R software. Different letters in the same row indicate statistically significant differences (P <0.05) between the mean values of each species.

Fatty acid groups	Steak[1]		Pate[2]		Salad[3]		Burger[3]		Smoked fillets[4]		Fillets in Olive oil[4]	
	Mean	CV	Mean	CV	Mean	CV	Mean	SD	Mean	CV	Mean	CV
SFA	23.61bc	1	12.2d	0	24.9b	20	41.9a	1.24	25.6b	2	17.8cd	6
MUFA	38.48b	5	29.6b	1	26.39b	87	35.4b	0.54	25.8b	10	70.4a	4
PUFA	37.91ab	6	57.45a	1	48.70a	37	22.71bc	0.73	48.60a	6	11.76c	18
n-9	31.04b	5	28.17b	2	23.19b	87	28.84b	0.48	13.27b	2	65.91a	6
n-6	11.74b	2	55.64a	2	35.09a	53	9.59b	0.28	9.68b	4	6.79b	2
n-3	24.96b	9	1.81d	8	13.13c	7	12.48c	0.42	37.55a	8	4.80d	42
18:2n-6	10.26b	3	55.28a	2	5.94bc	90	8.65bc	3	2.71c	29	6.12bc	4
ARA	0.86b	15	0.10b	9	0.31b	99	0.67b	0.02	4.27a	14	0.45b	11
EPA	5.68a	7	0.42c	6	1.19bc	61	2.25b	4	5.72a	19	0.91bc	51
DHA	12.40b	20	1.28c	10	8.46b	13	7.03bc	3	21.15a	22	1.94c	32
n-3/n-6	2.13b	9	0.03d	10	0.52d	77	1.30c	1	3.88a	8	0.71cd	43

[1]Greater amberjack, [2] Pikeperch, [3] Meagre, [4] Grey mullet

Table 6: Statistical changes of composition and main fatty acid groups in the processed products (and respective level of significance) when compared to respective raw fillets and the respective processed products in the proximate compositions.

	G. amberjack	pikeperch	meagre		grey mullet	
	steak	pate	salad	Burger	smoked fillets	fillets in olive oil
moisture	increase*	decrease*	increase**	decrease**	decrease**	decrease***
protein	-	decrease*	decrease**	decrease*	increase*	-
fat	decrease*	increase***	increase*	increase**	incease**	increase***
ash	-	increase*	decrease**	increase**	increase**	-
SFA	-	decrease**	-	increase***	decrease*	decrease*
MUFA	-	increase**	-	-	increase**	increase***
PUFA	decrease	-	-	decrease**	-	decrease***
n-9	decrease*	increase**	-	increase*	increase*	increase***
n-6	decrease*	increase***	increase*	decrease**	decrease*	decrease*
n-3	increase*	decrease***	decrease*	decrease*	decrease***	decrease***
18:2n-6	decrease	increase***	decrease**	decrease**	-	increase**
ARA	-	decrease**	decrease**	decrease*	decrease**	decrease***
EPA	increase*	decrease***	decrease**	decrease*	decrease*	decrease***
DHA	increase*	decrease***	decrease*	decrease*	increase**	decrease***
n-3/n-6	increase*	decrease***	-	increase*	-	decrease**

*P<0.05, **P<0.01, ***P<0.001

Besides fatty acids, the lipid quality of the products was evaluated through their sterol contents (**Table 7**). The findings of sterols were expected since squalene and 5Δ-avenasterol are of olive oil origin (Lou-Bonafonte et al., 2012), which is an additional material in fish salad and the fillets in olive oil, the b-sitosterol is a component of all plant oils (Wen-Sen et al., 2018) - therefore present in the 3 products that contain additional oil - and cholesterol is present in all animal lipids. The fact that the fish burger contains almost 40% more cholesterol than the rest of the products can be attributed to the addition of the Emmental cheese.

Table 7. Lipid quality of the 6 generated products (n=5) in aspects of sterol contents.

	Steak[1]		Pate[2]		Salad[3]		Burger[3]		Smoked fillets[4]		Fillets in Olive oil[4]	
(mg/100g)	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
squalene	0.04c	0.25	0.74	0.34	3.09b	0.29	0.09c	0.7	0.10c	0.8	72.0a	0.24
cholesterol	71.6b	0.15	67.5b	0.15	64.7b	0.04	104.8a	0.1	77.6b	0.16	49.0c	0.15
β-sitosterol	0	0	25.4a	0.19	2.31c	0.13	0	0	0	0	15.4b	0.10
Δ5 -avenasterol	0	0	0.68	0.82	0	0	0	0	0	0	0.96	0.14

[1]Great er amberjack, [2] Pikeperch, [3] Meagre, [4] Grey mullet,

4. Conclusions

Results indicated that processing had an effect on both the proximate composition and fatty quality of the products when compared to the raw fillet tissue. However, the effect depended on the processing method used as well as the inclusion of additional materials (such as olive oil) during the product formulation. Processing generally had a negative effect on nutritional quality reducing the proportion of essential fatty acids, i.e. EPA and DHA, of the majority of products when compared to the corresponding fish fillets.

The proximate composition and fatty acid quality varied in a great extent across the processed products. This was expected, since the initial variations of the species were furthermore altered by the different post-mortem processing (Sampels, 2015). Fillets in olive oil exhibited the most distinctive profile of all processed products, due to the addition of olive oil that resulted in increased fat content and proportion of n-9 and Monounsaturated Fatty Acids (MUFA) and respective reduction of PUFA proportion, when compared to the rest of the processed fish products.

In regard to the sterol contents, all products contained cholesterol, due to the presence of this particular molecule in the fish tissues that is the base of the products. High cholesterol due to the cheese presence was observed in the fish burgers. The olive oil-containing products showed presence of squalene and Δ5-avenasterol while in all plant-oil containing products β-sitosterol was found.

Regarding the nutritional composition of the species, it was altered by the effect of processing, which was more intense for products that required additional materials, i.e. olive oil for grey mullet's fillets in olive oil, and intense heat treatment, i.e. pikeperch pate, in their formulation. Considering that pate was also amongst the lowest rated products in terms of liking in all EU countries, an alternative product formulation should perhaps be considered.

5. References

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Deviations: In the DOW the description of this deliverable was mistakenly (by copy paste) written as the description of exactly the same as D28.6. The correct description is as following: "Report on results of quality evaluation study on basic quality characteristics of the developed products. The report will refer: to the total proximate composition of the products (protein, lipid moisture, inorganic content and carbohydrates), the energy contents of the selected products the quantitative nutritional value in aspects of fatty acids".

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