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USE OF ACID AND RE-ESTERIFIED VEGETABLE OILS IN FISH DIETS

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Clara Trullàs Huguet

SOTA LA DIRECCIÓ DELS DOCTORS:

Roser Sala Pallarés, Ramon Fontanillas Giralt i Alba Tres Oliver

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Roser Sala Pallarés, del Departament de Ciència Animal i dels Aliments de la Universitat Autònoma de Barcelona, **Ramon Fontanillas Giralt,** de Skretting Aquaculture Research Centre (ARC) i **Alba Tres Oliver**, del Departament de Nutrició i Bromatologia-XaRTA-INSA de la Facultat de Farmàcia de la Universitat de Barcelona,

Certifiquen:

Que la memòria titulada "**Use of acid and re-esterified vegetable oils in fish diets**", presentada per Clara Trullàs Huguet amb la finalitat d'optar al grau de Doctor en Veterinària, ha estat realitzada sota la seva direcció i, considerant-la acabada, autoritzen la seva presentació perquè sigui jutjada per la comissió corresponent.

I perquè consti als efectes oportuns, signen la present a Bellaterra, 17 de Desembre de 2015.

Dra. Roser Sala Pallarés Dr. Ramon Fontanillas Giralt Dra. Alba Tres Oliver

La present memòria de tesi ha estat realitzada gràcies a una beca predoctoral Formación de Personal Investigador (FPI) concedida pel *Ministerio de Ciencia e Innovación del Gobierno de España* i al finançament de la "Comisión Interministerial de Ciencia y Tecnología" (CICYT AGL2010-22008-C02) del *Ministerio de Economía y Competitividad del Gobierno de España*.

AGRAÏMENTS

"De totes les virtuts, l'esperança és la més important per a la vida. Perquè sense esperança, qui gosaria emprendre cap activitat? Qui tindria el coratge d'afrontar el futur, obscur, incert, imprevisible?"

Francesco Alberoni, *L'esperança*

He de reconèixer que no ha estat un camí fàcil. Durant aquests quatre anys, els moments de dubte, de vacil·lació i de defalliment han estat molts, incomptables. Però com diu la dita, les coses bones no són fàcils, i a més es fan esperar. Malgrat això, en moments en què l'espera es fa massa llarga o el camí es complica, l'esperança sorgeix del no res per donar-nos l'empenta que necessitem per seguir endavant amb allò que ens hem proposat.

En el meu cas, quan la pròpia esperança semblava no ser suficient, aquesta arribava en forma de bona notícia, de bon resultats, de bona proposta o de prova superada, entre moltes altres coses. Arribava per ajudar-me a retrobar el camí i a seguir endavant com si res. Sóc sincera quan escric que, de tot allò que m'ha anat portat l'esperança necessària durant aquests anys de doctorat les persones han estat, sense cap mena de dubte, el més important. I és per això que no puc tancar aquests pàgines sense dirigir-me a tothom qui, d'una manera o altra, ha estat caminant al meu costat per fer el camí més planer.

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"Algunes persones m'han dit que si haguessin pogut haurien escollit els seus pares. He de confessar que, si hagués pogut escollir, m'hauria dirigit a les mateixes persones que em posaren al món i em feren gran"

Josep Pla, *El quadern gris*

SUMMARY

The use of native vegetable oils (VO) in fish diets as substitutes of the traditionally used lipid source, fish oil (FO), has limitations due to their growing demand by feed, food and biofuel industries.

Acid VO are a rich-in-free fatty acid (FFA) by-product from the refining of crude VO that can be interesting alternatives to native VO. However, its use as lipid sources in monogastric animal diets appears to be controversial. Acid VO can be chemically re-esterified with glycerol in order to obtain re-esterified VO, which have different proportions of mono- (MAG), di- (DAG) and triacylglycerols (TAG). Moreover, re-esterified VO have a different molecular structure than their corresponding native VO and thus acquire new physico-chemical characteristics that can be interesting from a nutritional point of view. Thus, the aim of this dissertation was to investigate the use of acid and re-esterified VO in fish diets.

The experimental oils and diets characterization (***Chapters 1 to 7)***, showed that re-esterified VO have a higher proportion of MAG and DAG and a higher proportion of saturated fatty acids (SFA) in the sn-2 position of their acylglycerol molecules than the corresponding native VO.

In the first two trials (***Chapters 3 and 4***) the dietary use of palm or rapeseed acid (**A**) and re-esterified (low or high in MAG and DAG) oils (**EL** and **EH**, respectively) was assessed. In their effect on total fatty acid apparent digestibility coefficients (ADC) in rainbow trout (***Chapter 3***) and gilthead sea bream (***Chapter 4***) was evaluated in comparison with the corresponding native oils (N). Fish fed **A** diets had lower ADC than those fed N or **EL/EH** diets in both species. In rainbow trout, diets containing palm **EL/EH** did not result in different total fatty acid ADC than those containing palm N. For rapeseed, total fatty acid ADC were high (96.4-98.1%) in all cases, although that of fish fed the N diet was higher than that of fish fed the **EL** diet. In gilthead sea bream, fish fed the N palm oil diet had a lower total fatty acid ADC than the **EH** diet. Regarding rapeseed, fish fed **EL/EH** diets did not have different total fatty acid ADC than those fed the N diet. Overall, fatty acid digestibility of the experimental oils seemed to be more affected by their degree of unsaturation than by their positional distribution (molecular structure) or lipid class composition (TAG, MAG, DAG, FFA).

In the third trial (***Chapters 5, 6 and 7***), on the basis of the good results obtained with **A** and **EL/EH** diets, their dietary use when combined with 5% FO was evaluated. For this

purpose, the experimental oils were included in diets as a sole lipid source or in blends of **A** with N or the re-esterified (high in MAG and DAG) oil (**EH**). Their effects on fat and total fatty acid ADC, growth performance, plasma parameters, morphology of liver and intestine (***Chapters 5***), fillet quality (***Chapter 6***) and total and sn-2 fatty acid composition of liver, fillet and abdominal fat (***Chapter 7***) were assessed. Results showed no relevant effects of the experimental oils diets on the aforementioned parameters compared to the native oil diet. However, although fish final weights were high in all cases, those of fish fed rapeseed diets did not reach values obtained by fish fed the FO diet. Moreover, detrimental effects in fish growth were observed as more **A** was present in diets.

All things considered, the inclusion of acid and re-esterified VO in fish diets is an interesting alternative to the use of native VO provided that they are produced from a predominantly unsaturated source such as rapeseed.

RESUM

La utilització d'olis natius d'origen vegetal (VO) en dietes per peixos com a substituts de la font lipídica tradicionalment utilitzada, l'oli de peix (FO), es veu limitada degut a la creixent demanda per part de les indústries de pinsos, aliments i biocombustibles.

Els olis àcids són un subproducte ric en àcids grassos lliures provinent del procés de refinació d'olis vegetals i que poden ser fonts alternatives interessants a la utilització d'oli de peix (FO). Malgrat això, la seva utilització com a font lipídica en dietes per animals monogàstrics presenta controvèrsia. Els olis àcids vegetals poden ser re-esterificats químicament amb glicerol per produir olis vegetals re-esterificats, que tenen diferents proporcions de mono- (MAG), di- (DAG) i triglicèrids (TAG). A més, els olis vegetals re-esterificats tenen una estructura molecular diferent de la del seu corresponent oli natiu i per tant adquireixen noves propietats físico-químiques que poden ser interessants des d'un punt de vista nutricional. Per tot això, l'objectiu d'aquesta tesi va ser el d'investigar la utilització d'olis àcids i re-esterificats d'origen vegetal en dietes per peixos.

La caracterització dels olis experimental (***Capítols 1 al 7***), va mostrar que els olis vegetals re-esterificats tenen una major proporció de MAG i DAG, a més d'una major proporció d'àcids grassos saturats (SFA) en la posició 2 (sn-2) de les seves molècules d'acil glicerols que els seus corresponents olis natius.

Als dos primers experiments (***Capítols 3 i 4***) es va estudiar la utilització en dietes d'olis àcid (**A**) i re-esterificat (baix o alt en MAG i DAG) (**EL** i **EH**, respectivament). Els seus efectes sobre el coeficient de digestibilitat aparent (ADC) dels àcids grassos totals en truita irisada (***Capítol 3)*** i orada (***Capítol 4***) va ser avaluada en comparació al corresponent oli natiu (N). Els peixos alimentats amb la dieta **A** van tenir un ADC inferior que aquells alimentats amb les dietes N o **EL/EH** en ambdues espècies. En truita irisada, les dietes amb **EL/EH** de palma no van resultar en ADC dels àcids grassos totals diferents d'aquelles amb N. En la colza, els ADC dels àcids grassos totals van ser alts (96.4-98.1%) en tots els casos, tot i que aquells dels peixos alimentats amb N van resultar superiors als dels peixos alimentats amb la dieta **EL**. En orada, els animals alimentats amb la dieta N van mostrar un ADC dels àcids grassos totals inferior al de la dieta **EH**. Pel que fa a la colza, els peixos alimentats amb **EL/EH** no van presentar diferències en el ADC dels àcids grassos totals

respecte a aquells alimentats amb la dieta N. En general, la digestibilitat dels àcids grassos dels olis experimentals es va veure més afectada pel seu grau d'insaturació que per la seva distribució posicional (estructura molecular) o la seva composició en fraccions lipídiques.

Al tercer experiment, (***Capítols 5, 6 i 7***), en base als bons resultats obtinguts amb les dietes **A** i **EL/EH**, es va estudiar la seva utilització en dietes, en combinació amb un 5% de FO. Amb aquesta finalitat, els olis experimentals es van incloure en les dietes com a única fot lipídica o en combinacions de **A** amb N o amb l'oli re-esterificat (alt en MAG i DAG) (**EH**). Els seus efectes sobre el ADC del greix i dels àcids grassos totals, creixement, paràmetres plasmàtics, morfologia de fetge i intestí (***Capítol 5***), qualitat del filet (***Capítol 6***) i composició en àcids grassos totals i en sn-2 de fetge, filet i greix abdominal (***Capítol 7***) van ser avaluats. Els resultats no van mostrar efectes rellevants de les dietes experimentals sobre els paràmetres mencionats en comparació amb la dieta amb N. Tot i així, malgrat que els pesos finals dels peixos van ser alts en tots els casos, aquells dels animals alimentats amb dietes de colza no van arribar als dels peixo alimentats amb la dieta amb FO. A més d'això, es van observar efectes negatius en el creixement dels peixos en relació a un major contingut de **A** en les dietes.

Per tot això, la inclusió d'olis àcid i re-esterificat d'origen vegetal en dietes per peixos és una alternativa interessant a la utilització d'olis natius, sempre i quan provinguin d'una font principalment insaturada com la colza.

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ABBREVIATIONS

ADC: apparent digestibility coefficient(s)

ADG: average daily growth

ALT: alanine aminotransferase

ARA: arachidonic acid

AST: aspartate aminotransferase

CEL: carboxyl ester lipase

CF: condition factor

CM: chylomicrons

DAG: diacylglycerol(s)

DHA: docosahexaenoic acid

EFA: essential fatty acid(s)

EPA: eicosapentaenoic acid

FA: fatty acid(s)

FAS: fatty acid synthetase

FCR: feed conversion ratio

FFA: free fatty acid(s)

FO: fish oil

GGT: gamma-glutamyl transferase

HDL: high density lipoproteins

HL: hepatic lipase

HSI: hepatosomatic index

HUFA: highly unsaturated fatty acid(s)

LC: long-chain

LCAT: lecithin:cholesterol acyltransferase

LDL: low density lipoproteins

LHC: liquid holding capacity

LPL: lipoprotein lipase

MAG: monoacylglycerol(s)

MDA: malondialdehyde

MUFA: monounsaturated fatty acid(s)

NMR: nuclear magnetic resonance

PUFA: polyunsaturated fatty acid(s)

SFA: saturated fatty acid(s)

SGR: specific growth rate

TAG: triacylglycerol(s)

TBARS: thiobarbituric acid reactive substances

TPA: texture profile analysis

UFA: unsaturated fatty acid(s)

VLDL: very low density lipoproteins

VO: vegetable oil(s)

VSI: viscerosomatic index

WG: weight gain

CHAPTER 1

GENERAL INTRODUCTION

1.1. Fish consumption and aquaculture

Growing human population has been predicted to reach 9.1 billion by year 2050 (Miller, 2008). In light of this, doubts about the ability to provide the nutritious food needed for the global human population in the decades to come have arisen, turning it into a major social challenge (Olsen, 2011).

Although agricultural products provide the majority of food energy, aquaculture was pointed out to be the most promising future source of food protein for humans, accounting for about 17% of the global population's intake of animal protein. Fish represents a valuable source of animal protein, a commercial portion of fish providing around 50-60% of the daily protein requirements for an adult. It is usually low in saturated fats, carbohydrates and cholesterol, but it is a concentrated source of essential fatty acids (EFA). It has a particular role as a unique source of the long-chain omega-3 polyunsaturated fatty acids (n-3 LC-PUFA), particularly eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:5n-6, DHA) (FAO, 2014). These fatty acids (FA) are essential for human health and well-being because they have well-known beneficial effects in a range of human pathologies including cardiovascular and inflammatory diseases and are also key in processes such as neural development, immune response, hormone modulation etc. (Simopoulos, 1999; Calder, 2006; Mozaffarian and Rimm, 2006; Campoy et al., 2012; Delgado-Lista et al., 2012; Gil et al., 2012). Hence, health and well-being are increasingly influencing consumption decisions and fish has particularly importance in this respect. On the other hand, fish can be crucial for the nutrition of some densely populated countries where may be no alternative affordable food sources that provide as many essential nutrients as fish does.

All the factors mentioned above, added to the growing human population, are predicted to generate an increase in the demand of fish and fish products. In 2010, per capita apparent fish consumption in the world was 18.9 kg, although that of industrialized countries being 27.4 kg. Fish consumption is expected to have an overall growth of 4% by 2022, with an increase of 1.8 kg in the per capita consumption (FAO, 2014).

As a response to the increasing demand of fish worldwide, global fish production has grown steadily in the last five decades **(Figure 1.1.)**. In fact, aquaculture emerged as a solution to the existent gap between supply and demand of fish and fish products, arising

from the decline that world fishery landings had experimented since the late 1980s. This decline was mainly due to factors such as the unsustainable fishing and environmental events like the meteorological phenomenon El Niño, which led to a depletion of wild fisheries stocks (Pauly, 2002).

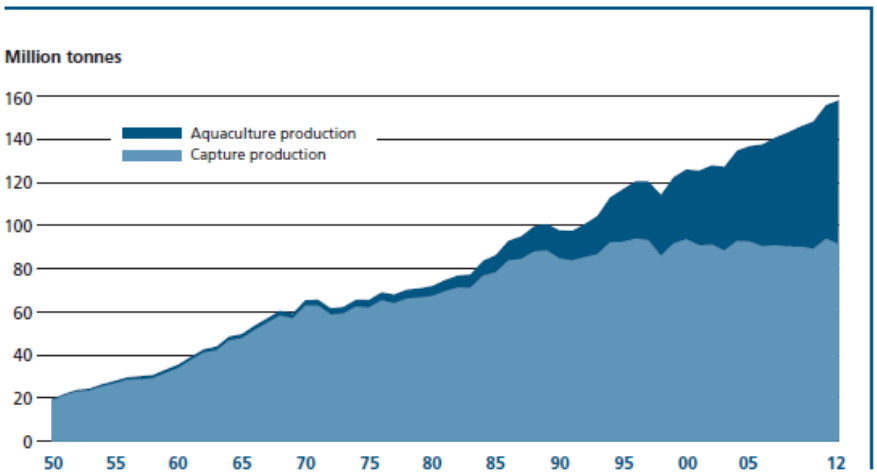

Figure 1.1. World capture fisheries and aquaculture production in the last five decades (FAO, 2014).

Aquaculture production expanded at an average annual rate of 10.8% in the period 1980-1990. During the period 1990-2000 it showed a high but slightly low rate (9.5%), that decreased to 6.2% between 2000 and 2012. Even though aquaculture production seemed to grow at a progressively slowing rate in the recent years, it still is one of the fastest-growing food-producing sectors and remains strong as a consequence of the already mentioned increasing demand for fish. In 2012, production attained an all-time high of 90.4 million tonnes, this representing a 42.2% of the total 158 million tonnes of fish produced by capture fisheries and aquaculture (FAO, 2014).

Regarding regions, 15 countries produced 92.7% of all farmed food fish worldwide. Asia (mainly China) accounted for more than 80% of the world aquaculture production by volume, but it was only the 4.32 % for Europe **(Table 1.1.)**.

Table 1.1. Evolution of the aquaculture production by region: percentage (%) and weight (t) of world total production (adapted from FAO, 2014).

		Year			
		1990	1995	2005	2012
Region					
Africa	*(%)*	0.62	0.45	1.46	2.23
	(t)	81.015	110.292	646.182	1.485.367
Americas	*(%)*	4.19	3.77	4.91	4.78
	(t)	548.479	919.571	2.176.740	3.187.319
Asia	*(%)*	82.61	88.90	88.46	88.39
	(t)	10.801.531	21.677.062	39.185.417	58.895.736
Europe	*(%)*	12.25	6.49	4.83	4.32
	(t)	1.601.649	1.581.359	2.137.340	2.880.641
Oceania	*(%)*	0.32	0.39	0.34	0.28
	(t)	42.005	94.238	151.466	184.191

Of the 66.6 million tonnes of farmed food fish produced in 2012, two-thirds were finfish species grown from inland aquaculture (38.6 million tonnes) and mariculture (5.6 million tonnes) **(Table 1.2.)**.

Table 1.2. World production of farmed fish species groups from inland aquaculture and mariculture in 2012 (adapted from FAO, 2014).

	Inland aquaculture	Mariculture	Quantity subtotal		Value subtotal	
	(Million tonnes)	*(Million tonnes)*	*(Million tonnes)*	*(Percentage by volume)*	*(US$ million)*	*(Percentage by value)*
Finfish	38.599	5.552	44.151	66.3	87.499	63.5
Crustaceans	2.530	3.917	6.447	9.7	30.864	22.4
Molluscs	0.287	14.884	15.171	22.8	15.857	11.5
Other species	0.530	0.335	0.865	1.3	3.512	2.5
Total	41.946	24.687	66.633	100	137.732	100

According to FAO (2014), aquaculture will remain one of the fastest-growing food-producing sectors in the future to come. Its contribution to the global fishery production is expected to rise 6% by 2022, which would represent 53% in terms of fish destined for

human consumption and it is projected to be about 60-70% beyond 2030 (Subasinghe et al., 2009).

In relation to the type culture environment, inland aquaculture (freshwater) makes the greatest direct contribution to the supply of affordable protein food, currently accounting for the 57.9% of farmed food fish production globally. Since 1980, its annual growth rate was of 9.2% and the 7.6% for mariculture.

1.2. Lipids in fish nutrition

Under intensive culture conditions, fish must be fed adequate quantities of diets that meet all their nutrient requirements for their adequate growth, health and welfare. This objective is achieved by selecting appropriate feed ingredients, deciding how they should be combined to meet the nutritional requirements of farmed aquatic animals, and processing the mixture of ingredients into a physical form suitable for practical use (NRC, 2011). It is important to bear in mind that digestion of nutrients present in the different feedstuffs, metabolic utilization or interactions among them may differ among species and are mainly related to natural feeding habits of species (Oliva Teles, 2012).

Lipids, proteins and carbohydrates comprise the major macronutrient classes required to provide the essential nutrients for both energy production and for development of cells and tissues that allow growth and maintenance of homeostasis in all vertebrate organisms. Dietary lipid is the densest form of energy available to fish at approximately 38.5 kJ g^{-1}. The other macronutrients, protein (23.6 kJ g^{-1}) and carbohydrate (17.3 kJ g^{-1}), are approximately half as energy-dense as lipid. Because of this energy density, lipid is the most efficient nutrient for maximizing energy intake (Bureau et al. 2002). In addition, the efficiency with which dietary lipid energy is converted to somatic lipid is also the greatest of the three macronutrients (Glencross et al. 2008). Hence, lipids are the main conventional energy sources in fish diets as carbohydrate utilization is not very efficient, particularly in carnivorous species, because their natural fish food usually does not include high dietary carbohydrate levels. Thus, the diets formulated for the aquaculture industry contain predominantly protein and lipid, with small amounts of vitamins and minerals. Moreover, as protein sources are the most costly ingredients in commercial diets, increasing the dietary lipid level might spare protein requirements (Sargent et al., 2002). Therefore, the

current trend in aquaculture is to use high-energy nutrient dense diets, and so the roles of lipids in fish nutrition have become more important in the recent years (Turchini et al., 2009). It is important to take into account, though, that there are great differences among fish species in their ability to use high dietary lipid levels (Oliva Teles, 2012).

1.2.1. Dietary fat

Lipid (oil or fat) in dietary formulations serves two important roles, as a high-density energy supply and as a source of EFA, to allow the rapid growth and development required in modern aquaculture production (Bell, 2008). Lipids are, by definition, a chemically heterogeneous group of hydrophobic compounds that are soluble in a range of organic solvents such as chloroform, hexane and diethyl ether (Gurr and Harwood, 1991; Higgs and Dong, 2000).

Animal lipids, including those from fish, can be divided into two broad groups (Sargent et al., 2002; NRC, 2011):

1) neutral lipids: soluble in non-polar solvents. It includes triacylglycerols (TAG), wax esters, sterols, steryl esters and free fatty acids (FFA).

2) polar lipids: can possess a wide range of solubility based on their non-lipid head groups. The major polar lipids are phospholiglycerides, sphingolipids, sulpholipids and glycolipids.

In terms of function, neutral lipids have a storage role while polar lipids have a predominantly structural function. Regarding their structure, most lipids are "complex", meaning that they contain FA usually esterified to alcohol groups in the case of acylglycerols (glycerides) and to amino groups in the case of sphingolipids (Christie, 2003). As most dietary lipids in feeds are complex lipids, FA usually comprise the bulk of dietary lipid intake and can be delivered in a variety of chemical forms including TAG, phospholipids, steryl and wax esters or FFA.

By definition, FA are aliphatic monocarboxylic acids derived from or contained in esterified form in an animal or vegetable fat, oil or wax. Natural FA commonly have a chain of 4 to 28 carbons (IUPAC, 1997). They can be classified based on the number of carbon atoms in their chain (chain length), their degree of unsaturation (number of double bonds within the chain) and the position of the unsaturated bonds relative to the methyl end of the FA chain (Rigaudy and Klesney, 1979). In relation to the chain length, FA can be

categorized as short-chain (less than 6 carbons), medium-chain (6-12 carbons), long-chain (more than 12 carbons) FA. The number of unsaturated bonds between carbon atoms present in a FA will classify it as saturated fatty acid (SFA; no unsaturated bonds) or unsaturated fatty acid (UFA; presence of unsaturated bonds). Unsaturated bonds in dietary UFA are usually in *cis*- configuration, although FA with *trans*- ethylenic bonds occasionally occur. UFA can, in turn, be subdivided into monounsaturated fatty acids (MUFA; a single unsaturated bond) and polyunsaturated fatty acids (PUFA, minimum one unsaturated bond). FA with four or more unsaturated bonds in their FA chain are often referred to as highly unsaturated fatty acids (HUFA) or long-chain PUFA (LC-PUFA), which is used for FA consisting of 20 carbons or more (Nichols, 2004). In addition, the structure of a particular PUFA can be defined by specifying the position of the first ethylenic bond relative to the methyl end of the FA (n- or "omega" nomenclature; ω). In this nomenclature, FA are described by the general formula X:Yn-z, where X is the chain length, Y is the number of ethylenic/double bonds, and n-z (or ωz) denotes the position of the first double bond relative to the methyl end of the aliphatic chain. Names and formulas of the common FA of animal and plant origin are presented in **Table 1.3.**

The lipid raw materials included in aqua feeds are traditional marine sources such as fish oil (FO) and refined fats and oils of terrestrial animal and vegetable origins (Bell and Koppe, 2011), which are mainly complex mixtures of TAG (>95%) along with small amounts of monoacylglycerols (MAG), diacylglycerols (DAG), FFA and phospholipids (De Silva et al., 2011; Gunstone, 2011a). Oils from vegetable origin (VO) can also contain small proportions of sterols, sterol esters, tocols (tocopherol and tocotrienol) and hydrocarbons (Gunstone, 2011a).

Table 1.3. The common fatty acids of animal and plant origin (adapted from Christie, 2003).

Systematic name	Trivial name	Notation
Saturated fatty acids		
ethanoic	acetic	2:0
butanoic	butyric	4:0
hexanoic	caproic	6:0
octanoic	caprylic	8:0
decanoic	capric	10:0
dodecanoic	lauric	12:0
tetradecanoic	myristic	14:0
hexadecanoic	palmitic	16:0
octadecanoic	stearic	18:0
eicosanoid	arachidic	20:0
docosanoic	behenic	22:0
Monoenoic fatty acids		
cis-9-hexadecenoic	palmitoleic	16:1n-7
cis-6-octadecenoic	petroselinic	18:1n-12
cis-9-octadecenoic	oleic	18:1n-9
cis-11-octadecenoic	*cis-vaccenic*	18:1n-7
cis-13-docosenoic	erucic	22:1n-9
cis-15-tetracosenoic	nervonic	24:1n-9
Polyunsaturated fatty acids		
9,12-octadecadienoic	linoleic	18:2n-6
6,9,12-octadecatrienoic	γ-linolenic	18:3n-6
9,12,15-octadecatrienoic	α-linolenic	18:3n-3
5,8,11,14-eicosatetraenoic	arachidonic	20:4n-6
5,8,11,14,17-eicosapentaenoic	EPA	20:5n-3
4,7,10,13,16,19-docosahexaenoic	DHA	22:6n-3

TAG constitute a major class of neutral lipid and consist of three molecules of FA, each with its own chain length and degree of unsaturation, esterified to the three alcohol groups of the carbon atoms of the glycerol molecule (**Figure 1.2.).** When esterified, these positions are termed by a stereospecific numbering system (sn): outer position (sn-1 and sn-3) and sn-2 (middle position) (Tocher, 2003; Berry, 2009). Each TAG may contain a mixture of different FA or the same FA esterified to all three positions of the glycerol

(Berry, 2009). At ambient temperature, TAG can be either solid, in which case they can be termed fats, or liquid in which case they can be termed oils (Tocher, 2003).

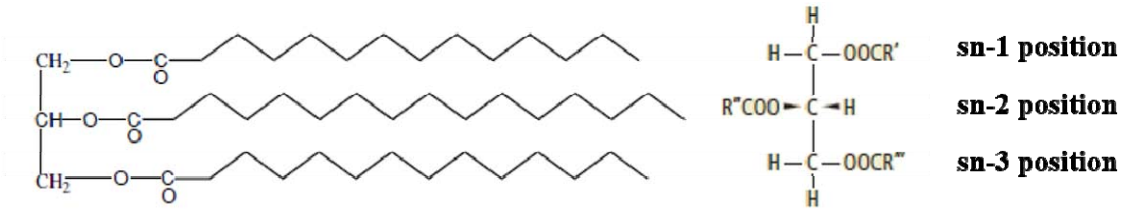

Figure 1.2. The structure of the triacylglycerol molecule. R', R'' and R''' are the fatty acids esterified to the glycerol molecule and sn represents the stereospecific numbering position (adapted from Sargent, 2002 and Christie, 2003).

In fish lipids, SFA and MUFA are preferentially located in the sn-1 and sn-3 positions in acylglycerols, whereas PUFA are preferentially located in the sn-2 position. However, many exceptions exist to this general rule (Sargent et al., 2002; Tocher, 2003).

1.2.2. Essential fatty acids

As already mentioned for humans and as in all vertebrates, EFA also play different roles in the physiological and biochemical processes within aquatic animals. Lipids in general, and EFA in particular, play important roles in cell synthesis, neural development, endocrine function and control, ionic regulation, immune function and reproduction (Glencross, 2009). There are several clinical signs that fish exhibit with dietary EFA deficiency, being poor growth rate and increased mortality some of the more obvious (Watanabe, 1982; Sargent et al., 1995; Ruyter et al., 2000).

The highly biologically active and essential LC-PUFA arachidonic acid (C20:4n-6, ARA), EPA and DHA are biosynthesized from linoleic (C18:2n-6) and α-linolenic (C18:3n-3) acids after a succession of desaturation and elongation reactions mediated by the activity of Δ5 and Δ6 desaturases **(Figure 1.3.)** (Tocher, 2003). The precursors linoleic and α-linolenic acids need to be supplied within the diets because they cannot be biosynthesized *de novo* by fish and other vertebrates (Cunnane, 2003).

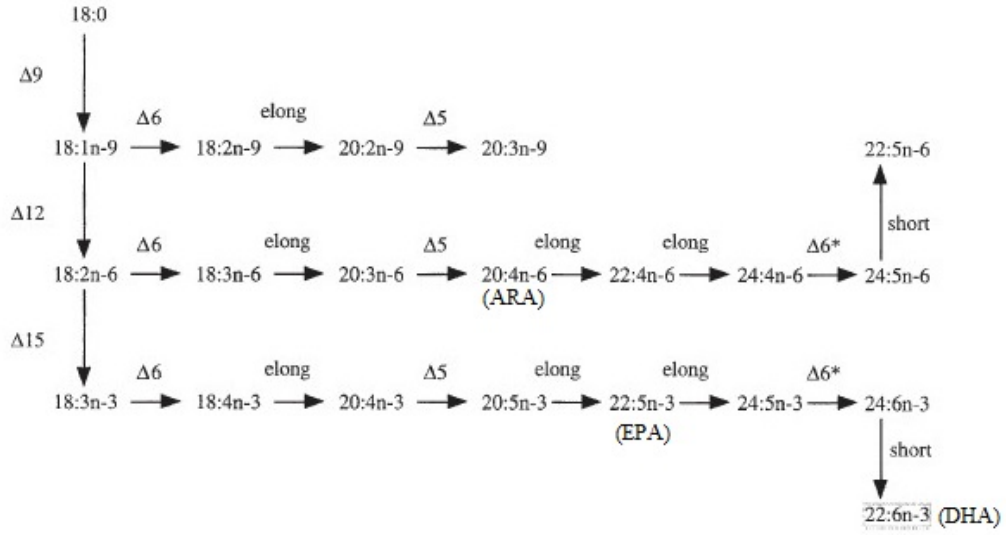

Figure 1.3. Pathways of biosynthesis of C_{20} and C_{22} HUFA from n-3, n-6 and n-9 C_{18} precursors. Δ5, Δ6, Δ6*, Δ9, Δ12, Δ15: Fatty acyl desaturases; Elong: Fatty acyl elongases; Short: chain shortening. Δ9 desaturase is found in all animals and plants whereas Δ12 and Δ15 desaturases are generally only found in plants and so 18:2n-6 and 18:3n-3 are "essential" fatty acids for many animals including many species of freshwater fish **(Tocher, 2003).**

Almost all fish, as with all vertebrates, potentially have the ability to convert linoleic and α-linolenic acids into the corresponding C20 and C22 n-6 and n-3 PUFA *in vivo.* However, marine carnivorous fish species lost their ability to desaturate and elongate the two PUFA linoleic and α-linolenic acids to the essential LC-PUFA due to specific deficiencies in desaturases and/or elongases, as they are very well supplied in their natural diets (Sargent et al. 2002). The implication is that such species, when farmed, have to be provided with these essential LC-PUFA through the feed. Freshwater and diadromous species, on the other hand, require generally less n-3 LC-PUFA in their diets than carnivore marine fish (Glencross, 2009; Tocher, 2010). Therefore, while linoleic and α-linolenic acids could satisfy the EFA requirements of freshwater fish, EPA and DHA are required to satisfy the EFA requirements of marine fish (Watanabe, 1982; Kanazawa, 1985).

1.2.3. Source of essential fatty acids in aqua feeds

Fish meal and fish oil (FO) have been the standard ingredients of bulk feeds for intensively farmed fish, above all salmonids and marine fish, for many years. They are

derived from industrial feed fisheries, e.g., anchovy, capelin, herring, sand eel, mackerel, and sardine fisheries (Sargent et al., 2002; Tacon et al., 2010) and their use was a perfectly sensible approach 30-40 years ago during the initial the development of intensive aquaculture. Both ingredients were readily accepted and digested by fish, had favourable nutrient compositions and represented the natural food for the fish (NRC, 2011).

The main reason fish meal and FO have been primarily used routinely in carnivorous fish feeds is that they have been affordable sources of amino acids and EFA, respectively, that support optimal growth and product quality. Although these ingredients have historically been more expensive than other sources of proteins or fats/oils, the high growth rates and feed efficiencies observed when fish meal and FO were used made them cost effective (Gaylord et al., 2010).

FO, the main lipid source in feeds, are highly polyunsaturated, characterized by high but variable levels of n-3 LC-PUFA, predominantly 20:5n-3 and 22:6n-3, with 20:4n-6 as the major n-6 PUFA, with palmitic acid (16:0) followed by stearic acid (18:0) as the predominant SFA, and containing substantial amounts of the monoene oleic acid (18:1n-9) (Tocher, 2003). Its primarily use in the 21st century is in feeds for aquaculture, primarily as a source of n-3 LC-PUFA, now prized for their health benefits in the human consumers. Although FO has also been used in terrestrial animal feeds (pigs and poultry) and small amounts were always used for direct human consumption (particularly cod liver oil), history demonstrates that, in terms of n-3 LC-PUFA, aquaculture represents arguably the best use of bulk FO to date (Tocher, 2015).

1.3. Lipid digestion, absorption and metabolism

Dietary lipid is well utilized in most fish species (Olsen and Ringo, 1997). In order to accomplish this, fish require an efficient system for lipid digestion, absorption and transport which has been reported to be, in general, similar to that in mammals (Sargent et al., 1989; Tocher, 2003). It is important to bear in mind, though, that the complexity of the intestinal tract and marked anatomical differences between different species of fish have made this a challenging area for metabolic and enzymatic studies (Tocher, 2003). Indeed, variation in anatomy and histomorphology of the digestive tract among fish species is greater than for any other phylum (Buddington and Kuz'mina, 2000a, b). In this section, the general processes of digestion, absorption and metabolism will be described

considering fish digestive tract possesses mouth, pharynx, oesophagus, stomach, midgut with pyloric ceca and distal or hindgut without specifically mentioning any species particularity. More detailed information on this topic can be found in the reviews by Tocher (2003) and Bakke et al. (2011).

1.3.1. Digestion

Digestion is the process of hydrolysis and solubilisation of ingested nutrient polymers into molecules and elements suitable for transport across the intestinal wall (Bakke et al., 2011). A condition for efficient digestion and absorption of a nutrient is, thus, solubility in water.

For lipids, digestion has been defined as the hydrolytic cleavage of dietary FA esters in the gastrointestinal tract (Olsen et al., 1998). Lipids are hydrophobic compounds and so they need to be solubilised and degraded to be ready for their uptake by the intestinal enterocytes. The digestion process in fish, as well as in mammals, comprises 3 main consecutive phases that have the final aim of converting lipids into more polar derivatives.

1. Emulsification of the lipids released from the feed in form of bulk fat globules into lipid droplets.
2. Hydrolysis of the FA ester bonds by pancreatic lipase.
3. Aqueous dispersion of lipolytic products in mixed micelles.

Prior to start describing each phase in detail, it is useful to mention that TAG and wax esters are the two main forms of neutral lipids available to fish in their natural environment (Cowey and Sargent, 1977) and TAG is generally the predominant lipid class in the natural diet of freshwater fish.

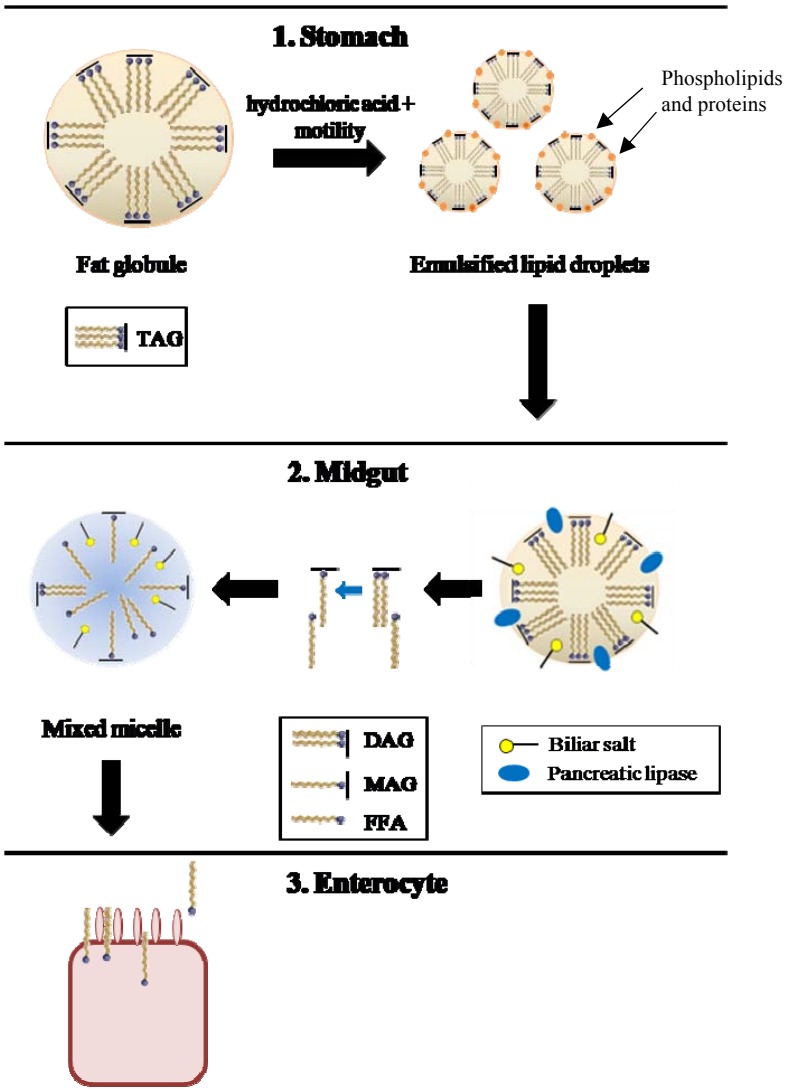

Figure 1.4. Schematic representation of lipid digestion in fish. Step 3 (Enterocyte) corresponds to a general representation of absorption. TAG: triacylglycerols; DAG: diacylglycerols; MAG: monoacylglycerols and FFA: free fatty acids.

The arrival of the feed to the stomach produces a distension that stimulates the secretion of hydrochloric acid and pepsinogen **(Figure 1.4.)**. In marine fish, the food is first coated with a mucous layer containing hydrochloric acid and pepsin that helps prevent dilution by the alkaline seawater as these species must continually drink to maintain their osmotic balance (Wedemeyer, 1996). The concerted action of gastric hydrochloric acid and motility release lipids and their hydrophobicity gives them a tendency to aggregate into droplets. Phospholipids and certain proteins from the feed will limit the size of the lipid droplets.

In mammals, once these droplets are formed, pre-duodenal lipases are able to start hydrolysis in a process of partial digestion. In fish, differently, there are no clear indications that gastric juice contains lipases (Smith, 2004). Although Olsen and Ringo (1997) suggested that fish gastric lipolytic activity may contribute to primary digestion in a ways similar to that of mammals, the physiological significance of gastric lipolytic activity in fish is unclear (Tocher; 2003). The product of stomach is a mixture of emulsified lipids, which is released through the pyloric sphincter into the alkaline midgut when it has the appropriate particle size and moisture level sufficient for further transport and processing (NRC, 2011).

In the midgut, as described for mammals in the duodenum, fat hydrolysis is helped by emulsification which is brought about by the action of bile salts; detergents or amphipaths that act as emulsifying agents and aggregate together to form micelles (Mc Donald et al., 2010). In fish, lipolytic activity is generally greatest in the proximal part of the intestine and the pyloric caeca if present, but can extend into the lower parts of the intestine with the activity decreasing progressively (Tocher, 2003). The breakdown of fats is achieved by digestive lipase from the pancreas.

Lipases can be broadly defined as enzymes that catalyse the hydrolysis of ester bonds in substrates such as TAG, phospholipids, cholesteryl esters and vitamin esters (Wong and Schotz, 2002). Moreover, there are several categories of lipase specificity. Substrate-specific lipases differentiate between esters such as TAG, DAG, MAG and phospholipids. Fatty acid-specific lipases show preference for particular FA or a type of FA (e.g., short-chain, PUFA, etc.). Lipases can also be regioselective (i.e. positional specificity) in that they distinguish between the external (sn-1 and sn-3 positions) and internal (sn-2 position) ester bonds (Kurtovic et al., 2009). Pancreatic lipase, secreted by the exocrine pancreas, is the main digestive lipolytic enzyme in higher vertebrates and has strong preference for acylglycerides over other lipids (Crenon et al., 1998; Lowe, 2002). Upon binding to the emulsified substrate at the lipid-water interface, the activity of pancreatic lipase greatly increases due to a conformational change in the enzyme (van Tilbeurgh et al., 1993, 1999). Both bile salts and colipase must be present to transform the enzyme to optimal conformation for activity. Colipase is a required cofactor for pancreatic lipase, being necessary for its activity during hydrolysis of dietary TAG in the presence of bile salts (Borgström et al., 1979). It interacts with the bile salt in the micelles to allow lipase to adsorb at bile salt-covered interfaces (Carriere et al., 1998; Lowe, 2002).

In mammals, pancreatic lipase specifically hydrolyses the primary (sn-1 and sn-3) ester bonds of TAG, being FFA and 2-MAG the main products of the hydrolysis (Mattson and Volpenhein, 1964). In fish, digestive lipase activity differs between species, and knowledge of characteristics and specificities of these enzymes is far from complete (Bakke et al., 2011). Kurtovic et al. (2009) suggested that freshwater fishes may have mainly a pancreatic lipase-type enzyme while marine fishes have a bile-acid-dependent carboxyl ester lipase (CEL), the latter having a broader substrate specificity. Authors suggested that these differences are likely to be a response to differences in diet, i.e., freshwater fish are primarily omnivorous, whereas marine fish are primarily piscivorous. Omnivorous fish consume vegetables, in which the main lipid class are TAG. Differently, piscivorous fish consume a wider variety of lipid classes such as wax esters that cannot be hydrolysed by pancreatic lipase.

Whether MAG are the main products of lipolytic action in fishes as in monogastric mammals, is not known. Some studies suggested that CEL is sn-1,3-specific (Tocher and Sargent, 1984; Gjellesvik et al. 1989; Bogevik et al., 2007), producing FFA and 2-MAG as end products of the hydrolysis. Indeed, the enzymes responsible for the synthesis of TAG from absorbed FA in the enterocytes (more detailed in section 1.3.2.) seem to preferentially use 2-MAG as substrate, rather than glycerol (Oxley et al. 2007), suggesting that 2-MAG are important end products (Bakke et al., 2011). This would lead to the possibility that CEL with sn-1,3-specific hydrolytic activity was the dominating enzyme in most fish species (NRC, 2011). However, the possibility of the existence of non-specificity of the lipase had also been previously suggested by some authors (Olsen and Ringo, 1997; Olsen et al., 1998).

Which seems to be clear is that digestive lipase most likely prefers certain FA in a particular position over another. Cases in which complete hydrolysis of TAG to FFA and glycerol if the FA in sn-2 position was an UFA were noted: MUFA (Leger, 1985), PUFA (Koven, 1994) or LC-PUFA (Kurtovic et al., 2009).

The FA products liberated by lipolysis are incorporated into primary micelles formed by bile acids and phospholipids, which also contain MAG and FA (Webster and Lim, 2002). The entry of these amphiphilic compounds expands the mixed micelle. As the micelles enlarge, they are transformed into secondary micelles that have the capacity to include more lipophilic compounds such as long-chain SFA, DAG, cholesterol-esters and fat-soluble vitamins. Then, secondary micelles turn into mixed micelles and are transported

from the lumen to the brush border, where they disintegrate once they reach the so-called unstirred water layer of the intestinal mucosa. The FA are then released from the micelles and the absorption across the epithelial membrane takes place (Bakke et al., 2011).

1.3.2. Absorption

Solubilised FA that have been released during digestion of dietary lipid are subsequently transported or otherwise absorbed across the apical (brush border) membrane of the enterocytes lining the post-gastric alimentary tract, exit the cells across their basolateral membrane, and enter the circulatory system.

In general, nutrients product of the action of digestive enzymes can enter the organism across the brush border by diffusion or facilitated transport down a concentration gradient or by active and energy-dependent transport against a concentration-gradient. Passage via paracellular pathway is also possible, but considered to be of minor importance in fish (Ferraris et al., 1990; Oxley et al., 2007). Facilitated and active transport takes place via specialized transporters unique for the nutrient or group of nutrients with similar characteristics. Both are saturable mechanisms.

Information about lipid transport across the intestinal mucosa in fish is limited, as the absorption of the products of lipid digestion has not been studied extensively (Tocher, 2003). However, present knowledge indicates that the processes are quite similar to those in other vertebrates (Leger 1985). FA released from the micelles are thought to be absorbed by diffusion or facilitated transport. Nonetheless, the involvement of transporters, as has been demonstrated in mammals (Iqbal and Hussein, 2009), has not been verified in fish (Bakke et al., 2011).

The rate of absorption of some individual FA may be more efficient than for others (Olsen et al., 1998). In mammals, the absorption of FA increases with decreasing chain length and with an increase in unsaturation (Carlier et al., 1991). In fish, it has been considered that the rate of lipid absorption is slower than that of mammals (Leger, 1985; Morais et al. 2005) as a consequence of the lower body temperatures (Kapoor et al., 1975).

Even though lipid absorption processes in fish mainly occur in the proximal regions of the intestine and pyloric ceca (Diaz et al. 1997; Hernandez-Blazquez et al. 2006; Tocher, 2003), coinciding with the highest lipolytic activity (Tocher, 2003), this may depend on lipid class, chain length and degree of unsaturation. Short- and medium-chain

FA, with a relatively high water solubility, are absorbed rapidly in the most proximal part of the intestine, leading to the hypothesis that they may not be incorporated into micelles (Røsjø et al. 2000; Denstadli et al. 2004). HUFA are also absorbed in proximal regions. Contrarily, due to the high degree of hydrophobicity and lower micellar solubility of SFA, they may not easily reach the brush border and may therefore not be as readily absorbed as FA with a similar chain length but with a certain degree of unsaturation (Bakke et al. 2011). As Sigurgisladottir et al. (1992) and Olsen et al. (1998) reported, the rates of absorption of Atlantic salmon fed FFA were: PUFA>MUFA>SFA, and short chain>longer chain.

1.3.3. Metabolism

1.3.3.1.1. Enterocyte triacylglycerol synthesis

Once inside the enterocyte, the absorbed FA are re-esterified with glycerol, partial acylglycerols (MAG and DAG) and lysophospholipids to resynthesize TAG and phosphoglycerides (Sargent et al., 1989) in the endoplasmic reticulum (Tocher et al., 2003). The fate of the FA inside the enterocyte seems to vary so that ARA, EPA, DHA and palmitic acid are preferentially esterified in the phospholipids while oleic (C18:1n-9) and estearic are incorporated mainly in TAG (Olsen et al., 1999; Pérez et al., 1999; Tocher et al., 2002).

In mammals, the re-esterification process can take place by two different pathways: the 2-MAG or the glycerol-3-phosphate pathways. The 2-MAG pathway is the predominant TAG synthetic pathway following digestion of TAG to 2-MAG by specific pancreatic lipase and subsequent absorption into the enterocyte. In this case, this pathway accounts for 80% of the synthesized TAG (Lehner and Kuksis, 1996; Linderborg and Kallio, 2005). However, when 2-MAG are not available, TAG are synthesized via the glycerol-3-phosphate or phosphatidic acid pathway (Mu and Hoy, 2004). In fish, even though the major pathway is still not completely clear, studies by Oxley et al. (2005, 2007) indicated that the 2-MAG pathway predominated over the glycerol-3-phosphate pathway in Atlantic salmon.

The newly formed TAG and phospholipids are grouped with proteins to form chylomicron (CM)-like and very-low-density lipoprotein (VLDL)-like particles (Caballero et al. 2003; Tocher, 2003). The lipid load and degree of unsaturation affects lipoprotein

production, with high dietary lipid and PUFA leading to the production of larger CM, whereas high dietary SFA result in the production of smaller VLDL particles (Tocher, 2003). These particles appear to exit the enterocytes by exocytosis (Hernández-Blázquez et al., 2006).

1.3.3.2. Transport

As in mammals, the transport of FA and other lipid-soluble components from the intestine to the peripheral tissues in fish is mediated mainly by lipoproteins (Caballero et al., 2003).

A portion of intestinal lipoproteins may be transported directly to the liver via the portal system (Tocher, 2003) but the majority of them are transported via the lymphatic system before appearing in the circulatory system and being delivered to the liver (Sheridan et al., 1985). However, it is not yet clearly understood which route the CM and VLDL take from the enterocytes (Turchini et al., 2009). Whether a lymphatic system exists in fish is still a matter of debate (Denstadli et al., 2011). A recent study by Rummer et al. (2014) indicated that there is no evidence that lymphatic system plays a role in lipid transport in fish. Authors concluded that this system in fish has different form and function than that of mammals.

The lipid that is not re-esterified is either absorbed directly into the circulation and catabolised (particularly short-chain SFA) by the process of β-oxidation or transported directly from the enterocyte to the peripheral tissues within the animal, bypassing the portal vein and liver (Denstadli et al., 2011). The adipose tissue is the primary site where TAG are stored, in contrast to the liver which plays a major role in the secondary processing of TAG and phospholipids, including modification of FA chain lengths and degree of unsaturation (Sargent et al. 1993).

Both marine and freshwater fish species have the major enzymes of lipoprotein metabolism present in mammals (Tocher, 2003), although some FFA can be transported bound to plasmatic albumin-like proteins without requiring incorporation into lipoproteins for transport.

Lipoproteins contain a core of TAG and cholesteryl esters covered by a surface layer of amphiphilic compounds such as phospholipids, cholesterol and apolipoproteins (Sethi et al., 1993). Apoproteins play important roles in lipoprotein clearance and metabolism (Mu and Hoy, 2004) and although studies in apoproteins in fish are few, they have similar compositions and probably the same metabolic functions as in mammalian systems. The proportions of the different lipoprotein classes and their lipid composition vary among fish species (Tocher, 2003, NRC, 2011). The latter can be affected also by dietary composition (Sheridan, 1985; Torstensen et al. 2000).

As mentioned above, exogenous lipids absorbed by the intestine are predominantly integrated into CM and VLDL (Sire et al. 1981; Caballero et al. 2003) whereas endogenous lipids are transported in VLDL, LDL and HDL (Sheridan, 1988; Babin and Vernier, 1989; Iijima et al., 1995) (**Figure 1.5.**). CM are produced exclusively in the intestine and, although some VLDL can be synthesized also in the intestine, the majority of them are synthesized in the liver (endogenous).

TAG in CM and VLDL are hydrolyzed by the enzyme lipoprotein lipase (LPL) at peripheral tissue sites. LPL is a glycoproteic enzyme belonging to a large family of lipases, including pancreatic, hepatic and endothelial lipases (Rader and Jaye, 2000), which is attached to the surface of the vascular endothelium and is present in trout in numerous tissues (Black et al., 1983, 1987). The hydrolysed TAG produce FFA, which can then be re-esterified and stored in adipose tissue or used as an energy source (oxidized) by peripheral tissues such as muscle and heart (Auwerx et al., 1992). As reported in humans, the activity of LPL can be regulated reciprocally between muscle and adipose tissue as to favour either energy production or storage of TAG (Brindley, 1985). As for hepatic lipase (HL), it serves a dual role in TAG hydrolysis and in ligand-binding for lipoprotein uptake into the liver of vertebrates (Martin et al., 1988; Holmes et al., 2011). In fish, LPL activity is also found in liver but that not correspond to the classical HL activity (Lindberg and Olivecrona, 1995, 2002).

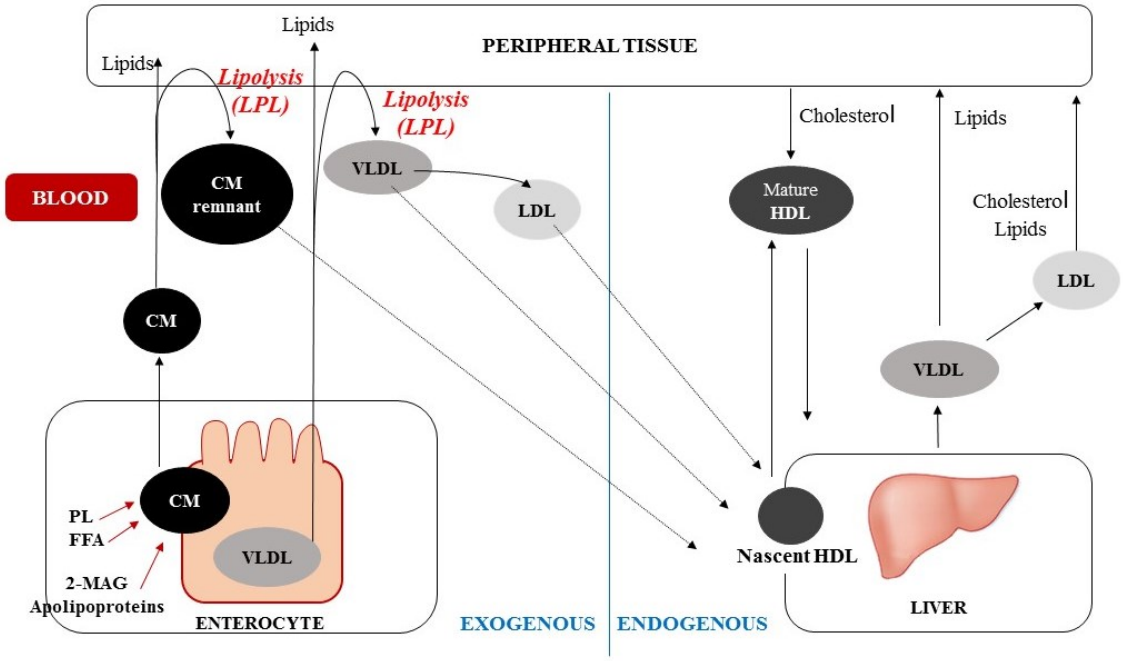

Figure 1.5. Proposed route of lipid transport by lipoproteins in fish. PL: phospholipids CM: chylomicrons; VLDL: very-low-density lipoprotein; LDL: low-density lipoprotein; HDL: high-density lipoprotein; FFA: free fatty acids and 2-MAG: 2-monoacylglycerols **(adapted from Torstensen and Tocher, 2011).**

After LPL has hydrolysed TAG from CM and VLDL, remnants of these two types of lipoprotein can be taken up by the liver but further hydrolytic action leads to the formation of LDL (Tocher, 2003), which are major transporters of cholesterol to peripheral tissues (Babin and Vernier, 1989; Lie, 1993) (**Figure 1.5.**). If excess surface constituents (phospholipids, cholesterol and apolipoproteins) are absorbed by the liver, they emerge as nascent HDL particles. They can take up free cholesterol from peripheral tissues which is then esterified by the action of the enzyme lecithin:cholesterol acyltransferase (LCAT) resulting in the production of mature HDL (Caballero et al., 2006). Thus, they provide a reservoir for cholesterol, accepting cholesterol from peripheral tissues and transporting it to the liver for excretion, degradation or reutilization. As in mammals, the relative proportions of the plasma lipoproteins in fish can vary among species, but is a constant characteristic of each species depending on the dietary status (Tocher, 2003).

Excess dietary FA are stored in the form of TAG in specific lipid storage sites. The primary site for long-term storage in many fish is the mesenteric adipose tissue, although some fish also store significant amounts of fat within the white (light) muscle and between

skin and muscle (Henderson and Tocher, 1987). Red (dark) muscle contains most of the lipid within the muscle fibres (Sheridan, 1994). Moderate amounts of lipid can also be stored in liver in many fish, although this is generally shorter-term storage (Tocher, 2003).

1.3.3.3. Lipogenesis

Lipogenesis refers to the biosynthetic reactions for the formation of new endogenous lipid. Although adipose tissue has some lipogenic capability in rainbow trout, liver is the principal site of lipogenesis in this species (Henderson and Sargent, 1985).

The key pathway is catalysed by the cytosolic fatty acid synthetase (FAS) multienzyme complex and their main products are the SFA palmitic (16:0) and stearic (18:0), which can be biosynthesized *de novo* by fish (Sargent et al., 1989). The rate of lipogenesis is regulated by a number of dietary/nutritional factors, especially in freshwater fish. In particular, dietary lipid suppresses lipogenesis (Tocher, 2003).

Lipid depots accumulated by fish that naturally consume diets rich in lipid and almost all predator marine fish will be derived largely or exclusively from dietary lipid. However, this does not mean that marine fish are not capable of biosynthesizing FA *de novo* (for instance by chain-elongating or chain-shortening dietary FA). The situation is different in freshwater fish since lipid-rich prey are less common in fresh water than in marine water and this is reflected in their substantial lipogenic activity (Henderson and Sargent, 1985).

Fish are capable of desaturating 16:0 and 18:0 to yield, by means of the Δ9 FA desaturase, 16:1n-7 (palmitoleic acid) and 18:1n-9, respectively (Fig. 1.3) . However, they lack Δ12 (ω6) and Δ15 (ω3) desaturases and therefore cannot form 18:2n-6 and 18:3n-3 from 18:1n-9. Thus, the presence of 18:2n-6 and 18:3n-3 is essential in their diets, as for all vertebrates. These dietary EFA can be desaturated further and elongated to form the physiologically essential C20 and C22 PUFA (20:4n-6, 20:5n-3 and 22:6n-3). The degree to which a specie can perform these conversions is dependent on the relative activities of FA elongases and desaturases, such as Δ6 and Δ5, in their tissues and these activities are related to the extent to which the species can or cannot readily obtain the end product 20:4n-6, 20:5n-3, and 22:6n-3 from their natural diets (Tocher, 2003).

1.3.3.4. Lipid mobilization

TAG stored in adipose tissue can be used when the energy requirements of the animal exceed the energy available from the diet and particularly when the energy requirements of the animal are very high (i.e. periods of starvation, reproduction, etc) (Tocher, 2003). In salmonids and various other fish species under a situation of high energy requirement, lipid mobilization from liver and adipose tissue is under β-adrenergic control with adrenalin and noradrenalin stimulating TAG hydrolysis and an increase in plasma FFA, as in mammals (Sheridan, 1994; Fabbri et al., 1998). Other hormones also stimulate lipid mobilization in depot organs and especially in liver, this including glucagon, cortisol, adrenocorticotropic hormone, growth hormone, somatostatin, thyroxine and prolactin, resulting in TAG breakdown and secretion of FFA, whereas insulin inhibits it (Sheridan, 1994). Mobilized FA are transported from adipose tissue stores mainly bound to the albumin-like protein in plasma (De Smet, 1978).

The catabolism of FA (β-oxidation) involves the sequential cleavage of two-carbon units, released as acetyl-CoA through a cyclic series of reactions catalysed by several distinct enzyme activities (Tocher, 2003). It can take place in the mitochondria and also in the peroxisomes (Frøyland et al., 1998), although peroxisomal β-oxidation accounts for less than 10% in Atlantic salmon (Frøyland et al., 1999).

Red muscle, liver, and heart are generally regarded as the most important tissues of β-oxidation in fish (Henderson and Tocher, 1987) and SFA and MUFA seem to be the preferred substrate (Henderson, 1996).

1.4. Factors affecting fat digestibility, absorption and metabolism

Formulation of practical diets of high nutritional value and availability to the fish is of high importance to the intensive aquaculture. Measurement of digestibility of dietary nutrients provides the first indication of their nutritional value (Cho and Kaushik, 1990) and aqua feeds are highly digestible because they contain mainly sources of protein and lipids (Sanz, 2009).

Regarding dietary lipid, its digestibility is generally assumed to be very high, even though there are certain differences in the utilization of the various FA. Molecules present in fats and oils have particular physicochemical properties that contribute to their behaviour and that can affect not only their digestibility but also their absorption and

metabolism. As indicated by Gunstone (2006), physicochemical but also nutritional properties of fats are limited by their composition of FA and the stereochemistry of the TAG. They are also affected by the lipid class composition of the fat, as has been reported for humans and other animal species**.**

1.4.1. Fatty acid chain length and degree of unsaturation

Digestibility of the individual FA in fish is highly dependent on both chain length and degree of unsaturation. In general, digestibility is known to decrease with increasing chain length and to increase with unsaturation (Ringø, 1989, 1991; Sigurgisladottir et al. 1992). This has been related to the melting point of the individual FA, with absorption decreasing with increasing melting point (Lied and Lambertsen 1982; Sigurgisladottir et al. 1992).

In practical diets, dietary lipids are mainly formed by mixtures of TAG, not individual FA. Digestibility of TAG appears to be dependent both on the rate of release of FA from TAG (lipolysis) and on the rate of their subsequent absorption.

Both chain length and degree of unsaturation may have an influence on lipolysis.The release of FA from TAG seems to decrease with increasing chain length (Lie and Lambertsen, 1985; Gjellesvik, 1991; Gjellesvik et al., 1992; Olsen and Ringø, 1997). Medium-chain lauric acid (12:0) was a good substrate for digestive lipase and so it was also efficiently absorbed, myristic (14:0) and palmitic acids being intermediate and stearic being relatively resistant to hydrolysis (Olsen et al., 1998). Also, the following specificity for lipolysis of dietary TAG: PUFA>MUFA>SFA has been suggested (Olsen and Ringø, 1997). Certainly, lipases in fish lumen are known to have high specificity towards PUFA (Lie et al., 1987; Gjellesvik et al., 1992; Koven et al. 1994). PUFA are efficiently released from TAG and thus might be absorbed at very high rates compared with LC-SFA and MUFA (Olsen et al., 1998). In view of all this, both the process of lipid digestion and absorption seem to favour the utilization of UFA over their more saturated counterparts, and shorter-chain FA over longer-chain FA (Lie et al. 1987; Lie and Lambertsen, 1991; Ringø and Olsen, 1991; Sigurgisladottir et al. 1992; Koven et al. 1994).

In terms of utilization of FA, studies suggest that SFA and MUFA are preferred over PUFA as substrates for β-oxidation in fish. Especially nonessential FA as palmitic, palmitoleic, oleic, cetoleic acid (22:1n-11) and linoleic acids seem to be preferentially mobilized during starvation, while DHA is oxidized at low rates (Kiessling and Kiessling 1993; Henderson 1996). Within dietary PUFA, Torstensen et al. (2000, 2004) reported that the degree of retention in white and red muscle in Atlantic salmon gradually decreased for the following FA: C18:1n-9>18:2n-6>22:1n-11>18:3n-3,whereas DHA is effectively retained in all tissues and lipoproteins

Therefore, nonessential FA were readily catabolized, whereas EFA were retained in increasing degree with decreasing dietary levels but also varying with tissue. In spite of this, the degree of retention or catabolism of a FA was dependent also on its dietary level and type of tissue and not only on the type of FA (Torstensen et al., 2004).

Finally, dietary FA composition seems to have an influence also on plasma lipids. It has been reported that SFA increase plasma cholesterol levels in mammals and humans (Grundy et al., 1990) and that high levels of oleic acid and linoleic acids in diets decrease plasma total cholesterol and LDL-cholesterol (Fernández and West, 2005; Grundy and Denke, 1990). Similar results are had been described also in fish (Richard et al., 2006).

1.4.2. Fatty acid position within the acylglycerol molecule

Several studies and reviews including both humans and animals have reported that, in addition to chain length and degree of unsaturation, the position of FA on TAG molecules may be an important determinant of digestibility and metabolism of oils and fats (Small, 1991; Bracco, 1994; Innis, 1997; Karupaiah and Sundram, 2007). It may influence lipid absorption from the intestine, metabolism in enterocytes, subsequent chylomicron metabolism and distribution into tissues (Mu and Høy, 2004; Berry, 2009). Therefore, nutritional, chemical and physical properties of fats and oils may be limited by the FA composition and their position within the TAG molecule (Gunstone, 2006).

As described before, during lipid digestion in humans and mammals, pancreatic lipase hydrolyses the external positions (sn-1 and sn-3) of TAG producing 2-MAG and FFA. 2-MAG will be incorporated into the micelles and directly absorbed, whilst the fate of FFA will depend on their nature: free MUFA or PUFA will be mainly incorporated into

micelles and absorbed, while LC-SFA however, are likely to end up unabsorbed (Hunter, 2001; Small, 1991). When released in free form, LC-SFA are likely to form insoluble soaps with divalent cations such as calcium and magnesium, which makes them unavailable for absorption (Hayes et al., 1994). In fish, this may be especially significance in marine species, as sea water is rich in calcium and magnesium and so fish have to drink large amounts of water (Olsen et al., 1998).

Thus, the fate of a FA after lipolysis will depend not only on its type but also on its position within the TAG molecule.

The superior absorption of FA when located in sn-2 has been demonstrated mainly in studies in which LC-SFA had been relocated from the external positions of dietary TAG to the sn-2 position in order to make the fat more digestible for human infants (Carnielli et al., 1995; Kennedy et al., 1999) or animals (Lien et al., 1993; Innis and Dyer, 1997; Lin and Chiang, 2010). Contrarily, studies in chickens (Smink, 2008; Vilarrasa et al., 2015c) and piglets (Vilarrasa et al., 2015a) fed diets containing oils with a high proportion of SFA in sn-2 did not obtain an improvement in digestibility and absorption.

In fish, it is not known if differences on the positional composition of dietary fats and oils would have similar effects as those observed in humans and mammals due to controversial of the specificity of digestive lipase. However, the fact that 2-MAG seemed to be the preferential substrate used in enterocytes to resynthesize TAG (Oxley et al., 2007) might suggest that similar effects of the FA position within TAG molecules to those observed in in humans and animals might be expected also in fish.

In terms of metabolism, it has been reported that the FA located in sn-2 in the ingested TAG has a high preservation during digestion, absorption and biosynthesis of TAG in the enterocytes of humans and animals (Innis et al., 1995; Kubow, 1996; López-López et al., 2001). In light of this, and as reviewed by Berry (2009), the presence of SFA in the sn-2 position of TAG in CM has been shown to slow down TAG lipolysis (Mattson et al., 1979; Redgrave et al., 1988) and the clearance of CM and CM remnants in rats (Mortimer et al., 1990; 1992) compared with when they are located in the external positions of TAG. A delayed CM remnant clearance may also contribute to the appearance of atherosclerosis (Lambert et al., 1996). Furthermore, it has been reported that SFA in sn-2 may have hypercholesteraemic effects, while for oleic acid and PUFA a reduction levels

of plasma LDL-cholesterol in mammals was observed (Grundy and Denke, 1990; Fernandez and West, 2005). FA located in sn-2 may be preferentially transported to the liver due to the positional specificity of LPL for the sn-1 and sn-3 positions, at least in mammals. Because the hepatocyte is the major site of action of LDL metabolism, when SFA are located in sn-2 they may elevate LDL-cholesterol concentrations more than if they were located in the external positions (Botham, 2008).

1.4.3. Lipid class composition

1.4.3.1. Mono- and diacylglycerols

Although edible fats and oils from both animal and vegetable origin are mainly constituted of TAG (Flickinger and Matsuo, 2003) they contain MAG and DAG as minor components (D'Alonzo et al., 1982; Destaillats et al., 2010). They can also be produced by chemical or enzymatic routes, generally obtained from the glycerolysis of TAG, the hydrolysis of TAG or the direct esterification of glycerol with FA (Fregolente et al., 2010).These partial acylglycerols have been achieving increasing popularity from different perspectives in the recent years (Martin et al., 2014). The most popular function of these lipids is related to their amphiphilic nature (they combine a hydrophilic and a hydrophobic portion) and surface-active properties, being well-known as emulsifier ingredients in the food, pharmaceutical, cosmetic (Kaewthong et al., 2005) and plastic (Coteron et al., 1998) industries.

In the food grade area, purified MAG and mixtures of MAG and DAG represent the major emulsifying agents in the worldwide market due to their affordable price and proper performance (Fregolente et al., 2008). Partial acylglycerols are also attractive lipids in the formulation of potential vehicles of drugs and bioactive compounds of poor solubilisation (Pouton and Porter, 2008), as well as valuable precursors for the production of diverse structured lipids (Pfeffer et al., 2007; Wang et al., 2013). For instance, DAG can be used not only like emulsifiers together with MAG, but with a nutritional purpose. As reported by many studies and reviewed by Rudkowska et al. (2005), oil rich in DAG exhibits beneficial effects mainly at the level of a reduction of body mass in both humans (Taguchi et al., 2001; Kim and Park, 2011) and animals (Murase et al., 2002). Their presence produces increased β-oxidation, enhanced body weight loss, suppression of body fat accumulation and decreased postprandial serum TAG levels. In fish, however, a study in

rainbow trout fed a diet containing a higher amount of DAG than the rest did not report reduction in fat accumulation (Skall Nielsen et al., 2005).

In spite of their rising use and taking into account their excellent emulsifying properties, a few studies focusing on their possible effects on lipid digestibility have been performed in animals. Garrett and Young (1975) reported the efficiency of 2-MAG of oleic acid (mono-olein) in increasing the absorption of free SFA (palmitic acid) in broiler chickens. In a similar way, the dietary addition of MAG to tallow produced an increase in SFA and total FA digestibility in weanling pigs (Jones et al., 1992). In contrast, Vilarrasa et al. (2015a) did not obtain significant differences in total FA apparent digestibility or in animal performance in piglets fed a diet containing palm oil rich in MAG and DAG. Therefore, the possible effects of partial acylglycerols on digestion and absorption in animals remain still unclear.

1.4.3.2. Free fatty acids

A high content of FFA, and especially free SFA, has been related to low digestibility values in different animal species when present in diets (Wiseman et al., 1991; Powles et al., 1994; Vilà and Esteve-García, 1996). This was probably because, as previously reported, they have high melting points and thus low solubility, along with tendency to form insoluble soaps in the intestine.

In fish, however, there are very few studies assessing the effects of feeding diets with a high content of FFA. In spite of this, three studies evaluating the dietary incorporation of rich-in-FFA palm oils (palm fatty acid distillates) did not report differences in feed efficiency when compared to a native oil diet (Bahurmiz and Ng, 2007; Ng et al., 2010; Ailyu-Paiko and Hashim, 2012). Moreover, Ng et al. (2010) observed an easier absorption when FA where in free form than when being part of TAG molecules. Authors suggested an increased luminal micellar solubilisation of FFA and a lower melting point of palmitic acid when presented as a FFA than when in TAG as possible causes for these results.

1.5. Fish oil replacement

1.5.1. The fish oil issue

Feed is the largest production cost in commercial aquaculture and thus improving feed efficiency in industrial systems has always been a priority (Naylor et al., 2000; 2009). Feeds for farmed carnivorous species were traditionally based on fishmeal and fish oil (FO). Back in the 1980s, most of the feed resources needed for the cultivation of both carnivorous and omnivorous fish, as well as crustaceans, originated from pelagic forage ("feed") fisheries (Olsen, 2011).

The major characteristic of FO is the high level of n-3 LC-PUFA, essential for the optimal growth and health of farmed fish (Turchini et al., 2009) (**Table 1.4.**). Thus, its use as major ingredient in aquafeeds, together with fish meal, was good for the supply of these n-3 LC-PUFA (Sargent et al., 2002). However, it was also an unsustainable practice due to it relied on finite marine resources derived from capture fisheries (Naylor et al., 2000; Tacon and Metian, 2009). Furthermore, the growing demand of FO not only for aquaculture but also for terrestrial animal nutrition and human consumption through the production of high-quality food (IFFO, 2013) (**Figure 1.6.**) generated a steady increase in its price (FAO, 2014).

Table 1.4. Fatty acid composition (% total fatty acids) of fish oils, vegetable oils and animal fats used in fish feed formulations (adapted from Turchini et al., 2009).

	Fatty acid (%)								
Oils/fats	SFA	MUFA	Linolenic	ARA	α-linolenic	EPA	DHA	n-6 PUFA	n-3 PUFA
Fish oils									
Anchovy	28.8	24.9	1.2	0.1	0.8	17.0	8.8	1.3	31.2
Capelin	20.0	61.7	1.7	0.1	0.4	4.6	3.0	1.8	12.2
Menhaden	30.5	24.8	1.3	0.2	0.3	11.0	9.1	1.5	25.1
Herring	20.0	56.4	1.1	0.3	0.6	8.4	4.9	1.4	17.8
Cod liver	19.4	46.0	1.4	1.6	0.6	11.2	12.6	3.0	27.0
Vegetable oils									
Crude palm	48.8	37.0	9.1	-	0.2	-	-	9.1	0.2
Soybean	14.2	23.2	51.0	-	6.8	-	-	51.0	6.8
Canola/rapeseed	4.6	62.3	20.2	-	12.0	-	-	20.2	12.0
Sunflower	10.4	19.5	65.7	-	-	-	-	65.7	0.0
Cottonseed	45.3	17.8	51.5	-	0.2	-	-	51.5	0.2
Groundnut	11.8	46.2	32.0	-	-	-	-	32.0	0.0
Corn	12.7	24.2	58.0	-	0.7	-	-	58.0	0.7
Linseed	9.4	20.2	12.7	-	53.3	-	-	12.7	53.3
Animal fats									
Beef tallow	47.5	40.5	3.1	0.4	0.6	-	-	3.1	0.6
Pork lard	38.6	44.0	10.2	-	1.0	-	-	10.2	1.0
Poultry fat	28.5	43.1	19.5	-	1.0	-	-	19.5	1.0

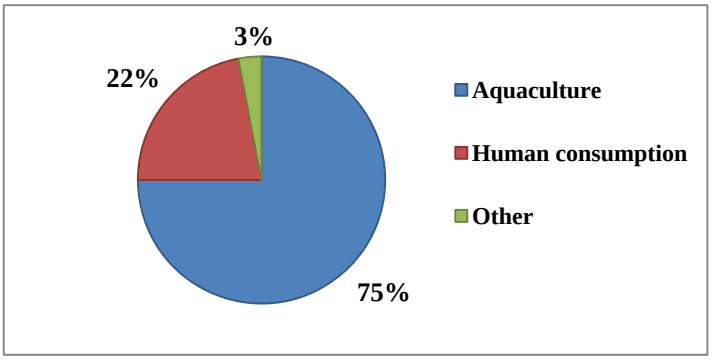

Figure 1.6. Major fates of fish oil in 2012 (adapted from Tocher, 2015).

As a consequence, finding a way to uncouple the need of aquaculture for wild fisheries became urgent (Turchini et al., 2009) and was one of the main priorities of the sector already in the late 1990s (Naylor et al., 2000). Hence, the growth of aquaculture was directed to the dependence upon developing more sustainable feeds with alternative ingredients, generally derived from terrestrial agriculture (Gatlin et al., 2007; Tocher, 2009). In light of this, even though aquaculture still represents the best use of bulk FO to date (around 75% of total global supply), the use of FO in aquaculture remained relatively stable over the last decade (IFFO, 2013) and thus the proportion of fisheries production converted into FO is in decline (FAO, 2014). This is in part due to the increased regulation, the more stringent quotes on raw materials and, to a lesser extent, the rising use of seafood and fish by-products to produce FO (FAO, 2012; 2014).

1.5.2. Vegetable oils as fish oil replacers

Intensive research activities have been conducted globally in order to evaluate alternative lipid sources and the substantial amount of research in this area has been reviewed by Miller et al. (2008), Glencross (2009) and Turchini et al. (2009; 2011c). The challenge for fish production is to maintain, if not improve, the recognised benefits of fish for human health while simultaneously seeking to maximize sustainability, fish health and economic benefits.

To be a viable alternative to FO, a candidate ingredient must possess certain characteristics such as nutritional suitability, ready availability and ease of handling, shipping, storage and use in feed production. In addition, feeds are selected on the basis of fish health and performance, consumer acceptance, minimal pollution and ecosystem stress, and human health benefits. Finally, competitive pricing is essential for the adoption of non-fish alternatives in feeds (Naylor et al., 2009), the decision to use them being usually dictated by their current market price compared with FO (Turchini et al., 2009).

For years, VO have received considerable attention as substitutes for FO because of their steady rising production since the early 2000s (USDA, 2015) (**Figure 1.7.**) high availability and economically viable costs. Therefore, several studies have been carried out to investigate a wide diversity of VO as possible sustainable partial substitutes for FO in compounded fish feeds. The most common VO used in fish feed production have been

soybean, linseed, rapeseed, sunflower, palm oil and olive oil (Nasopoulou and Zabetakis, 2012), of which palm and soybean were the most produced in 2014-2015 (**Figure 1.8.**). Each of these oils has been trialled as a lipid source in aquaculture feeds with varying success.

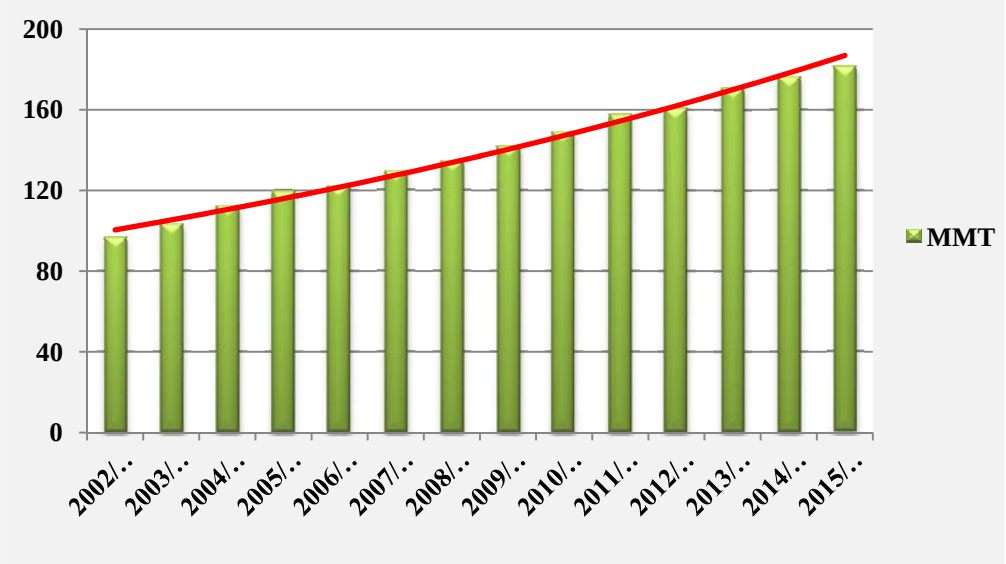

Figure 1.7. World major vegetable oils (copra, cottonseed, palm kernel, peanut, rapeseed, soybean and sunflower) production (Million Metric Tonnes, MMT) between 2002/2003 and 2015/1016 (adapted from USDA, 2015).

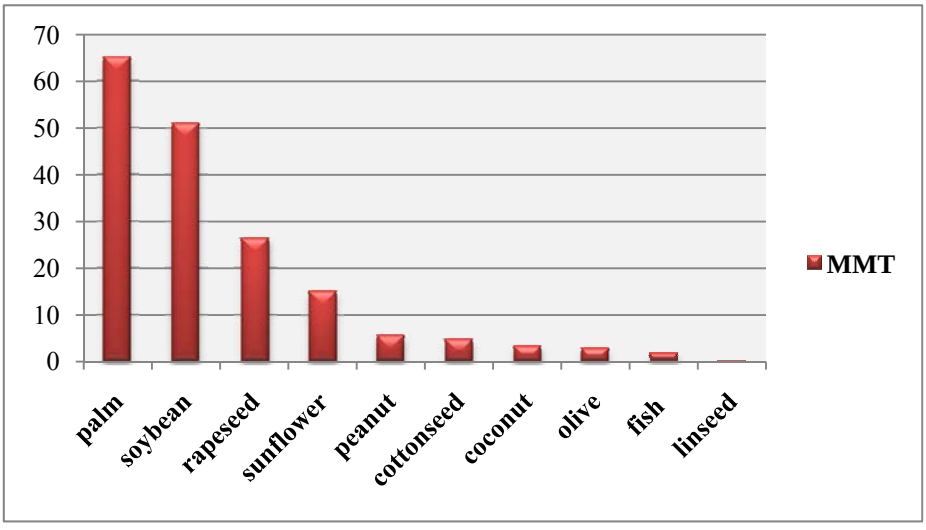

Figure 1.8. World major vegetable oils production (Million Metric Tonnes, MMT) in the period 2014-2015 (adapted from FAO, 2015 and USDA, 2015).

1.5.2.1. Effects of the replacement

The replacement of FO by VO in aquafeeds changes the dietary FA composition, which might limit their dietary inclusion. Changes may influence the fish lipid digestibility and metabolism (transport, uptake, lipogenesis, FA desaturation, FA elongation, eicosanoid synthesis and catabolism), as well as the tissue lipid composition. This may have direct consequences on fish overall performance, health and/or final product quality.

Although digestibility can vary to a large extent depending on the FA composition of the dietary lipid source, numerous studies assessing the use of VO, either singly or blended, have reported irrelevant effects on lipid digestibility and growth performance (Caballero et al., 2002; Pettersson, 2009; Bell et al., 2010; Sun et al., 2011; Masiha et al., 2013; Qingyuan et al., 2014). Contrarily, many of these studies have encountered changes in the FA composition of fillets. Certainly, the dietary FA composition is mirrored in the fish organs and lipid stores (Olsen et al., 1999; Bell et al., 2001, 2003; Caballero et al., 2002; Tocher et al., 2003; Torstensen et al., 2004, 2005; Fonseca-Madrigal et al., 2005; Nanton et al., 2007; Stubhaug et al., 2007; Fountoulaki et al., 2009; Pettersson et al., 2009; Yildiz et al., 2015). This effect have been reported in almost all finfish species studied to date, from carnivorous to herbivorous species and from marine cold water to tropical freshwater species fed with VO, terrestrial animal fats or blends (Izquierdo et al., 2003, 2005; Regost et al., 2003; Turchini et al., 2003, 2006, 2007; Menoyo et al., 2004; Montero et al., 2005; Mourente et al., 2005; Francis et al., 2006, 2007; Ng et al., 2006; Bahurmiz and Ng, 2007; Mørkøre et al., 2007).

The FA composition of VO might pose a problem and limits the sole use of these alternative lipid sources. They are abundant in n-6 and n-9 PUFA, but most of them are relatively poor sources of n-3 PUFA compared to FO and, as previously mentioned, n-3 HUFA are essential for the growth of healthy fish (Turchini et al., 2009; Sales and Glencross, 2011). Thus, replacement of FO by VO would be possible when FA are present in the diets in sufficient quantities to meet their EFA requirements (Turchini et al., 2011a; Nasopoulou and Zabetakis, 2012).

Changes in the FA composition of the whole fish, organs and fillet in fish fed VO will have a direct effect on the fillet nutritional quality, particularly related to the contents

of n-3 LC-PUFA (Rosenlund et al., 2011). Even though this is the most pronounced effect of replacing FO, authors have reported changes also on physico-chemical fillet quality aspects. Literature indicates limited effects on fillet texture and gaping and changes in liquid holding capacity and color in flesh of fish fed diets containing VO (Bjerken et al., 1997; Regost et al., 2004; Ng and Bahurmiz, 2009). However, except the overall agreement on the reduction in oxidation values in fillets of fish fed VO, results on physico-chemical quality are rather scarce and in some cases not consistent (Rosenlund et al., 2011).

1.5.2.2. The vegetable oils issue: possible alternatives

Most of the VO studied as possible FO substitutes in aquafeeds are used for human food consumption and some of them also for animal feed, as well as for the production of useful materials such as personal care products, paint and lubricants in the oleochemical industry. Moreover, in recent years, VO have been burnt to make electricity and have been converted to methyl esters for use as biofuels, including ethanol and biodiesel (mainly rapeseed, sunflower, palm and soybean) (Demirbas, 2006; Gunstone, 2011a).

The worldwide rising use of conventional food grains and oilseeds to produce biofuel for use as a "greener" petroleum substitute arose as a possible solution to the increasing petroleum costs and concern for the climate (FAO, 2008). However, it has led to less grains and crops being available for food and feed. This fact, together with the increased demand in order to feed a population that is also increasing, has resulted in increased prices (Behr and Gomes, 2010; Gunstone, 2011a; Tacon et al., 2011). Certainly, in recent years, the price of VO has become a serious issue (Gunstone, 2011b).

Given the above and in relation with aquaculture, finding suitable and economically interesting alternatives to the commonly used VO in fish nutrition will be the next step in view of the growing competition among industries.

1.5.2.2.1. Industrial fatty by-products: acid oils

Crude or unrefined oils extracted from oilseeds (native oils) generally consists of TAG (generally>95%) along with DAG, MAG and FFA; minor components such as phospholipids, sterols and sterol esters, tocols (tocopherols and tocotrienols) and hydrocarbons; and agricultural residues and compounds resulting from the environment in

which the plant was grown (Gunstone, 2011b). They are refined to remove most of these and eliminate undesirable odour, flavour and colour but at the same time retain the beneficial components such as vitamins, provitamins and antioxidants (Gunstone, 2011b; Ng and Gibon, 2011).

Refining can be chemical or physical, the principal difference between the two routes being the removal of FFA. In physical refining most of the FFA are removed during the deodorization process, while in chemical refining most FFA are removed during the alkali neutralization process (Haslenda and Jamaludin, 2011) (**Figure 1.9.**). A significant amount of "waste" by-products are produced from crude oil refining processes. Moreover, with the expected increase in the consumption of edible oils, the production of these by-products was also expected to rise (Dumont and Narine, 2007). Some of these compounds, however, are valuable in their own right and may be recovered for subsequent use (Gunstone 2011b). In order to convert the "waste" products to value added materials, however, economically feasible chemical modification, identification and separation techniques had to be developed (Dumont and Narine, 2007).

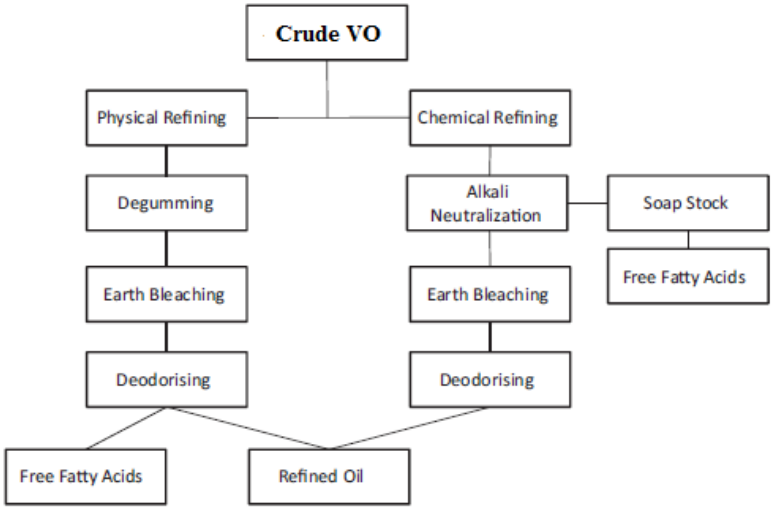

Figure 1.9. Vegetable oil refining process. VO: vegetable oil **(adapted from Junior et al., 2012).**

Acid VO are the most important quantitatively by-product obtained from the refining of crude VO that could represent an economically interesting alternative to these native oils (Parini and Cantini, 2009). Acid VO are rich in FFA, the amount of which depends on the refining process utilized. Those coming from the physical method having a higher FFA content (>90%) than those from the chemical refining (40-60%). As reported by Nuchi et

al. (2009) in the framework of a project on the characterization of fat co- and by-products from the food chain used in feeds and their effects on animal production (Feeding Fats Safety, http:// www.ub.edu/feedfat/), acid VO from chemical refining seemed to be valuable for feeding uses. Furthermore, because of their price is lower than that of their original native oil, they could be an economically interesting alternative to the conventional oils. Even though acid VO could have detrimental effects on digestibility due to their richness in FFA, as previously reported, their inclusion in diets for fattening pigs did not produce differences in digestibility or growth when compared to the inclusion of native oils (Vilarrasa et al., 2015b).

1.5.2.2.2. Structured lipids: re-esterified vegetable oils

Structured lipids can be defined as TAG that have been modified by the incorporation of new FA, restructured to change the positions of FA or the FA profile from the natural state or synthesized to yield novel TAG (Sahin et al., 2005).

The food industry utilises fats and oils with a wide range of functional properties such as melting point, crystallisation characteristics, solid fat content, plasticity, consistency, oxidative stability, etc. Many of the natural edible fats and oils do not possess these properties naturally, resulting in the development of various modification processes. Modification can be carried out either physically, by fractionation or blending, or chemically by hydrogenation or interesterification (Azadmard-Damirchi and Dutta, 2008).

The synthesis of structured lipids allows for the supply of the desired FA and TAG profiles. Interesterification is also used to achieve more efficient delivery of specific FA for nutritional and medical purposes, allowing for the introduction of essential FA into oils, which may be useful in the treatment of certain clinical disorders (Hamam et al., 2005). However, as previously mentioned, not only the type of FA but also its position on TAG will determine whether or not it will be absorbed.

Interesterification reactions involve FA redistribution between and within TAG molecules until a thermodynamic equilibrium is reached (Idris and Mat Dian, 2005; Karabulut et al., 2004). The resulting products maintain the FA profile and saturation degree of the starting blends (Karabulut et al., 2004; Rodrigues and Gioielli, 2003) but present a different TAGs stereochemistry which results in new physico-chemical

characteristics and nutritional properties (Klinkesorn et al., 2004; Xu et al., 2006). There are two types of interesterification reactions, depending on the catalyst: enzymatical and chemical (Sahin et al., 2005).

- Enzymatic interesterification uses commercial lipases from plant, animal and microbial sources. It is driven under milder temperature conditions (Rodrigues and Gioielli, 2003; Criado et al., 2007), undergoes fewer deleterious side reactions and produces fewer by-products (Chu et al., 2001; Wang et al., 2006) than the chemical reaction. However, its most important characteristic is the regiospecificity. For instance, lipases may hydrolize the sn-1 and sn-3 positions, incorporating FA at these sites without changing the FA in the sn-2 position (Wang et al., 2006).
- Chemical interesterification: FA are exchanged within (intra) and among (inter) TAG until a thermodynamic equilibrium is reached producing, in theory, a complete randomization of acyl groups in TAG (Coenen, 1974; Ferrari et al., 1997). Thus, it modifies the physical properties of lipids by rearranging the distribution of FA on the glycerol backbone without changing FA profiles (Xu et al., 2006). For instance, in the case of palm oil, it increases the proportion of palmitic acid located at the sn-2 position of TAG (Vilarrasa et al., 2014). Industrial chemical interesterification processes are performed at high temperatures and can be carried out using sodium methoxide (0.2-0.3%) or an alkali metal (0.1-9.2%) as a catalyst (Anderson, 1996).

Chemical interesterification has some advantages over enzymatic modification, which include lower catalyst cost, use of existing and more available industrial procedures and equipment, and shorter reaction times (Konishi et al., 1993). However, it depends on the initial quality of the oil. FFA and water concentrations need to be less than 0.1 and 0.01%, respectively. If these levels are exceeded, more catalyst is required because it is used up initially by residual water and neutralizing FFA (Posorske et al.,1988). Chemical interesterification has the potential to be applied to the nutritional improvement of fats and oils, mainly to increase the proportion of specific FA in specific positions on the glycerol backbone to improve their bioavailability.

Depending on the nature of the substrates, there are three reactions associated with interesterification: acidolysis, alcoholysis and transesterification (Marangoni and Rousseau, 1995; Xu et al., 2006):

- Acidolysis: reaction between fatty esters and an acid (usually FFA).
- Transesterification: ester-ester interchange.
- Alcoholysis: reaction between a fatty ester and an alcohol. Within alcoholysis reactions, that occurring between a TAG and a glycerol molecule is called glycerolysis. It has been long used for the industrial production of partial acylglycerols (MAG and DAG) (Bornscheuer, 1995), widely used as emulsifiers by different industries.

Glycerol is the main by-product of the biodiesel production; about 10% of the weight of biodiesel is generated in glycerol (Quispe et al., 2013) that has a number of applications in pharmaceutical and food industry (Meurer et al., 2012). The current large amount of glycerol generated may become an environmental problem since it cannot be disposed of in the environment.

A way to take advantage of the acid VO and to reduce, at the same time, the load of these "waste" by-products is to produce **re-esterified vegetable oils** through a glycerolysis-like reaction. In this case, the glycerolysis reaction occurs between an **acid VO** (not a native VO) and glycerol (**Figure 1.10.**). Therefore, the FFA present in the acid VO are re-esterified in the molecule of glycerol during the process in order to form TAG (**Figure 1.11.**), being a way to give both acid VO and glycerol an added value.

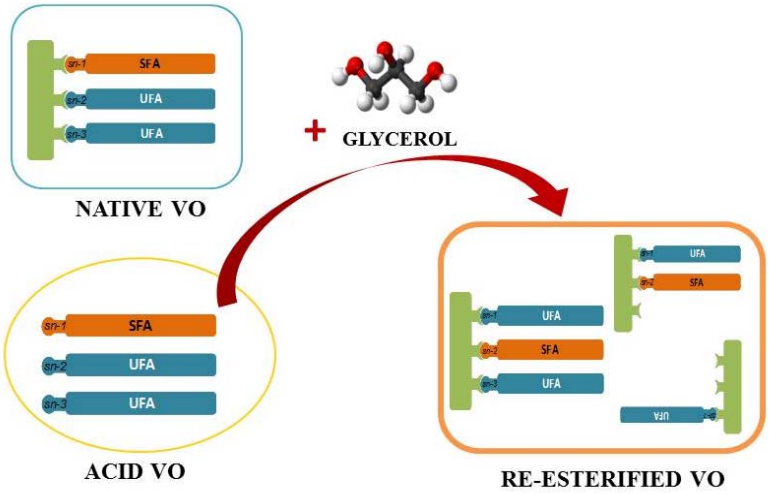

Figure 1.10. Esterification reaction between an acid vegetable oil (VO) and glyvcerol (glycerolysis-like reaction). SFA: saturated fatty acid; UFA: unsaturated fatty acid **(adapted from Parini, personal communication).**

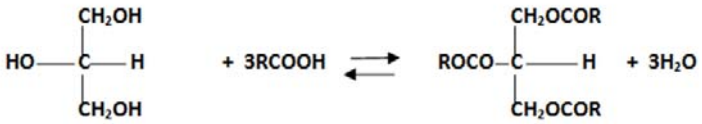

Figure 1.11. Esterification reaction (FFA + Glycerol → TAG) (adapted from Parini, personal communication).

In esterification reactions, performed in a stirred reactor, both the conditions of the process and the type of oil used as starting point will determine the characteristics of the final product. Re-esterified oils used in the present study were obtained with conditions of 190-250ºC and a residual pressure of 1-3 mm Hg, the reaction, which is a balance that can be shifted towards the end products by removing the water from the product, leads to a final mixture of TAG and partial acylglycerols (**Figure 1.12.**). Along with setting specific conditions, the final amount of MAG and DAG can be achieved by determining the glycerol-to-FFA ratio before carrying out the reaction. Hence, glycerolysis reactions can, to a certain extent, favour the formation of partial acylglycerols depending on the reaction conditions such as the glycerol-to-FFA ratio, as well as time, temperature, pressure and the glycerol-to-FFA ratio (Parini, personal communication).

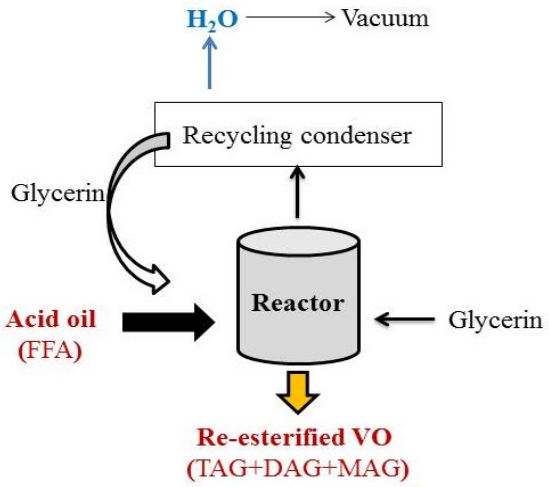

Figure 1.12. Diagram of the esterification reaction process (adapted from Parini, personal communication).

CHAPTER 2

HYPOTHESES AND OBJECTIVES

In fish nutrition, lipids are a source of available energy and essential fatty acids for regular growth, health, reproduction and numerous physiological functions. Moreover, given that fish diets have high lipid contents and thus are energy-dense, the role of lipids in aquaculture has gained importance over time.

The present PhD dissertation is part of a project (ref. AGL2010-22008-C02) aimed at improving the nutritive value of vegetable acid oils by means of re-esterification and at evaluating the use of re-esterified vegetable oils in four monogastric animal species: poultry, swine, dogs and fish.

The need to replace fish oil (FO), the traditional lipid sources used in fish diets, has been explained in the General introduction section. As mentioned, oils from vegetable origin (VO) first received a lot of attention and were deeply studied as sustainable and economically interesting FO substitutes, with good results. However, the growing competition for VO among the food, the feed and the biofuel industries reduced their availability and caused a rise in their prices. Therefore, finding alternative sources to these conventional VO became the next step.

Acid VO are a rich-in-free fatty acid (FFA) by-product from the refining of crude VO that could represent an economically interesting alternative to these native VO and that seemed to be promising for feeding uses. Although some authors pointed out at the high FFA content of these oils as responsible for their low nutritive values, others found them to be potential lipid sources in monogastric animal diets. Hence, its use as lipid sources in animal diets seems to be quite controversial. Moreover, acid VO can be chemically re-esterified with glycerol in order to obtain **re-esterified VO**, constituted of different proportions of mono- (MAG), di- (DAG) and triacylglycerols (TAG). Because MAG and DAG have an emulsifying effect, re-esterified VO could have a higher nutritive value, and thus represent an advantageous lipid source when compared to native VO. In addition, the esterification reaction implies a rearrangement of the FA present in the oil within the TAG molecule. For instance, re-esterified VO would incorporate higher proportions of SFA in the sn-2 position of TAG than the native VO. Hence, these oils acquire new physico-chemical characteristics that can be interesting from the nutritional point of view. To the best knowledge of the authors and to-date, there are no studies in the literature reporting the use of randomly re-esterified VO in fish diets.

Given all the above, the **overall aim** of this PhD dissertation is to assess the use of acid and re-esterified VO as lipid sources in fish diets. We hypothesize that, by means of the chemical esterification with glycerol, acid VO will be given an added value. Likewise, re-esterified VO can be advantageous lipid sources with respect to the native VO, providing nutritional benefits and resulting in improved digestibility and absorption of fat.

For this purpose, different trials with the following **specific objectives** were carried out:

- To assess the effects of dietary re-esterified oils produced from two VO (palm and rapeseed) with different degree of unsaturation and with different MAG and DAG content (low and high), on total fatty acid (FA) digestibility in rainbow trout and gilthead sea bream. *Trials 1 and 2* - ***Chapters 3 and 4***.

- To assess the effects of dietary acid and re-esterified (with a high in MAG and DAG content) rapeseed oils on:

  - total fat and total fatty acid digestibility, growth performance, feed utilization, biometrical parameters, plasma biochemical parameters and morphology of liver and intestine in rainbow trout. *Trial 3* - ***Chapter 5***.
  - fillet FA composition and fillet physico-chemical parameters as indicators of the final product quality in rainbow trout. *Trial 3* - ***Chapter 6***.
  - total FA composition and sn-2 FA composition of liver, fillet and abdominal fat in rainbow trout. *Trial 3* - ***Chapter 7***.

  In order to find the more efficient dietary option, both nutritional and economically, the effects of the inclusion of graded levels of the acid oil with the native or the re-esterified oils on the aforementioned parameters (***Chapters 5, 6 and 7***) were also assessed.

CHAPTER 3

VEGETABLE RE-ESTERIFIED OILS IN DIETS FOR RAINBOW TROUT: EFFECTS ON FATTY ACID DIGESTIBILITY

Vegetable re-esterified oils in diets for rainbow trout: effects on fatty acid digestibility

Trullàs, C.[1*], Fontanillas, R.[2], Tres, A.[3], Sala, R.[1]

[1]Departament de Ciència Animal i dels Aliments, Servei de Nutrició i Benestar Animal (SNiBA), Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain;

[2]Skretting Aquaculture Research Center (Skretting ARC), Sjøhagen 3, Stavanger 4016, Norway;

[3]Departament de Nutrició i Bromatologia-XaRTA-INSA, Facultat de Farmàcia, Universitat de Barcelona, 08028 Barcelona, Spain.

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Abstract

The present study aimed at determining the effect of re-esterified oils with different monoacylgycerol (MAG) content, produced from two different vegetable sources with different degree of saturation, palm and rapeseed, on fatty acids digestibility in rainbow trout. Re-esterified oils were obtained from a chemical esterification process using acid oils (free fatty acids (FFA)-rich by-products from the refining of vegetable oils) and glycerol (by-product of biodiesel production). This process, which produces the formation of triacylgycerols (TAG), reduces the content of FFA present in the acid oil and generates a redistribution of the fatty acids in the glycerol molecule. This redistribution could increase the amount of saturated fatty acids (SFA) located at the sn-2 position of acylglycerols. Moreover, it allows to obtain fats with a certain proportion of MAG, known for being good emulsifiers. Therefore, a higher nutritive value of re-esterified oils than that of acid oils might be expected. A 21-day feeding trial where triplicate groups of rainbow trout were fed nine experimental diets formulated to contain a 21% of different experimental oils was carried out. For each source, four different types of oil were used: native, re-esterified low in MAG, re-esterified high in MAG and acid. A commercial fish oil was used for the control diet. Although re-esterified oils had better apparent digestibility coefficients (ADC) of SFA than their corresponding acid oils diets, no improvement in SFA digestibility was observed in rainbow trout fed re-esterified oils diets compared to those fed native oils diets, not even when a high content of MAG was present. Although this improvement did not occur, both palm and rapeseed re-esterified oils could be incorporated as a fat source in diets for rainbow trout without negatively affecting fatty acids digestibility values. The study concluded that fatty acids digestibility in the experimental oils was more affected by their degree of saturation than by their positional distribution and lipid class composition of the oils.

3.1. Introduction

Nowadays, aqua feeds account for about 75% of the global consumption of fish oil (FO). Its production depends on the availability of wild fisheries, which has decreased since the mid-1990s. In spite of the progressive drop-off in the use of FO by aquaculture, its global demand and price have been increasing due to both the rapid expansion of aquaculture sector and its growing use by the nutraceutical industry. Thus, the price of FO is expected to rise 70% from 2010 to 2030 (FAO, 2014). As a consequence, the use of vegetable oils

(VO) as an alternative source of energy to replace FO in commercial fish feeds has increased. VO are renewable sources produced in large volumes that have a lower price than FO, being palm, soybean, rapeseed and sunflower the most produced (Gunstone, 2011).They are mainly used for food and feed, although its use as feedstock for energy production has increased steadily (Behr and Gomes, 2010; Jayasinghe and Hawboldt, 2012).

Refining is an industrial procedure necessary to render VO to an edible form that has bland flavour and odour, clear appearance, light colour, stability to oxidation and suitability for frying (Brooks et al., 2013; FAO, 1994). It removes compounds other than triacylglycerols (TAG) in order to obtain a TAG-rich oil (by 99%). Some of these other compounds are valuable and can be recovered for subsequent use (Nuchi et al., 2009). They can be used for the production of special feed "technical" lipids such as calcium soaps, hydrogenated lipids, re-esterified, mono- and diglyceride oils which can satisfy specific nutritional requirements (Parini and Cantini, 2008). Acid oils are free fatty acids (FFA)-rich by-products generated from the refining process (Nuchi et al., 2009). They can be considered a cheaper alternative to the use of vegetable native oils. However, studies performed in broiler chickens (Blanch, 1996; Wiseman and Salvador, 1991) reported that acid oils have a lower energy value than that of native oils, which has been related to a lower digestibility due to their high FFA content. The nutritive value of these oils might be improved by means of a chemical esterification process, which generates the formation of TAG and thus reduces the content of FFA. These TAG are formed after the reaction of FFA from vegetable acid oils with glycerol (Parini and Cantini, 2009), the latter being a by-product of biodiesel production. As this TAG synthesis is not selective, it can result in a different fatty acid positional distribution within the TAG molecule than their corresponding native oils. In native VO, SFA (palmitic and stearic) are mainly located in the external positions of the TAG molecules (sn-1 and sn-3) while the sn-2 position contains a high proportion of unsaturated fatty acids (oleic, linoleic and linolenic) (Hunter, 2001; Karupaiah and Sundram, 2007). During lipid digestion in mammals, pancreatic lipase hydrolyses the external positions of the TAG, being 2-monoacyglycerols (MAG) and FFA the main products of the lipid digestion process. FFA that are mono- (MUFA) or polyunsaturated (PUFA) fatty acids will be mainly incorporated into micelles and absorbed. However, impaired digestibility is found for free long chain SFA due to its hydrophobicity, high melting point and the possibility to form insoluble soaps in the gut and thus be lost in

faeces (Berry, 2009; Hunter, 2001; Small, 1991). The fatty acid located in sn-2 remains bound to the glycerol molecule as 2-MAG, which is directly absorbed (Schulthess et al., 1994). In fish, the predominant type and specificity of pancreatic lipase is still quite controversial and seem to vary greatly among species. Even so, a bile salt-dependent pancreatic lipase with sn-1,3-specific hydrolytic activity has been pointed as the main lipolytic enzyme in different species (Bogevik et al., 2007; Gjellesvik et al., 1992; Tocher, 2003). Thus, when a SFA is esterified in the sn-2 position, it may have a superior absorption, as it has been described in rats (Renaud et al., 1995), piglets (Innis et al., 1995), broiler chickens (Smink et al., 2008) and human infants (Kennedy et al., 1999). It could then be expected that the distribution of FA in chemically re-esterified oils resulted in a higher content of SFA in sn-2 position in the TAG and this could result in a higher digestibility of these oils compared to their native counterparts.

Another important difference between re-esterified and native oils could be the proportion of the different lipid classes -TAG, diacylglycerols (DAG) and MAG- that are present in the new re-esterified oil (Parini and Cantini, 2009). Chemical esterification process allows obtaining fats with the same fatty acid profile, but with different content of TAG, DAG and MAG according to the process conditions (i.e. proportions of FFA and glycerol). As it has been pointed out, lipid digestion aims to reduce large lipid molecules (TAG and DAG) to smaller ones (MAG and FFA) for their absorption. Of these lipid classes, MAG have been long known as good emulsifiers due to their amphiphilic nature and surface-active properties (Cruz-Hernandez et al., 2012; Hess et al., 1995; Martin et al., 2014), so digestibility values might improve when a major MAG content is present in the dietary fat.

To the best knowledge of the authors and to-date, there are no studies in the literature reporting the use of randomly re-esterified VO in fish diets. A higher nutritive value of re-esterified oils than of acid oils might be expected as a result of the reduction of the amount of FFA that takes place during esterification. Similarly, changes in their physicochemical properties compared to their corresponding native oils could be obtained due to both the higher amount of SFA in sn-2 and their higher proportion of MAG. Thus, the present study aims at determining the effect of re-esterified oils with different MAG content, produced from palm and rapeseed acid oils, on fatty acids digestibility in rainbow trout (*Oncorhynchus mykiss*) as a first step to determine if they can be suitable fat sources for fish diets.

3.2. Materials and methods

3.2.1. Experimental diets

Nine experimental diets were formulated to contain 48% protein and 21% lipid using the same ingredient composition except for the added lipid source. Oils used for the experimental diets came from two different vegetable sources with different degree of saturation, palm (P) and rapeseed (R). For each source, four different types of oil were used: native oil (N), re-esterified oil low in MAG (EL), re-esterified oil high in MAG (EH) and acid oil (A), all resulting in eight experimental diets (Table 3.1).

Table 3.1. Ingredient formulation and proximate composition of the experimental diets.

	Diets[a]								
	FO	PN	PEL	PEH	PA	RN	REL	REH	RA
Ingredient composition (g kg^{-1})									
Wheat[b]	162.1	162.1	162.1	162.1	162.1	162.1	162.1	162.1	162.1
Wheat gluten[c]	206.9	206.9	206.9	206.9	206.9	206.9	206.9	206.9	206.9
Soya concentrate[d]	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0
Fish meal North Atlantic[e]	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0
Fish oil South America[f]	210.0	0	0	0	0	0	0	0	
Experimental oils[g]	0	210.0	210.0	210.0	210.0	210.0	210.0	210.0	210.0
Yttrium premix[h]	1	1	1	1	1	1	1	1	1
Mineral and vitamin premix[h]	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Proximate composition (g kg^{-1})									
Dry matter (g kg^{-1})	937.7	947.9	948.2	948.2	941.3	945.6	941.0	940.9	940.5
Crude protein	481.5	489.3	483.8	474.4	482.6	485.2	489.8	471.3	471.0
Crude fat	198.8	219.5	213.8	209.9	214.3	211.9	203.7	204.6	212.6
Ash	89.2	88.4	84.3	76.9	94.1	88.5	83.0	83.2	69.7
Gross energy (kJ g^{-1})	23.2	23.4	23.5	23.4	23.7	23.5	23.3	23.3	23.4
Digestible energy (kJ g^{-1})[i]	18.5	17.3	16.6	17.1	16.3	17.9	18.2	18.4	17.8

[a]Experimental diets nomenclature: FO: fish oil (control diet); PN: palm native oil; PEL: palm re-esterified oil low in MAG; PEH: palm re-esterified oil high in MAG; PA: palm acid oil; RN: rapeseed native oil; REL: rapeseed re-esterified oil low in MAG; REH: rapeseed re-esterified oil high in MAG and RA: rapeseed acid oil.
[b]Statkorn, Norway.
[c]Cerestar Scandinavia AS, Denmark.
[d]Selecta, Brasil.
[e]Welcon AS, Norway.
[f]Holtermann ANS, Norway.
[g]Experimental oils.
[h]Skretting standard vitamin and minerals premix, according to requirement data from NRC (2011), Trow Nutrition, The Netherlands.
[i]Values were determined by calculating the apparent digestibility coefficient (ADC) of the gross energy of diets using the formula proposed by Maynard & Loosli (1979), prior to multiplying this value to the gross energy of the corresponding diet.

Commercial fish oil was used for the control diet (FO). Native, acid and re-esterified oils were provided by SILO S.p.a. (Firenze, Italy). Both native palm and rapeseed oils were crude oils. In the process of re-esterification, the proportion fatty acid:glycerol was fixed to obtain a re-esterified oil with a high MAG content (EH oil) and a re-esterified oil with a low MAG content (EL oil). The free fatty acidity was determined following the ISO 660:1996 method. Glycerol was calculated according to the following stoichiometric formula: glycerol weight =fatty acid weight · free fatty acid acidity · glycerol molecular weight/fatty acid molecular weight. Once the proportion fatty acids:glycerol was established, both components were put in the reactor at 190-205ºC of temperature and 1-3 mmg Hg of pressure during 4-6 hours. Feeds were produced at the Skretting Feed Technology Plant (Aquaculture Research Center; Stavanger, Norway) as extruded pellets. Yttrium oxide (Y_2O_3) was added to the diets as an inert marker for the apparent digestibility of fatty acids measure. The ingredient formulation and proximate composition of the diets are shown in Table 3.1. Nutrient composition of experimental diets was determined by standard procedures (AOAC, 2005): moisture (934.01), ash (942.05), crude protein (968.06) and crude lipid (920.39). Gross energy of dried feed was determined using an adiabatic bomb calorimeter (IKA-Kalorimeter system C4000, Jankel-Kunkel, Staufen, Germany). Yttrium was analyzed according to Austreng et al. (2000).

3.2.2. Fish, experimental conditions and sampling

All the procedures were conducted in accordance with the Animal Protocol Review Commitee of the Universitat Autònoma de Barcelona (UAB) and following the European Union Guidelines for the ethical care and handling of animals under experimental conditions (2010/63/EU). The trial was carried out at the Skretting Aquaculture Research Center in Mozzecane, Italy. A total of 567 rainbow trout with a mean initial body weight of 412.7±54g were randomly distributed into 27 cylindro-conical tanks of 600 l of capacity (21 fish per tank) in an open freshwater system with a continuous water flow of 24l min^{-1}. Water temperature (14.3ºC) and dissolved oxygen levels (7.15±0.2mg/l) were maintained constant throughout all the experimental period. The tanks were subjected to a 24h light photoperiod. Fish were fed the experimental diets for 21 days. Each diet was randomly assigned to three replicate tanks and was fed twice a day by automatic feeders, adjusted to provide the 2.5% of biomass daily. Uneaten feed was collected by filtering effluent water from each tank. Collectors were emptied after each meal and feed intake was recorded daily. At the end of the experimental period, all the fish from each tank were anaesthetized

with clove oil (Phytosynthese, Za de Mozac-Volvic, France; 0.04ml/l) and faecal samples were collected from the hindgut by manual stripping. After faecal stripping, fish were put into tanks supplied with freshwater to recover from anaesthesia. Faecal samples were pooled by tank and stored at -20°C prior to analysis of yttrium oxide, fatty acid composition and gross energy.

3.2.3. Fatty acid composition

Fatty acid composition of oils, diets and faeces were determined by gas chromatography-flame ionization detector (GC-FID). For experimental oils, the fatty acid methyl esters (FAME) were previously obtained as described by Vilarrasa et al. (2014). For diets and faeces, FAME were obtained by direct methylation, according to Meier et al. (2006) and analyzed using an HP 5890A gas chromatograph. In both cases, fatty acid methyl esters were identified by comparison of their retention times with those of known standards, and quantified by internal normalization (FAME peak area/total FAME area, in %).

3.2.4. Lipid class composition of the experimental oils and diets

Lipid class composition (TAG, DAG, MAG and FFA) of oils and diets were determined by size-exclusion chromatography on an Agilent 1100 series HPLC chromatograph equipped with a Refractive Index Detector (RID) set at 35Cº. Oils were melted at 55ºC prior to analysis, and a solution of approximately 10 mg of oil/ml of tetrahydrofurane was prepared. The solution was filtered through a Nylon filter (0.45μm) and injected (20μL loop) to the chromatograph equipped with two Styragel columns (StyragelHR 1 and Styragel HR 0.5) of 30cm x 0.78cm i.d., filled with a spherical styrenedivinylbenzene copolymer of 5μm particle size (Water Associates, Milford, MA, USA), connected in series and placed in an oven set at 35°C. The mobile phase consisted of tetrahydrofuran at 1ml/min. For diets, fat was previously extracted with diethyl ether following the method 2003.05 from AOAC (2005).

The different isomers of DAG (1(3),2-DAG and 1,3-DAG) and MAG (1(3)-MAG and 2-MAG) fractions were determined by high-resolution ^{1}H-nuclear magnetic resonance Spectroscopy (^{1}H-NMR) according to a procedure adapted from Sacchi et al. (1997). Briefly, about 6mg of each oil were dissolved in deuterated chloroform and placed into 5-mm-diameter NMR tubes. Conventional one-dimensional ^{1}H-NMR spectra were collected under routine conditions on a Bruker 600 MHz spectrometer (Bruker; Billerica, MA, USA), equipped with a triple-channel TXI probe. All experiments were recorded at 298K, using a recycle delay of 3s and 4 scans per sample. After Fourier transformation and base-

line correction, the areas of the selected H2 proton signals of the spectrum were quantified by area integration. 2-MAG were distinguished from 1(3)-MAG and 1(3),2-DAG from 1,3-DAG species by area integration of the individual resonances corresponding to the central CH at the sn-2 position in each type of compound. These species were detected in the area covering 5.3-3.8ppm, clearly differentiating the H2 protons belonging to: 1(3),2-DAG (5.05ppm), 1,3-DAG (4.03ppm), 2-MAG (4.88ppm) and 1,(3)-MAG (3.89ppm) derivatives. The degree of saturation and the chain length of fatty acids did not influence the chemical shift values.

3.2.5. Sn-2 fatty acid composition of the experimental oils

For the determination of the composition of fatty acids at the sn-2 position of the experimental oils, they were hydrolysed by pancreatic lipase (EC 3.1.1.3 from porcine pancreas Type II, Sigma-Aldrich Co.; St. Louis, MO, USA). 2-MAG were then separated from free fatty acids and other acylglyceride forms (such as 1, (3)-MAG) by thin layer chromatography using 20 x 20cm Silica gel 60 plates (Merck, Darmstadt, Germany) impregnated in boric acid (5% in methanol) and chloroform/acetone 90:10 (v/v) as mobile phase. Both 1-monoolein and 2-monoolein standards (Sigma-Aldrich Co) were also spotted to control the separation and were visualized under UV light after spraying with 2,7-dichlorofluorescein. The fatty acid composition of scrapped 2-MAG was determined after obtaining the corresponding methyl esters as described for the fatty acid composition of the oils (see section 2.3). Results were expressed as internal normalization (sn-2 FAME peak area/total sn-2 FAME area, in %). Then, the distribution of each fatty within glycerol positions (sn-2 and sn-1, (3) of TAG, DAG and MAG) was calculated, focusing the calculation on the fraction of each fatty acid located at the sn-2 position (% of each fatty acid at the sn-2 position relative to its content in the oil). For this calculation, results from the fatty acid composition and the composition of fatty acids at sn-2 that had been expressed in area% (FAME area% and sn-2 FAME area%) were calculated in mol% (FAME mol% and sn-2 FAME mol%). Then, an adaptation of the formula suggested by Mattson and Volpenhein (1961) was applied: % of each fatty acid esterified to the sn-2 position relative to its content in the oil =(sn-2/Total) x *a* x 100, where sn-2 is the percentage of a specific fatty acid at the sn-2 position (sn-2 FAME mol%), Total is the percentage of that fatty acid in the oil (FAME mol%) and *a* is the ratio between the moles of fatty acid located at the sn-2 position and the moles of total fatty acid. The original study by Mattson and Volpenhein (1961) was performed on native oils, thus they were

constituted by TAG and so *a* was 0.33. But since in our study oils were a mixture of TAG, DAG, MAG and FFA, *a* was calculated for each oil taking into account its lipid class composition, its average molecular weight (according to the total fatty acid composition of the oil) and the glycerol-to-fatty acid ratio for each molecular species. It was equal to 0.30 in FO, 0.29 in PN, 0.25 in PEL, 0.16 in PEH 0.12 in PA, 0.32 in RN, 0.29 in REL, 0.17 in REH and 0.12 in RA diets. Finally, the % of each fatty acid esterified to the sn-2 position (relative to its content in the oil) was multiplied by its total content in the oils (FAME area (%)) to obtain the % of each fatty acid esterified to the sn-2 position relative to the total fatty acid amount.

3.2.6. Digestibility calculations

Apparent digestibility coefficient (ADC) of fatty acids was calculated as: ADC (%) = 100 − [100 × (Y in feed/Y in faeces) · (FA in faeces/FA in feed)] (Maynard & Loosli, 1979), where FA = fatty acid ($mg \cdot kg^{-1}$) and Y = yttrium ($mg \cdot kg^{-1}$). ADC of gross energy (GE) was calculated following the formula above and was then used to calculate the digestible energy (DE) of the diets.

3.2.7. Statistical analysis

Data were subjected to a one-way analysis of variance (ANOVA) and the significance of the differences between means was tested by Tukey's test. Values are given as means±standard error mean of triplicate pooled samples, each containing faeces samples from 21 fish. Differences were considered significant when $P<0.05$. All statistics were performed by means of the General Lineal Model (Proc GLM) of SAS® software version 9.2 (SAS Institute Inc., Cary, NC, USA).

3.3. Results

3.3.1. Characterization of experimental oils and diets

Because scarce information about re-esterified oils is available, a previous characterization of the experimental oils was necessary.

Fatty acid composition of experimental oils and diets

The fatty acid composition of the experimental oils is the typical described for these oils (Table 3.2). Palm oils were rich in SFA (46.6-52.8%), being palmitic acid (C16:0) the predominant fatty acid, while MUFA and PUFA accounted for 40% and 10%, respectively. Rapeseed oils were richer in MUFA (57.4-65.5%) than palm oils, mainly due to the abundance of oleic acid (C18:1n9). Their PUFA content was also higher (27.3-31.9%),

while the amount of SFA ranged from 7.2 to 10.8%. Differences in the fatty acid composition between the four types of oil (N, EL, EH, A) within each source (P and R) were low, as expected. The diets reflected the fatty acid composition of the oils, although some differences were observed mainly related to the highly unsaturated fatty acid composition (Table 3.2). These differences were probably due to the fatty acids coming from the lipid fraction of the fish meal included as one of the main ingredients in the experimental diets.

Lipid class composition of experimental oils and diets

The lipid class composition of oils and diets is shown in Table 3.3. As expected, native oils (FO, PN and RN) were basically composed of TAG in more than 80%, with a very low MAG content (<1%). The percentages of FFA were higher in FO (9.6%) and in PN (6.2%) diets than in RN diet (1.7%). Also, a low content of DAG was generally observed, being higher in PN (10.6%) than in FO (4.1%) and in RN (2.5%). In acid oils (PA and RA), FFA accounted for more than 50%, followed by TAG (by 30%), DAG (by 12%) and a very low MAG content (<4%). EL re-esterified oils resulted in a MAG proportion of about 6%, together with 50-60% TAG and 33-39% DAG. In EH oils, MAG increased to 27%, TAG decreased to 23% and DAG was 46-48%. In both re-esterified oils, EL and EH, a low content of FFA was observed (1.3 - 5%).

Regarding the distribution of fatty acids in MAG and DAG molecules, we found that they were mainly located at the sn-1 and sn-3 positions. 1(3)-MAG constituted the major MAG isomer in most of the oils, being present in about 90% in all the re-esterified oils, although a 50% was found in FO and RN. Similarly, 1,3-DAG constituted the highest DAG isomer (66-77%) present in vegetable oils. As observed for the fatty acid composition, the lipid class composition of the diets was a reflection of that of the oils.

Fatty acid composition of the sn-2 position of experimental oils

In Table 3.4, the proportions of both the individual and the main categories of fatty acids located at the sn-2 position are presented. As expected, higher MUFA and PUFA were present in the sn-2 position in native VO than in the rest of oils. Regarding re-esterified oils, an increase in the content of SFA located at the sn-2 position was clearly observed in both palm and rapeseed EL and EH oils compared to the native oil (Table 4). Regardless of the VO source, when EL and EH oils were compared, a higher sn-2 position was observed in EL oils.

Table 3.2. Fatty acid composition (area %) of the experimental oils and diets.

	Oils[a]									*Diets*[a]								
	FO	PN	PEL	PEH	PA	RN	REL	REH	RA	FO	PN	PEL	PEH	PA	RN	REL	REH	RA
Fatty acids (%)																		
C14:0	7.9	1.2	1.3	1.4	1.3	TR	TR	TR	TR	6.9	1.6	1.9	1.9	1.7	1.5	TR	TR	TR
C16:0	16.5	40.3	39.5	40.5	44.5	4.4	7.4	7.4	6.1	15.5	38.3	36.5	36.4	40.2	7.7	9.1	8.8	7.8
C18:0	2.2	4.5	8.5	8.4	6.0	2.0	2.7	2.6	2.4	1.9	3.8	6.8	7.0	4.9	1.9	2.3	2.3	2.1
C16:1n-7	6.2	TR	TR	TR	TR	TR	TR	TR	TR	5.6	1.0	TR	1.0	TR	1.4	TR	TR	TR
C18:1n-9	12.1	38.6	39.6	39.0	37.1	60.7	53.0	52.3	54.7	10.9	30.6	32.3	31.8	30.9	44.4	41.7	42.4	44.5
C18:1n-7	2.5	TR	TR	TR	TR	3.2	3.5	3.5	3.4	2.6	1.9	1.5	1.7	1.7	3.4	3.7	3.5	3.3
C20:1n-9	7.6	TR	TR	TR	TR	1.2	TR	TR	1.0	6.6	1.3	1.3	1.4	1.3	2.7	2.2	2.1	2.3
C18:2n-6	1.9	11.4	8.1	7.8	8.5	19.1	25.8	26.0	23.6	5.8	13.3	11.3	10.9	11.3	17.8	24.6	25.5	22.7
C18:3n-3	2.2	TR	TR	TR	TR	8.1	5.0	5.8	7.4	1.6	TR	TR	TR	TR	6.2	4.9	5.4	6.3
C18:4n-3	3.4	ND	ND	ND	ND	ND	ND	ND	ND	2.8	TR	TR	TR	TR	TR	TR	TR	TR
C20:4n-6	TR	ND	ND	ND	ND	ND	ND	ND	ND	6.6	1.2	1.3	1.4	1.2	2.7	2.1	2.1	2.3
C20:5n-3 (EPA)	10.5	TR	ND	ND	ND	ND	ND	ND	ND	8.4	1.3	1.1	1.2	1.1	2.2	1.4	1.3	1.6
C22:5n-3	1.3	TR	ND	ND	ND	ND	ND	ND	ND	1.0	TR	TR	TR	TR	TR	TR	TR	TR
C22:6n-3 (DHA)	10.2	TR	ND	ND	ND	ND	ND	ND	ND	8.3	1.9	1.5	1.5	1.3	2.5	1.8	1.7	1.8
ΣSFA[b]	26.9	46.6	50.7	51.7	52.8	7.2	10.8	10.7	9.2	24.3	43.7	45.2	45.3	46.9	11.1	12.2	11.8	10.7
ΣUFA[c]	73.1	53.4	49.3	48.3	47.2	92.8	89.2	89.3	90.8	71.3	55.0	53.5	53.3	51.8	86.8	85.5	86.7	87.7
ΣMUFA[d]	30.2	40.2	41.0	40.3	38.4	65.5	58.3	57.4	59.7	35.6	35.6	36.9	37.0	35.6	54.2	49.6	49.8	52.2
ΣPUFA[e]	42.9	13.2	8.3	8.0	8.8	27.3	30.9	31.9	31.1	35.7	19.3	16.6	16.3	16.2	32.6	35.9	36.8	35.5
Σn-6PUFA[e]	2.7	11.5	8.1	7.8	8.5	19.1	25.9	26.0	23.7	12.9	14.5	12.6	12.3	12.5	20.6	26.8	27.6	25.0
Σn-3PUFA[e]	40.2	1.7	TR	TR	TR	8.1	5.0	5.8	7.4	22.7	4.8	3.9	4.0	3.7	12.0	9.1	9.2	10.5
SFA:UFA	0.4	0.9	1.0	1.1	1.1	0.1	0.1	0.1	0.1	0.3	0.8	0.8	0.8	0.9	0.1	0.1	0.1	0.1

[a]Experimental oils and diets nomenclature as in experimental diets (Table 3.1).
TR = trace (<1 g/100 g fatty acids).
ND not detected.
[b]SFA: saturated fatty acids. It includes other SFA of small quantity.
[c]UFA: unsaturated fatty acids. It includes other UFA of small quantity.
[d]MUFA: monounsaturated fatty acids. It includes other MUFA of small quantity.
[e]PUFA: polyunsaturated fatty acids. It includes other PUFA of small quantity; n-6 PUFA: omega 6 polyunsaturated fatty acids; n-3 PUFA: omega 3 polyunsaturated fatty acids.

Table 3.3. Lipid class composition of the experimental oils and diets.

	Oils[a]								
	FO	PN	PEL	PEH	PA	RN	REL	REH	RA
Lipid classes (%)									
ΣTAG[b, c]	86.0	82.3	59.6	23.6	28.8	95.6	49.9	23.3	32.2
ΣDAG[b, c]	4.1	10.6	33.0	48.1	12.1	2.5	39.2	46.3	12.1
1 (3), 2-DAG[b, d]	40.0	28.6	24.7	22.9	37.9	33.3	24.3	28.5	25.6
1, 3-DAG[b, d]	60.0	71.4	75.3	77.1	62.1	66.7	75.7	71.5	74.4
ΣMAG[b, c]	0.3	0.9	5.7	27.0	3.7	0.2	5.8	27.5	2.3
1(3)-MAG[b, d]	50.0	75.0	87.5	93.1	89.5	50.0	90.9	91.6	90.9
2-MAG[b, d]	50.0	25.0	12.5	6.9	10.5	50.0	9.1	8.4	9.1
ΣFFA[b, c]	9.6	6.2	1.7	1.3	55.4	1.7	5.1	2.9	53.4
Diets[a]									
Lipid classes (%)									
ΣTAG[b, c]	91.9	82.1	61.1	30.7	34.3	94.5	54.2	26.9	40.1
ΣDAG[b, c]	3.2	10.1	30.8	44.3	12.3	2.9	35.9	45.3	11.0
ΣMAG[b, c]	0.4	0.8	4.5	23.2	3.5	0.2	4.7	25.0	1.6
ΣFFA[b, c]	4.5	6.9	3.6	1.8	49.9	2.4	5.2	2.8	47.3

[a]Experimental oils and diets nomenclature as in Table 3.1.
[b]TAG (triacylglycerols), DAG (diacylglycerols), MAG (monoacylglycerols) and FFA (free fatty acids).
[c]Values determined by size-exclusion chromatography. Values are given as wt% of the total lipid classes (TAG, DAG, MAG and FFA).
[d]Values determined by ^{1}H-NMR. Values of each isomer are given as wt% of the total corresponding fraction (DAG or MAG).

Table 3.4. Selected fatty acid composition of the sn-2 position of the experimental oils.

	Oils[a]								
sn-2 (%)	FO	PN	PEL	PEH	PA	RN	REL	REH	RA
C16:0	42.5 (7.0)	7.8 (3.1)	20.93 (8.3)	11.9 (4.8)	3.7 (1.6)	2.7 (0.12)	19.5 (1.4)	11.7 (0.9)	6.8 (0.4)
C18:0	17.1 (0.4)	8.3 (0.4)	25.1 (2.1)	17.1 (1.4)	6.1 (0.4)	4.1 (0.08)	30.9 (0.8)	16.6 (0.4)	8.0 (0.19)
C18:1n-9	23.8 (2.9)	49.0 (18.9)	27.9 (11.0)	19.7 (7.7)	21.2 (7.9)	28.8 (17.5)	29.3 (15.6)	16.5 (8.6)	12.2 (6.7)
C18:2n-6	44.3 (0.8)	55.5 (6.3)	32.0 (2.6)	20.9 (1.6)	21.8 (1.9)	52.0 (9.9)	31.4 (7.7)	18.9 (4.9)	13.9 (3.3)
ΣSFA	38.0 (10.0)	8.0 (3.7)	21.4 (10.9)	12.5 (6.4)	4.0 (2.1)	3.2 (0.2)	29.8 (2.5)	13.4 (1.5)	6.9 (0.7)
ΣMUFA	23.4 (7.1)	47.8 (19.2)	28.3 (11.6)	19.8 (8.0)	21.9 (8.3)	27.3 (17.9)	29.9 (17.4)	16.7 (9.5)	11.8 (7.0)
ΣPUFA	28.7 (12.1)	52.3 (6.9)	32.3 (2.6)	21.0 (1.7)	21.5 (1.9)	51.0 (13.9)	29.5 (9.1)	18.9 (6.0)	14.0 (4.3)

[a]Experimental oils nomenclature as in Table 3.1.

Values are given as the % of each fatty acid at the sn-2 relative to its content in the oil. Values in brackets are given as the % of each fatty acid at the sn-2 position relative to the total fatty acid amount.

3.3.2. Apparent digestibility of fatty acids

ADC of total and individual fatty acids are given in Table 3.5 for palm and in Table 3.6 for rapeseed. As expected, lower digestibility values were obtained for palm diets in relation to FO diet. In palm, ADC of total fatty acids of acid oil (PA: 69.6%) was 10 points lower than that of native oil (PN: 79.5%), although it reached higher values when re-esterified (PEL: 78.4% and PEH: 81.3%). In spite of this improvement, it did not result in higher values when compared to PN. Similarly, no significant differences in the ADC of C16:0, C18:0 or total SFA were obtained between re-esterified oils and the native oil, although these ADC values improved when PA was re-esterified to PEL or PEH.

In PA diet, a significantly lower ADC was found for all the fatty acids, being especially evident for SFA, which indicated the strong effect that this type of fatty acids had on total fatty acids ADC. In fact, in all diets, SFA showed the lowest ADC, making evident the effect the chain length and the degree of saturation have on fatty acids digestibility.

On the other hand, rapeseed diets reached high ADC (>96%) in all cases, being similar (RA) or higher (RN, REL and REH) than FO diet. Significant differences ($P<0.05$) were found among diets, although ADC of the different fatty acids were very similar among them. No differences were obtained in total fatty acids between re-esterified oil high in MAG (REH: 97.8%) and native oil (RN: 98.1%), being higher than in acid oil (RA: 96.4%). ADC of both individual and total SFA were significantly lower ($P<0.05$) in RA than in the rest of diets, although they were higher than in palm diets, reaching values above 85%.

When comparing results for palm (Table 3.5) with results for rapeseed (Table 3.6), the role of the degree of saturation of fatty acids on digestibility was clearly observed. ADC of the total fatty acids in rapeseed diets, low in SFA, were higher (96.4-98.1%) than in palm diets (69.6-81.3%), rich in SFA.

Table 3.5. Apparent digestibility coefficient (ADC %) of selected fatty acids in rainbow trout fed the experimental palm diets.

	Diets[a]				
	FO	PN	PEL	PEH	PA
Fatty acid			*ADC (%)*		
C14:0	94.6±0.6a	80.9±1.6b	79.1±1.5b	80.7±0.6b	73.5±1.0c
C16:0	90.5±0.8a	61.7±2.8b	62.1±2.5b	66.6 ±1.0b	47.7±2.3c
C18:0	85.4±1.1a	59.7±3.0b	57.1±2.9bc	62.1±1.0b	51.0±2.1c
C18:1n-9	97.4±0.2a	92.1±0.5b	92.5±0.6b	94.1±0.3b	87.6±0.6c
C18:2n-6	94.2±0.6a	95.2±0.3a	94.0±0.4a	94.9±0.1a	90.0±0.4b
C18:3n-3	96.6±0.3a	94.0±0.2b	93.3±0.1b	93.5±0.6b	89.1±0.2c
C20:4n-6	96.1±0.3a	90.8±0.8b	90.7±0.6b	92.2±0.4b	86.4±0.6c
C20:5n-3 (EPA)	99.3±0.0a	95.1±0.4b	93.9±0.1b	94.8±0.4b	90.4±0.4c
C22:6n-3 (DHA)	98.5±0.1a	93.1±0.4b	92.1±0.5b	92.1±0.1b	89.8±0.5c
ΣSFA	91.1±0.8a	62.2±2.8b	61.8±2.5b	66.3±1.1b	49.1±2.2c
ΣMUFA	95.9±0.3a	92.3±0.5b	92.0±0.5b	93.7±0.3b	87.3±0.6c
ΣPUFA	97.2±0.3a	94.6±0.5b	93.7±0.4b	94.3±0.1b	92.6±2.4c
Σn-6 PUFA	95.6±0.4a	94.9±0.3a	93.7±0.4a	94.6±0.1a	89.7±0.5b
Σn-3 PUFA	98.8±0.1a	94.4±0.4b	93.3±0.3b	93.7±0.2b	90.0±0.5c
Total FA	95.4±0.4a	79.5±1.5b	78.4±1.4b	81.3±0.6b	69.6±1.4c

[a]Experimental diets nomenclature: abbreviations as in Table 3.1.
Values represent mean±SEM of triplicate pooled samples from 21 fish. Values in the same row with different letters are significantly different (P<0.05).

Table 3.6. Apparent digestibility coefficient (ADC %) of selected fatty acids in rainbow trout fed the experimental rapeseed diets.

	Diets[a]				
	FO	RN	REL	REH	RA
Fatty acid			*ADC (%)*		
C14:0	94.6±0.6c	98.0 ± 0.4a	97.4 ± 0.2ab	96.7 ± 0.1ab	96.1±0.4bc
C16:0	90.5±0.8b	95.4 ± 0.4a	94.9 ± 0.5a	95.3 ± 0.3a	91.4±0.2b
C18:0	85.4±1.1b	93.3 ± 0.7a	93.2 ± 0.8a	93.4 ± 0.3a	85.2±0.2b
C18:1n9	97.4±0.2b	98.9 ± 0.2a	97.5 ± 0.3b	98.7 ± 0.1a	97.4±0.1b
C18:2n6	94.2±0.6b	98.2 ± 0.1a	97.4 ± 0.2a	98.1 ± 0.1a	97.3±0.1a
C18:3n3	96.6±0.3c	99.0 ± 0.1a	98.0 ± 0.1b	98.8 ± 0.0ab	98.2±0.1b
C20:4n6	96.1±0.3a	97.3 ± 0.4a	95.8 ± 0.6a	97.4 ± 0.4a	96.2±0.1a
C20:5n3 (EPA)	99.3±0.0a	98.6 ± 0.0b	97.8 ± 0.1c	98.3 ± 0.1b	97.7±0.1c
C22:6n3 (DHA)	98.5±0.1a	97.2 ± 0.1a	94.2 ± 0.8b	94.9 ± 0.1b	94.8±0.3b
ΣSFA	91.1±0.8b	95.1 ± 0.5a	94.6 ± 0.6a	94.8 ± 0.3a	89.8±0.2b
ΣMUFA	95.9±0.3c	98.6 ± 0.2a	97.4 ± 0.2b	98.5 ± 0.1a	97.2±0.1b
ΣPUFA	97.2±0.3bc	98.4 ± 0.1a	97.1 ± 0.8c	97.9 ± 0.1ab	97.2±0.2bc
Σn-6 PUFA	95.6±0.4b	98.0 ± 0.1a	97.2 ± 0.1a	98.0 ± 0.1a	97.1±0.2a
Σn-3 PUFA	98.8±0.1a	98.7 ± 0.1a	97.1 ± 0.2c	97.8 ± 0.1b	97.3±0.1bc
Total FA	95.4±0.4d	98.1 ± 0.2a	96.9 ± 0.3bc	97.8 ± 0.1ab	96.4±0.1cd

[a]Experimental diets nomenclature: abbreviations as in Table 3.1.
Values represent mean±SEM of triplicate pooled samples from 21 fish. Values in the same row with different letters are significantly different (P<0.05).

3.4. Discussion

Characterization of experimental oils and diets

Results from the present study indicated that 1(3)-MAG was the main MAG isomer present in the oils, and especially in re-esterified oils, while 2-MAG only represented the 7-12.5% of the total MAG in the oils. The predominance of 1(3)-MAG over 2-MAG could be related to the acyl migration of FA in sn-2 to sn-1 or sn-3 positions (Destaillats et al., 2010; Martin et al., 2014). As described by Cruz Hernandez et al. (2012), primary esters (sn-1(3)) are more stable than secondary esters (sn-2). In fact, the higher content of 1,3-DAG than 1,2-DAG present in all the experimental oils would support the existence of an acyl migration process. Moreover, the proportion of 1,3-DAG obtained in our study (60-77%) is in agreement with Taguchi et al. (2001), who reported that 70% of DAG present in edible oils are in 1,3 configuration.

The chemical esterification reaction did not affect the fatty acid composition or the degree of saturation of the original oil, as observed in interesterification reactions (Berry, 2009; Farfán, 2013). But, as expected, the distribution of fatty acids in the three glycerol positions varied between re-esterified and native oils, increasing the content of SFA located at the sn-2 position. In palm, the amount of SFA located at the sn-2 position increased a 1.7-2.9 fold in re-esterified oils compared to PN, while in rapeseed the increase was even higher (7.5–12.5 fold). In both cases, the rise was more clearly observed in EL than in EH. In EL oils, approximately between 20 and 30% of each fatty acid was located at the sn-2 position. In the chemical esterification process of FFA with glycerol, fatty acids are randomly esterified to the various glycerol positions. Then, theoretically, in a re-esterified oil containing 100% TAG, a 33% of each fatty acid would be expected at the sn-2 position. However, the values obtained in the present study were slightly lower, which could be explained by the fact that our oils contained a certain proportion of DAG and MAG. In addition, acyl migration phenomenon might have had an influence. It is also important to bear in mind that our starting material was not 100% FFA because acid oils already contained some TAG, DAG and MAG, fact that makes it difficult to obtain a complete random redistribution of fatty acids. Moreover, we cannot ignore the possible glycerolysis that can occur simultaneously during re-esterification as a result of the presence of these esterified forms and free glycerol (Noureddini and Medikonduru, 2007).

As previously indicated, EL oils had a higher SFA content in the sn-2 position than EH oils in both palm and rapeseed, which was probably due to the lipid class composition of these

experimental oils: EH oils were richer in MAG and poorer in TAG than EL oils. This, in addition to the fact that MAG and DAG were found to be mainly 1(3), resulted in EH oils having less sn-2 positions of the glycerol esterified to FA in general, and thus in SFA.

Apparent digestibility of fatty acids

Results from the present study showed differences between the oil source (palm or rapeseed). In palm, chemically re-esterification process resulted in an improvement of the digestibility compared to acid oil. However, this improvement in fatty acid digestibility was not obtained if compared to native oils. Similar results were reported by Smink et al. (2008) and Vilarrasa et al. (2014) in broiler chicks fed randomized native and randomly re-esterified palm oils, respectively.

One of the aims of performing the esterification process was to obtain a higher proportion of SFA located at the sn-2 position of the acylglycerols (TAG, DAG and MAG) in order to improve both SFA and overall digestibility. Although a notable increase of SFA in sn-2 was observed in both palm re-esterified oils (PEH and PEL), no significant improvement in the apparent digestibility values were observed between re-esterified and native diets.

Contrarily to what we obtained, studies carried out in chickens (Lin et al., 2010), rats (Lien et al., 1997), piglets (Innis et al., 1995) and human infants (Filer et al., 1969) found that SFA, and particularly palmitic acid, were better absorbed when located at the sn-2 position of dietary TAG. All these studies, though, were performed using completely randomized fats in which SFA, and specifically palmitic acid, were located at the sn-2 position in a proportion of minimum 33.9% of their total content. As this proportion is higher than those obtained in the present study (21.4% in PEL and 12.5% in PEH for total SFA), it is feasible to consider that the content of SFA in sn-2 position present in our re-esterified oils was maybe not enough to have a clear effect on digestibility.

Another possible cause of the lack of improvement in digestibility by re-esterified palm diets compared to native palm could be related to the lipase specificity. The studies mentioned above were conducted in non-aquatic species. In fish, luminal lipase specificity with regards to sn-acyl position seems to vary depending on the species (Bogevick et al., 2008; Kurtovic et al., 2009). Although a sn-1,3-specific lipase has been pointed as the main lipolytic enzyme for different species, studies in rainbow trout have obtained controversial results. For instance, Tocher (2003) and Gottsche et al. (2005) found specific 1,3-hydrolytic activity in crude intestinal isolates of rainbow trout. But the presence of both

non-specific and specific lipase activity has also been suggested for this species (Skall Nielsen et al., 2005). In fact, the non-specificity of the lipase had been previously described in other species (Olsen and Ringo, 1997; Olsen et al., 1998). Thus, in the present study, the absence of improved SFA digestibility of PEH and PEL compared to PN could be explained by the possible existence of a non-specific lipase activity, which would make the effects of the SFA located at the sn-2 position on digestibility less important.

Regarding acid oils, the lowest ADC of PA was probably caused by the low absorption of free long chain SFA such as C16:0 and C18:0, related to their higher melting points compared to MUFA and PUFA. In addition, a certain presence of "soaps", formed by a long chain SFA bound in form of salts by divalent ions present in the intestine, could be affecting. It is important to remark that this effect can be present in freshwater species, although it has been reported to be more significant in marine water, rich in calcium and magnesium (Olsen et al., 1998).

Only the study by Ng et al. (2010) has reported an improvement of individual and total SFA digestibility in fish fed diets with a high content of palm FFA, contrarily to what was observed in the present study. To explain the results, the authors suggested a higher polarity and a lower melting point of FFA compared to TAG, and the fact that FFA bypass the need to be hydrolyzed by pancreatic lipase. Nonetheless, this study was performed using a palm fatty acid distillate (about 80% FFA), while we used a palm acid oil (55.4% FFA). In light of the results of the present study, we would expect lower digestibility values when using an oil with a very high content in long-chain FFA, as a consequence of the formation of insoluble soaps. However, the differences in digestibility observed between the two studies might be related to other factors. More studies including FFA-rich oils with a different content in FFA should be performed in order to elucidate the possible reasons for these differences.

Comparing ADC values of EH and EL oils in palm, no difference was observed between them, although MAG are good emulsifiers. This lack of a MAG effect could be due to the predominance of 1(3)-MAG over 2-MAG together with the lipase specificity. During digestion, free SFA would be well absorbed provided there is a MAG emulsifying effect. If now considering a sn-1,3 specific lipase as the main lipolytic enzyme, it would hydrolyze 1(3)-MAG to FFA and so it would reduce the emulsifying effect of these 1(3)-MAG. As

this effect was reduced, SFA released from MAG would end up being lost in faeces due to their poor absorption as FFA, contributing to a decrease in digestibility.

In addition, the MAG effect was expected to be more evidently observed in EH diets compared to EL diets. However, taking into account that EL oils have a higher content of TAG than EH oils, EL would end up having a higher amount of 2-MAG, together with FFA, as final products after the action of lipase. Therefore, as MAG in EH diet are mainly 1(3)-MAG which could be hydrolyzed by lipase, the possible differences between EL and EH due to the MAG content would be compensated. Nevertheless, it is clear that further studies are required to elucidate the specific mechanisms that take place during lipid digestion in rainbow trout.

On the other hand, the good results obtained with rapeseed are in accordance with studies reporting high digestibility values when fish were fed diets containing rapeseed oils (Dernekbaşı, 2012; Turchini et al., 2013). In the present study, a few differences in ADC were found among diets, indicating that all rapeseed oils were well digested regardless of the amount of FFA, the fatty acid composition of the sn-2 position and the MAG content. Although a lower digestibility was observed in RA diet, this was similar as that obtained for FO diet. Thus, although the position of fatty acids within the glycerol molecule has been reported as a major determinant of its digestibility (Berry, 2009; Bracco, 1994), it seems that the chain length and especially the degree of saturation play a more predominant role in rainbow trout. This is also clear when observing the overall results, considering that ADC obtained in palm diets, rich in SFA, were lower than ADC obtained in rapeseed diets, rich in MUFA. This fact concurs with the generally held view that the absorption of fatty acids increases with decreasing chain length and with an increase in unsaturation (Olsen et al., 1998) and so lipid digestibility decreases with the increasing incorporation of dietary SFA. As Hua and Bureau (2009) suggested, SFA can be incorporated at levels up to approximately 23% of dietary total fatty acids without negatively affecting lipid digestibility, which could explain the results obtained in the present study, where the content of SFA was above this value in palm diets and below it in rapeseed diets. Accordingly, it has been observed that digestibility increases with the dietary incorporation of a vegetable oil such as rapeseed, rich in MUFA and with little SFA (Torstensen and Tocher, 2011).

In conclusion, the results of the present study indicate that the chemical esterification of vegetable acid oils reduces the amount of FFA and incorporates part of the SFA to the sn-2

position. Therefore, re-esterified oil diets have better apparent digestibility coefficients of SFA than their corresponding acid oils diets. However, no improvement in SFA digestibility was observed in rainbow trout fed re-esterified oils diets if compared to those fed with native oils diets, and not even when an increased level of MAG was present. Although this improvement did not occur, both palm and rapeseed re-esterified oils could be incorporated as a fat source in diets for rainbow trout without negatively affecting fatty acids digestibility values.

All things considered, it seems that fatty acids digestibility in the experimental oils is more affected by their degree of saturation than by their positional distribution and lipid class composition of the oil. Nevertheless, further investigations regarding the effects of re-esterified oils on growth, fillet final quality and fillet fatty acid composition should be performed in order to widely elucidate the suitability of the incorporation of these oils in fish diets. In addition, an assessment of the long term impact of the use of re-esterified oils on fish health should be performed.

CHAPTER 4

FATTY ACID DIGESTIBILITY IN GILTHEAD SEA BREAM FED DIETS CONTAINING NATIVE, RE-ESTERIFIED OR ACID VEGETABLE OILS

Fatty acid digestibility in gilthead sea bream fed diets containing native, re-esterified or acid vegetable oils

Trullàs, C.[1*], Fontanillas, R.[2], Tres, A.[3], Sala, R.[1]

[1]Departament de Ciència Animal i dels Aliments, Servei de Nutrició i Benestar Animal (SNiBA), Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain;

[2]Skretting Aquaculture Research Center (Skretting ARC), Sjøhagen 3, Stavanger 4016, Norway;

[3]Departament de Nutrició i Bromatologia-XaRTA-INSA, Facultat de Farmàcia, Universitat de Barcelona, 08028 Barcelona, Spain.

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Abstract

Re-esterified vegetable oils are obtained from a chemical esterification reaction between vegetable acid oils and glycerol. Due to their properties, it is expected that they have a higher nutritive value than their corresponding acid oils and a better digestibility than their native counterparts. The aim of the present study was to determine the effect of re-esterified oils with a different monoacylglycerol (MAG) and diacylglycerol (DAG) content, produced from palm or rapeseed, on fatty acid digestibility in gilthead sea bream (*Sparus aurata*). Triplicate groups of fish were fed nine experimental diets containing different oils during 28 days. For each source, four different types of oil were used: native, re-esterified low or high in MAG and DAG and acid. A commercial fish oil was used for the control diet. Diets containing re-esterified oils had better apparent digestibility coefficients (ADC) of total fatty acids than acid oil diets. Re-esterified oils do not negatively affect apparent digestibility coefficients of fatty acids when compared to their corresponding native oils and could be incorporated as a source of energy in diets for gilthead sea bream. An improvement in digestibility compared to the native oil diet was only obtained in palm re-esterified oil high in MAG and DAG.

4.1. Introduction

In view of the increasing global demand of fish oil (FO), its decreasing availability and its large use by the aquaculture industry (FAO, 2014) oils from vegetable origin have been widely studied as sustainable and economically valuable FO substitutes in aqua feeds (Bell et al., 2001; Ng et al., 2003; Turchini et al., 2009; Yildiz et al., 2014). As extensively reported, certain vegetable oils (VO) are considered good alternatives to FO in diets for salmonids and freshwater fish (Bell et al., 2001; Caballero et al., 2002; Fountoulaki et al., 2009; Dernekbaşı, 2012). However, in marine fish, many studies have shown the limitation of the inclusion of VO in diets as a sole lipid source due to the low ability of these species to synthesize long-chain polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) from their C18 precursors (Watanabe, 1982). In gilthead sea bream (*Sparus aurata*), oils from different vegetable sources can be included in diets as an efficient source of energy without affecting feed utilization (Izquierdo et al., 2003; 2005; Benedito-Palos et al., 2008; Fountoulaki et al., 2009).

Supplies of VO are approximately 100 times higher than those of FO (Bimbo, 1990) and its use as feedstock for energy production by the biofuel industry has greatly increased since the early 2000s. Thus, the livestock and the biofuel industries have undergone a competition for crop feedstocks, with the consequence of the rise of feed grains and oilseed prices. This has also led to a steadily increase of the amount of by-products derived from the production of biofuels (Taheripour et al., 2011). On the other hand, the refining process of VO also generates a considerable amount of fat by-products (Junior et al., 2012), their incorporation in animal diets being a potential way to reutilize them as a cheaper alternative to priced crops. In fact, studies have shown that some of the compounds are valuable and can be recovered for its subsequent use in animal nutrition (Dumont and Narine, 2007; Nuchi et al., 2009). This is the case of acid oils, a free fatty acid (FFA)-rich by-product. However, as described by Wiseman and Salvador (1991) in broiler chickens, acid oils have a lower nutritive value than that of native oils as a consequence of their high content in FFA. Even so, their nutritive value might be increased when chemically re-esterified with glycerol, a by-product derived from the biodiesel production process (Parini and Cantini, 2009; Vilarrasa et al., 2014; Trullàs et al., 2015). The chemical esterification process does not change the fatty acid composition and the degree of saturation of the processed oil in relation to the original oil. However, the resulting fat has a different positional distribution of fatty acids in the glycerol molecule compared to that in native oils since the chemical esterification process is not regioselective. This means that part of the saturated fatty acids (SFA) present in the oil could be incorporated in the sn-2 position of acylglycerols (Vilarrasa et al., 2014; Trullàs et al., 2015). As it is widely known in mammals, the main products of the hydrolysis by pancreatic lipase during lipid digestion are FFA and 2-monoglycerides (MAG). While 2-MAG are directly absorbed (Schulthess et al., 1994) the rate of absorption of FFA depends on their chain length and degree of saturation (Small, 1991). In fact, free long-chain SFA have a poorer absorption than mono- (MUFA) and PUFA as a consequence of their hydrophobicity, their high melting points and their tendency to form insoluble soaps in the gut (Hunter, 2001).

In VO, SFA are found predominantly in the external positions (sn-1 and sn-3) of the triacylglycerols (TAG) (Berry, 2009) so these SFA are converted to FFA during digestion with the risk of ending up unabsorbed. Then, having more SFA in sn-2 in re-esterified oils could result in a higher digestibility of these oils compared to their native counterparts.

In marine fish, there are indications that the dominant digestive enzyme could be a carboxyl ester lipase-type (CEL) (Kutovic et al., 2009), also known as bile salt-activated lipase (Gjellesvik et al., 1989; 1994; Iijima et al., 1998, Nolasco et al., 2011). This enzyme is also present in mammals (Hui and Howles, 2002) and shows strict dependence on bile-salts for hydrolytic activity on insoluble lipid substrates (Gjellesvik et al., 1994). CEL seems to be able to hydrolyze a wide range of lipid classes and is also likely to have a role in hydrolyzing monoglycerides, as reported in mammals (Gjellesvik, 1994; Kurtovic, 2009). Nonetheless, it has been established that the 2-MAG pathway is the predominant for TAG resynthesis in the enterocytes of gilthead sea bream (Caballero et al., 2006; Oxley et al., 2007). Thus, this leads to the possibility that it may possess certain sn-1,3 hydrolytic activity (Bogevik et al., 2008; Bakke et al., 2011).

On the other hand, re-esterified VO can have different proportions of lipid classes –TAG; diacylglycerols, DAG and MAG – which can be used to obtain a final product with specific desired characteristics (Parini and Cantini, 2009). For instance, when a major content of both MAG and DAG is present in the dietary fat, digestibility values might improve because of the emulsifying effect of these partial acylglycerols (Martin et al., 2014).

To the best knowledge of the authors, there is only one study reporting the use of chemically re-esterified VO in fish diets to-date (Trullàs et al., 2015), in which the improvement in fatty acids digestibility of re-esterified oils compared to acid oils was clearly shown in rainbow trout (*Oncorhynchus mykiss*). Re-esterified oils resulted in similar apparent digestibility coefficients (ADC) than those of native VO and were therefore considered potentially suitable for being incorporated as a source of energy in diets for this species. However, there is a wide diversity in the digestive physiology and differences in digestive lipase specificity could exist among fish species (Kurtovic, 2009; Bakke et al., 2011).

The present study aims at giving palm and rapeseed acid oils from the refining industry added value by transforming them to re-esterified oils with different contents of MAG and DAG, and to assess their effect on fatty acid digestibility in gilthead sea bream as a first step to determine if they can be appropriate energy sources for diets for this species.

4.2. Materials and methods

4.2.1. Experimental diets

Nine experimental diets were formulated to contain 48% protein and 24% lipid using the same ingredient composition except for the added lipid source. Oils used for the experimental diets originated from two different vegetal sources with different degree of saturation, palm (P) and rapeseed (R). For each source, four different types of oil were used: native oil (N), re-esterified oil low in MAG (EL), re-esterified oil high in MAG (EH) and acid oil (A), all resulting in eight experimental diets (Table 4.1).

Table 4.1. Ingredient formulation and proximate composition of the experimental diets.

	Diets[a]								
	FO	PN	PEL	PEH	PA	RN	REL	REH	RA
Ingredient composition (g kg^{-1})									
Wheat[b]	162.1	162.1	162.1	162.1	162.1	162.1	162.1	162.1	162.1
Wheat gluten[c]	206.9	206.9	206.9	206.9	206.9	206.9	206.9	206.9	206.9
Soy protein concentrate[d]	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0
North Atlantic fish meal[e]	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0
South American fish oil[f]	210.0	0	0	0	0	0	0	0	
Experimental oils[g]	0	210.0	210.0	210.0	210.0	210.0	210.0	210.0	210.0
Yttrium premix[h]	1	1	1	1	1	1	1	1	1
Mineral and vitamin premix[h]	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Proximate composition (g kg^{-1})									
Dry matter (g kg^{-1})	937.7	947.9	948.2	948.2	941.3	945.6	941.0	940.9	940.5
Crude protein	481.5	489.3	483.8	474.4	482.6	485.2	489.8	471.3	471.0
Crude fat	198.8	219.5	213.8	209.9	214.3	211.9	203.7	204.6	212.6
Ash	89.2	88.4	84.3	76.9	94.1	88.5	83.0	83.2	69.7
Gross energy (kJ g^{-1})	23.1	23.3	23.4	23.3	23.6	23.4	23.2	23.2	23.3

[a]Experimental diets nomenclature: FO: fish oil (control diet); PN: palm native oil; PEL: palm re-esterified oil low in MAG and DAG; PEH: palm re-esterified oil high in MAG and DAG; PA: palm acid oil; RN: rapeseed native oil; REL: rapeseed re-esterified oil low in MAG and DAG; REH: rapeseed re-esterified oil high in MAG and DAG and RA: rapeseed acid oil.
[b]Statkorn, Norway.
[c]Cerestar Scandinavia AS, Denmark.
[d]Selecta, Brasil.
[e]Welcon AS, Norway.
[f]Holtermann ANS, Norway.
[g]Experimental oils.
[h]Skretting standard vitamin and minerals premix, according to requirement data from NRC (2011). Trow Nutrition, The Netherlands.

A commercial fish oil was used for the control diet (FO). Native, acid and re-esterified oils were provided by SILO S.p.a. (Firenze, Italy). In the process of esterification, the level of the different lipid classes present in the oil (TAG, DAG and MAG) was previously established by fixing the proportion fatty acid:glycerol. The free fatty acidity was determined following the ISO 660:1990 method. Glycerol was calculated according to the

following stoichiometric formula: glycerol weight = fatty acid weight · free fatty acid acidity · glycerol molecular weight/fatty acid molecular weight. Once the proportion fatty acids:glycerol was established, both components were put in the reactor at 190-250ºC and 1-3 mm Hg of pressure for 4 – 6 hours. Feeds were produced at the Skretting Feed Technology Plant (Aquaculture Research Center; Stavanger, Norway) as extruded pellets. Yttrium oxide (Y_2O_3) was added to the diets as an inert marker for apparent digestibility (AD) of fatty acid measure. The ingredient formulation and proximate composition of the diets are shown in Table 4.1. Nutrient composition of experimental diets was determined by standard procedures (AOAC, 2005): moisture (934.01), ash (942.05), crude protein (968.06) and crude lipid (920.39). Gross energy of dried feed was determined using an adiabatic bomb calorimeter (IKA – Kalorimeter system C4000, Jankel – Kunkel, Staufen, Germany). Samples were analyzed for yttrium by inductively coupled plasma optical emission spectroscopy (ICP OES) (Perkin Elmer spectrometer, model Optima 4300DV) with a previous digestion in a CEM microwave (CEM MARSXpress).

4.2.2. Fish, experimental conditions and sampling

All the procedures were conducted in accordance with the Animal Protocol Review Commitee of the Autonomous University of Barcelona (UAB) and following the European Union Guidelines for the ethical care and handling of animals under experimental conditions. The trial was carried out at the Institute of Agrifood Research and Technology (IRTA), Sant Carles de la Ràpita, Spain. A total of 702 gilthead sea bream (Piscimar, Spain) with a mean initial body weight of 296±7.2 g were randomly distributed into 27 cylindro-conical tanks of 400l of capacity in a recirculation seawater system IRTAmar®. Water temperature (21.5ºC), salinity (36.1±1.20g·l^{-1}) and dissolved oxygen levels (6.1±1.06 mg·l^{-1}) were maintained constant throughout all the experimental period. The tanks were subjected to a photoperiod of 12h light and 12h dark. Following an adaptation period of a week, fish were fed the experimental diets during 28 days. Each diet was randomly assigned to three replicate tanks and was fed to satiation twice a day by automatic feeders. Feed was supplied in excess of appetite (20%) of measured feed intake. Uneaten feed was collected by filtering effluent water from each tank one hour and a half after each meal so that feed intake could be recorded daily. At the end of the experimental period, all the animals were euthanized in excess anaesthetic (2-phenoxyethanol) and faeces were collected from the hindgut after laterally opening the peritoneal cavity. Faecal

samples were pooled by tank and stored at −20°C prior to analysis of yttrium oxide and fatty acid composition.

4.2.3. Fatty acid composition

Fatty acid composition of oils, diets and faeces were determined by gas chromatography – flame ionization detector (GC-FID). For experimental oils, the fatty acid methyl esters (FAME) were previously obtained as described by Vilarrasa et al., (2014) and analyzed using an Agilent 4890D gas chromatograph. For diets and faeces, FAME were obtained by an adaptation of the method of Sukhija and Palmquist (1988) and analyzed using an HP 6890 gas chromatograph (Agilent Technologies). In both cases, fatty acid methyl esters were identified by comparison of their retention times with those of known standards, and quantified by internal normalization (FAME peak area/total FAME peak area, in %). Fatty acid composition of oils and diets is shown in Table 4.2.

4.2.4. Lipid class composition of the experimental oils and diets

Lipid class composition (TAG, DAG, MAG and FFA) of oils and diets is shown in Table 4.3. and was determined following the procedure described by Darnoko et al. (2000), adapted to these samples as reported in Trullàs et al. (2015).

4.2.5. Sn-2 fatty acid composition of the experimental oils

The composition of fatty acids located at the sn-2 position of the acylglycerols (TAG, DAG and MAG) of the experimental oils is shown in Table 4.4. and was determined as described in Trullàs et al. (2015).

4.2.6. Digestibility calculations

ADC of fatty acids was calculated as: ADC (%) = 100 − [100 × (Y in feed/Y in faeces) · (FA in faeces/FA in feed)] (Maynard and Loosli, 1969), where FA = fatty acid ($mg \cdot kg^{-1}$) and Y = yttrium ($mg \cdot kg^{-1}$).

Table 4.2. Fatty acid composition of the experimental oils and diets.

	Oils[a]									*Diets*[a]								
	FO	PN	PEL	PEH	PA	RN	REL	REH	RA	FO	PN	PEL	PEH	PA	RN	REL	REH	RA
Fatty acid (%)																		
C14:0	7.9	1.2	1.3	1.4	1.3	0.1	0.1	0.1	0.1	7.0	1.6	1.7	1.8	1.6	0.5	0.7	0.7	0.6
C16:0	16.5	40.3	39.5	40.5	44.5	4.4	7.4	7.4	6.1	19.0	38.6	36.0	35.4	40.2	6.8	8.9	8.7	8.1
C18:0	2.2	4.5	8.5	8.4	6.0	2.0	2.7	2.6	2.4	2.8	4.2	7.6	7.4	5.4	2.2	2.5	2.4	2.5
C16:1n-7	6.2	0.4	0.2	0.2	0.2	0.2	0.3	0.3	0.3	6.5	0.8	0.7	0.8	0.6	0.6	0.7	0.8	0.7
C18:1n-9	12.1	38.6	39.6	39.0	37.1	60.7	53.0	52.3	54.7	17.5	33.5	34.8	34.9	33.3	53.1	45.4	45.2	47.8
C18:1n-7	2.5	0.9	0.8	0.8	0.7	3.2	3.5	3.5	3.4	2.8	1.0	0.9	1.0	0.8	2.8	3.1	3.1	3.1
C20:1n-9	7.6	0.2	0.1	0.1	0.1	1.2	0.9	0.9	1.0	6.0	1.2	1.1	1.2	1.0	1.8	1.7	1.7	1.7
C18:2n-6	1.9	11.4	8.1	7.8	8.5	19.1	25.8	26.0	23.6	8.9	13.6	11.8	11.8	12.0	20.7	26.9	27.0	24.2
C18:3n-3	2.2	0.4	0.2	0.2	0.3	8.1	5.0	5.8	7.4	2.5	0.8	0.7	0.9	0.7	7.3	5.3	5.8	6.7
C20:4n-6	0.8	ND	ND	ND	ND	ND	ND	ND	ND	0.7	ND	ND	ND	ND	ND	ND	ND	ND
C20:5n-3 (EPA)	10.5	0.3	ND	ND	ND	ND	ND	ND	ND	11.3	1.3	1.0	1.1	1.0	0.9	1.1	1.1	0.9
C22:6n-3 (DHA)	10.2	0.7	ND	ND	ND	ND	ND	ND	ND	11.3	2.0	1.4	1.5	1.4	1.4	1.7	1.7	1.4
ΣSFA[b]	26.9	46.6	50.7	51.7	52.8	7.2	10.8	10.7	9.2	30.1	45.3	47.4	46.6	48.7	10.7	13.3	12.9	12.6
ΣUFA[c]	73.1	53.4	49.3	48.3	47.2	92.8	89.2	89.3	90.8	69.9	54.7	52.9	53.7	51.5	89.3	86.7	87.1	87.4
ΣMUFA[d]	30.2	40.2	41.0	40.3	38.4	65.5	58.3	57.4	59.7	34.5	36.9	38.0	38.4	36.4	58.9	51.6	51.4	54.0
ΣPUFA[e]	42.9	13.2	8.3	8.0	8.8	27.3	30.9	31.9	31.1	35.3	17.8	14.9	15.2	15.1	30.4	35.1	35.7	33.4
Σn-6 PUFA[e]	2.7	11.5	8.1	7.8	8.5	19.1	25.9	26.0	23.7	9.9	13.6	11.8	11.8	12.1	20.8	27.0	27.1	24.3
Σn-3 PUFA[e]	40.2	1.7	0.2	0.2	0.3	8.2	5.0	5.8	7.4	25.3	4.2	3.1	3.5	3.1	9.6	8.1	8.6	9.1
SFA:UFA	0.4	0.9	1.0	1.1	1.1	0.1	0.1	0.1	0.1	0.4	0.8	0.9	0.9	0.9	0.1	0.2	0.1	0.1

ND not detected.

[a]Experimental oils and diets nomenclature as in experimental diets (Table 4.1).

[b]SFA: saturated fatty acids. It includes other SFA of small quantity.

[c]UFA: unsaturated fatty acids. It includes other UFA of small quantity.

[d]MUFA: monounsaturated fatty acids. It includes other MUFA of small quantity.

[e]PUFA: polyunsaturated fatty acids. It includes other PUFA of small quantity; n-6 PUFA: omega 6 polyunsaturated fatty acids; n-3 PUFA: omega 3 polyunsaturated fatty acids.

Table 4.3. Lipid class composition of the experimental oils and diets.

Oils[a]									
	FO	PN	PEL	PEH	PA	RN	REL	REH	RA
Lipid classes (%)									
ΣTAG[b, c]	86.0	82.3	59.6	23.6	28.8	95.6	49.9	23.3	32.2
ΣDAG[b, c]	4.1	10.6	33.0	48.1	12.1	2.5	39.2	46.3	12.1
1 (3), 2-DAG[b, d]	40.0	28.6	24.7	22.9	37.9	33.3	24.3	28.5	25.6
1, 3-DAG[b, d]	60.0	71.4	75.3	77.1	62.1	66.7	75.7	71.5	74.4
ΣMAG[b, c]	0.3	0.9	5.7	27.0	3.7	0.2	5.8	27.5	2.3
1(3)-MAG[b, d]	50.0	75.0	87.5	93.1	89.5	50.0	90.9	91.6	90.9
2-MAG[b, d]	50.0	25.0	12.5	6.9	10.5	50.0	9.1	8.4	9.1
ΣFFA[b, c]	9.6	6.2	1.7	1.3	55.4	1.7	5.1	2.9	53.4
Diets[a]									
Lipid classes (%)									
ΣTAG[b, c]	91.9	82.1	61.1	30.7	34.3	94.5	54.2	26.9	40.1
ΣDAG[b, c]	3.2	10.1	30.8	44.3	12.3	2.9	35.9	45.3	11.0
ΣMAG[b, c]	0.4	0.8	4.5	23.2	3.5	0.2	4.7	25.0	1.6
ΣFFA[b, c]	4.5	6.9	3.6	1.8	49.9	2.4	5.2	2.8	47.3

[a]Experimental oils and diets nomenclature as in Table 4.1.
[b]TAG (triacylglycerols), DAG (diacilglycerols), MAG (monoacylglycerols) and FFA (free fatty acids).
[c]Values determined by size-exclusion chromatography. Values are given as wt% of the total lipid classes (TAG, DAG, MAG and FFA).
[d]Values determined by ^{1}H-NMR. Values of each isomer are given as wt% of the total corresponding fraction (DAG or MAG).

Table 4.4. Selected fatty acid composition of the sn-2 position of the experimental oils.

	Oils[a]								
sn-2 (%)	FO	PN	PEL	PEH	PA	RN	REL	REH	RA
C16:0	42.5 (7.0)	7.8 (3.1)	20.93 (8.3)	11.9 (4.8)	3.7 (1.6)	2.7 (0.12)	19.5 (1.4)	11.7 (0.9)	6.8 (0.4)
C18:0	17.1 (0.4)	8.3 (0.4)	25.1 (2.1)	17.1 (1.4)	6.1 (0.4)	4.1 (0.08)	30.9 (0.8)	16.6 (0.4)	8.0 (0.2)
C18:1n-9	23.8 (2.9)	49.0 (18.9)	27.9 (11.0)	19.7 (7.7)	21.2 (7.9)	28.8 (17.5)	29.3 (15.6)	16.5 (8.6)	12.2 (6.7)
C18:2n-6	44.3 (0.8)	55.5 (6.3)	32.0 (2.6)	20.9 (1.6)	21.8 (1.9)	52.0 (9.9)	31.4 (7.7)	18.9 (4.9)	13.9 (3.3)
ΣSFA	38.0 (10.0)	8.0 (3.7)	21.4 (10.9)	12.5 (6.4)	4.0 (2.1)	3.2 (0.2)	29.8 (2.5)	13.4 (1.5)	6.9 (0.7)
ΣMUFA	23.4 (7.1)	47.8 (19.2)	28.3 (11.6)	19.8 (8.0)	21.9 (8.3)	27.3 (17.9)	29.9 (17.4)	16.7 (9.5)	11.8 (7.0)
ΣPUFA	28.7 (12.1)	52.3 (6.9)	32.3 (2.6)	21.0 (1.7)	21.5 (1.9)	51.0 (13.9)	29.5 (9.1)	18.9 (6.0)	14.0 (4.3)

Values are given as the % of each fatty acid at the sn-2 relative to its content in the oil. Values in brackets are given as the % of each fatty acid at the sn-2 position relative to the total fatty acid amount.

[a]Experimental oils nomenclature as in Table 4.1.

4.2.7. Statistical analysis

Data were subjected to a one-way analysis of variance (ANOVA) and the significance of the differences between means was tested by Tukey's test. Values are given as means±standard error of a pooled samples each containing faeces samples from 26 fish, analysed in triplicate. Differences were considered significant when $P<0.05$. All statistics were performed by means of the General Lineal Model (Proc GLM) of SAS® software version 9.2 (SAS Institute Inc., Cary, NC, USA).

4.3. Results

Characterization of experimental oils and diets

As a consequence of the lack of information about chemically re-esterified oils from vegetable origin in the literature, the characterization of the experimental oils was necessary and was already reported in Trullàs et al. (2015). Briefly, differences in the fatty acid composition among the four types of oil (N, EL, EH, A) within each source (P and R) were low (Table 4.2). Regarding the different lipid classes (Table 4.3), native oils (FO, PN and RN) were mainly composed of TAG (> 80%) and acid oils consisted of FFA in more than 50%. EL re-esterified oils resulted in MAG and DAG proportions of about 6% and 33-39%, respectively, which increased to 27% MAG and 46-48% DAG in EH oils. 1(3)-MAG constituted the major MAG isomer in most of the oils, and especially in re-esterified and acid oils. Similarly, 1,3-DAG constituted the highest DAG isomer (66-77%). No formation of TAG polymers was observed. In relation to the fatty acid composition of the sn-2 position (Table 4.4.), an increase in the content of SFA located in sn-2 was clearly observed in both palm and rapeseed EL and EH oils, and especially in EL, compared to the native oil.

Apparent digestibility of fatty acids

The nine experimental diets were well accepted and survival rates were over 97% with all of them. Apparent digestibility coefficients (ADC) of total and individual fatty acids are given in Table 4.5. for palm and in Table 4.6. for rapeseed. Significantly lower digestibility values were obtained in palm diets in relation to FO diet. When comparing among palm diets, differences in digestibility were also obtained. ADC of total fatty acids of acid oil diet (PA: 49.6%) was lower than that of native oil diet (PN: 61.8%), but it reached higher values when re-esterified (PEL: 65.7% and PEH: 74.4%). In fact, in PA diet, ADC of both

total fatty acids and total SFA were significantly lower than in the rest of diets. As observed, ADC of total SFA seemed to be largely determined by ADC of C16:0, which represented approximately a 40% of the total fatty acids in palm oils and diets (Table 4.2). Compared to PA, higher digestibility values were also obtained in re-esterified oils diets (PEL and PEH) for total MUFA and PUFA.

When comparing digestibility values of re-esterified oils diets with PN diet, differences were obtained between PEL and PEH. In PEL, ADC of both individual and groups of fatty acids did not present significant differences compared to PN in any case, although they were numerically higher in most cases. Contrarily, ADC of total fatty acids, as well as of both individual and total SFA and MUFA, were significantly higher in PEH than in PN, which was especially remarkable in SFA. If compared to FO, PEH diet obtained similar ADC of total MUFA and PUFA, but not of total SFA.

When PEL and PEH diets were compared between them, only numerically differences in the ADC of the different individual and total fatty acids were observed. However, digestibility values in PEH were, in general, higher than in PEL. Once more, these differences were particularly notable in both individual and total SFA (PEL: 47.8% and PEH: 62.3% for total SFA).

In rapeseed, digestibility values were higher than those obtained in palm. Similarly, ADC of total fatty acids in REL and REH diets were higher when compared to RA, but similar to values of RN and FO diets. No significant differences were observed in ADC of fatty acids between REL and REH diets.

Table 4.5. Apparent digestibility coefficient (ADC %) of selected fatty acids in gilthead sea bream fed the experimental palm diets.

	Diets[a]				
	FO	PN	PEL	PEH	PA
Fatty acid			*ADC (%)*		
C14:0	88.1 ± 0.9a	51.6 ± 3.7cd	57.8 ± 2.3bc	66.0 ± 2.2b	44.3 ± 1.7d
C16:0	82.7 ± 1.0a	42.4 ± 2.9c	47.6 ± 5.0bc	62.6 ± 2.2b	23.8 ± 4.3d
C18:0	75.3 ± 0.9a	37.7 ± 3.2c	43.3 ± 5.8bc	58.4 ± 2.6b	26.6 ± 3.7c
C18:1n-9	88.4 ± 0.7a	77.1 ± 2.4cd	81.6 ± 1.0bc	86.6 ± 0.9ab	71.7 ± 1.0d
C18:2n-6	89.7 ± 1.1a	84.4 ± 1.8b	85.7 ± 1.1ab	86.8 ± 0.8ab	78.3 ± 0.2c
C18:3n-3	92.9 ± 0.7a	72.9 ± 3.4b	75.5 ± 3.0b	76.9 ± 1.1b	67.3 ± 3.0b
C20:4n-6	96.4 ± 0.4	ND	ND	ND	ND
C20:5n-3 (EPA)	97.3 ± 0.3a	78.8 ± 2.7b	77.3 ± 4.9b	74.5± 1.3b	69.6 ± 4.4b
C22:6n-3 (DHA)	96.7 ± 0.4a	82.8 ± 2.2b	80.9 ± 4.1b	78.4 ± 1.4b	74.0 ± 2.1b
ΣSFA	82.9 ± 1.0a	42.0 ± 3.0c	47.8 ± 5.0bc	62.3 ± 2.2b	25.2 ± 4.1d
ΣMUFA	89.3 ± 0.31a	75.7 ± 2.5cd	80.5 ± 1.1bc	85.0 ± 1.1ab	70.6 ± 0.7d
ΣPUFA	85.4 ± 1.6a	78.8 ± 2.5ab	81.0 ± 2.1a	80.7 ± 1.1a	72.2 ± 0.8b
Σn-6 PUFA	90.1 ± 1.0a	83.9 ± 1.8b	85.5 ± 1.3ab	86.1 ± 0.8ab	77.9 ± 0.2c
Σn-3 PUFA	96.6 ± 0.4a	79.6 ± 2.6b	78.6 ± 4.1b	76.8 ± 1.3b	71.1 ± 3.0b
Total FA[b]	89.3 ± 0.1a	61.8 ± 2.6c	65.7 ± 2.8bc	74.4 ± 1.6b	49.6 ± 2.2d

Values represent mean ± SEM of pooled samples from 26 fish analyzed in triplicate. Values in the same row with different letters are significantly different (P<0.05), according to ANOVA.
[a]Experimental diets nomenclature: abbreviations as in Table 4.1.
[b]Total FA: total fatty acids.

Table 4.6. Apparent digestibility coefficient (ADC %) of selected fatty acids in gilthead sea bream fed the experimental rapeseed diets.

	Diets[a]				
	FO	RN	REL	REH	RA
Fatty acid			*ADC (%)*		
C14:0	88.1 ± 0.9a	69.0 ± 2.0b	71.0 ± 3.9b	64.2 ± 2.8b	56.1 ± 1.5b
C16:0	82.7 ± 1.0a	76.0 ± 3.5a	83.2 ±2.7a	83.7 ± 0.4a	57.1 ± 1.2b
C18:0	75.3 ± 0.9ab	67.2 ± 1.8b	80.8 ± 3.1a	81.4 ± 0.1a	40.4 ± 2.0c
C18:1n-9	88.0 ± 0.7ab	90.0 ± 0.8ab	92.7 ± 1.2ab	93.2 ± 0.2a	77.6 ± 1.7b
C18:2n-6	88.0 ± 1.1a	91.5 ± 1.1a	94.0 ±0.8a	94.3 ± 0.2a	83.6 ± 1.2a
C18:3n-3	91.9 ± 0.7a	93.7 ± 4.0a	94.7 ± 0.6a	95.5 ± 0.3a	86.0 ± 1.2a
C20:4n-6	95.8 ± 0.4	ND	ND	ND	ND
C20:5n-3 (EPA)	97.3 ± 0.3a	81.8 ± 0.1b	78.3 ± 3.1b	75.4 ± 2.8b	73.8 ± 0.1b
C22:6n-3 (DHA)	96.7 ± 0.4a	84.3 ± 1.2b	82.5 ± 2.2b	79.5 ± 2.1bc	72.1± 2.2c
ΣSFA	82.9 ± 0.9a	81.2 ± 8.6a	82.1 ± 3.3a	82.9 ± 0.8a	51.6 ± 0.8b
ΣMUFA	89.6 ± 0.3ab	89.2 ± 1.3ab	91.9 ± 1.4ab	92.4 ± 0.2a	76.4 ± 1.7b
ΣPUFA	85.4 ± 1.6bc	90.0 ± 1.3ab	92.1 ± 1.1a	92.2 ± 0.5a	84.5 ± 1.1c
Σn-6 PUFA	88.4 ± 1.0a	91.2 ± 1.0a	93.8 ± 0.9a	94.1 ± 0.3a	83.1 ± 1.3a
Σn-3 PUFA	96.6 ± 0.4a	89.9 ± 3.2ab	90.0 ±1.2ab	89.5 ± 0.9ab	85.2 ± 0.3b
Total FA[b]	89.3 ± 0.1a	89.7 ± 1.5a	90.6 ± 1.1a	91.1 ± 0.4a	78.3 ± 1.2b

Values represent mean ± SEM of triplicate pooled samples from 26 fish. Values in the same row with different letters are significantly different (P<0.05).
[a]Experimental diets nomenclature: abbreviations as in Table 4.1.
[b]Total FA: total fatty acids.

4.4. Discussion

In relation to lipid classes, the predominance of 1(3)-MAG and 1(3)-DAG over the rest of MAG and DAG isomers has been related to the higher stability of primary esters (sn-1(3)) than of secondary esters (sn-2) (Cruz Hernandez et al., 2012), which could cause acyl migration of fatty acids from sn-2 to sn-1 or sn-3 positions of MAG and DAG (Destaillats et al., 2010; Martin et al., 2014).

Regarding the higher content of SFA located at the sn-2 position of re-esterified oils compared to the native oil, it was more noticeable in EL than in EH oils. This was probably due to EH oils had more partial acylglycerols (MAG and DAG) and these were mainly 1(3)-MAG and DAG, so less SFA in sn-2 position were present.

The fatty acid composition and degree of saturation of re-esterified oils compared to native oils were unchanged, as many studies on interesterification reactions had previously described (Marangoni and Rousseau, 1998; Scheeder et al., 2003; Berry, 2009; Farfán, 2013).

For more details on the composition of re-esterified oils, readers are addressed to our previous trial in rainbow trout, in which the same oils were used and their characterization was already discussed (Trullàs et al., 2015).

Apparent digestibility of fatty acids

Results indicate that the dietary form of the lipid may be more influential on fatty acid digestibility than the fatty acid composition, as suggested by Ng et al. (2010). Indeed, as observed, esterification of FFA from acid oils with glycerol improves the ADC of fatty acids in both palm and rapeseed.

The higher nutritive value of acid oils when re-esterified to the glycerol molecule could be due to several factors. The lowest fatty acid digestibility of acid oils could be related to a feedback inhibition on the lipase activity caused by FFA, as suggested by Bogevik et al. (2008) for Atlantic salmon. Thus, the richness in FFA of acid oils in our study could reduce the activity of lipase, resulting in a lower hydrolysis of the TAG and the partial acylglycerols present in these oils, which would have a negative effect on its digestibility. On the other hand, in chicks, secretion of bile salts seems to be stimulated by the presence of TAG and 2-MAG, and not FFA, in the intestine (Sklan, 1979). Although no information on this topic is available in fish, it has to be taken into account that bile salts seem to be a requirement for the hydrolytic activity of lipase in many marine species (Gjellesvik et al.,

1989; Iijima et al., 1998; Nolasco et al., 2011). Then, in the present study, an impairment in lipase activity would arise in animals fed acid oils, this producing a lower fatty acids emulsification than in the other experimental oils and leading to a reduction in the absorption of fat. Moreover, as already mentioned, divalent ions present in the intestine have a tendency to bind to long-chain free fatty acids and especially free SFA. Then, in acid oils, a high subsequent formation of insoluble salts would occur in fish, being detrimental in terms of fatty acid digestibility (Ringø, 1991; Olsen et al., 1998).

Comparing re-esterified oils with native oils, the effects on digestibility varied between the oil source (palm and rapeseed) and also between the type of re-esterified oil (PEL and PEH). In palm, contrarily to what was expected, the increase in the content of SFA in sn-2 did not improve digestibility, as no differences in ADC of fatty acids between PEL and PN were observed. Nevertheless, although not significant, higher numerically digestibility values were obtained in PEL than in PN diets, especially for the most quantitatively important fatty acids (SFA and MUFA). Better absorption of SFA when located at the central position of TAG had been reported in chickens, rats, piglets and human infants (Filer et al., 1969; Innis et al., 1995; Lien et al., 1997; Lin et al., 2010). However, in these studies, the minimum percentage of SFA in sn-2 position was 33.9%, so it is possible that the lower content of SFA in sn-2 obtained in re-esterified oils in the present study (maximum 21.4%) was insufficient to have a clear effect on digestibility.

Differently, an improvement in digestibility values were obtained in PEH diet compared to PN diet, although they did not always reach those obtained in the control diet (FO). These results seem to point out a positive effect of the partial acylglycerols on digestibility, since PEH had a high content of MAG and DAG. The emulsifying role of partial acylglycerols as amphiphilic molecules in digestion has been widely reported in studies in both human and animal nutrition (Hayes et al., 1994; Da Costa, 2003). Therefore, in the present study, having more MAG and DAG in PEH would have probably helped incorporating a higher amount of hydrophobic FFA in the core of mixed micelles during digestion than in the rest of diets. However, in rainbow trout (Trullàs et al., 2015) no differences among PN, PEL and PEH were found. Differences observed between species could be related to factors such as variations in their digestive physiology but also to the effect of the different water temperatures at which the two species were reared (14.3 ºC in rainbow trout and 21.5ºC in gilthead sea bream) during the whole experimental period. As reported by Vilarrasa et al. (2014) for palm oils, a re-esterified palm oil high in MAG and DAG had an expanded

melting range if compared to its corresponding native oil, which means that this oil has a higher solid fat content than the rest of experimental oils at a given temperature. As observed by the aforementioned authors, a high solid fat content was slightly detrimental in terms of crude fat and fatty acids apparent absorption. Then, water temperature would have had an effect on the melting point of the oils in fish diets. As temperature was higher in sea bream than in rainbow trout studies, the solid fat content of PEH would have been lower in sea bream, producing a beneficial effect on its digestibility.

Compared to rapeseed, ADC of fatty acids in palm diets were lower, which was expected due to the widely known fact that the process of lipid digestion and absorption seem to favor the utilization of unsaturated fatty acids over their more saturated counterparts (Sigurgisladottir et al. 1992; Olsen et al., 1998). In fact, as reported by many authors, high palm oil levels in fish diets significantly reduce fatty acids digestibility, especially in cold water (Torstensen et al., 2000; Ng et al., 2010). In the present study, experimental oils were the only source of fat in the experimental diets, in which the level of SFA of the total fatty acids (45-48%) exceeded the level of SFA (23%) of the total fatty acids up to which digestibility starts to decrease, as described by Hua and Bureau (2009).

In rapeseed oil diets, in spite of the improvement of digestibility of fatty acids when re-esterified, and contrarily to palm, no differences in ADC among RN, REL and REH diets were obtained. This is in accordance with the previous results obtained in rainbow trout (Trullàs et al. 2015) and could probably be a consequence of the strong effect that the degree of unsaturation had on digestibility, which could have masked both the possible effect of MAG-DAG and the effect of the increased content of SFA in sn-2. Therefore, digestibility of fatty acids of the experimental oils seemed to be more affected by their degree of saturation than by their positional distribution and lipid class composition of the oil. In fact, as observed in palm diets, ADC of individual SFA set the trend of both ADC of total SFA and total fatty acids, clearly showing the importance of the degree of saturation on the overall digestibility.

Independently from the source (palm or rapeseed) and the type of oil (native, re-esterified or acid), digestibility values obtained in the present study for gilthead sea bream were lower than those described in rainbow trout (Trullàs et al., 2015) (mean of 14.3±5.81 for

palm and 9.8±5.56 for rapeseed, for total fatty acids). Different faeces collection methods were used, -euthanasia followed by collection of faeces directly from the intestine in sea bream and stripping in rainbow trout- and this could have been related to the differences obtained between species. In spite of this, in a study in sea bream by Fernández et al. (1996), values obtained by stripping were very close to those obtained from sampling the content of the final segment of the intestine by dissection. One of the main differences between gilthead sea bream and rainbow trout is their habitat. In marine fish such as gilthead sea bream, the salinity of sea water results in a constant need to drink large amounts of water, rich in calcium and magnesium, to compensate what they lose through osmosis. Insoluble salts can be formed in the presence of these divalent ions and free long-chain fatty acids in the intestine. Thus, this might be affecting digestibility in marine fish in a greater extent than in freshwater fish. In fact, Ringø (1991) found a difference of a 7% in lipid digestibility in Arctic charr when fish were maintained in freshwater compared to sea water.

In conclusion, results from the present study corroborated that the esterification of FFA with glycerol improves the nutritive value of vegetable acid oils. Hence, both palm and rapeseed re-esterified oils could be incorporated as a source of energy in diets for gilthead sea bream without negatively affecting apparent digestibility coefficients of fatty acids when compared to their native oils.

This improvement in digestibility became significantly higher only in rich-in SFA (palm) re-esterified oils high in MAG and DAG, this showing a possible emulsifying effect of the presence of partial acylglycerols. However, this improvement would not reach apparent digestibility coefficients as those found in oils that are mainly unsaturated, such as rapeseed and fish oil. It is important to take into account that all palm experimental diets presented low total fatty acids digestibility values (under 75%) in gilthead sea bream. Therefore, the inclusion of re-esterified palm oils, irrespectively of their type, as a dietary source of energy should be done in combination with oils with a higher degree of unsaturation, as it had been done with native palm oil in previous studies (Benedito-Palos et al., 2008; 2010).

Further studies regarding the inclusion of these oils in diets for gilthead sea bream should be carried out in order to study their effect on growth, metabolism and fillet quality in this species.

CHAPTER 5

ACID AND RE-ESTERIFIED RAPESEED OILS AS ALTERNATIVE VEGETABLE OILS FOR RAINBOW TROUT DIETS: EFFECTS ON LIPID DIGESTIBILITY AND GROWTH

Acid and re-esterified rapeseed oils as alternative vegetable oils for rainbow trout diets: effects on lipid digestibility and growth

Trullàs, C.[1*], Fontanillas, R.[2], Tres, A.[3], Barroeta, A.C. [1], Sala, R.[1]

[1]Departament de Ciència Animal i dels Aliments, Servei de Nutrició i Benestar Animal (SNiBA), Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain;

[2]Skretting Aquaculture Research Center (Skretting ARC), Sjøhagen 3, Stavanger 4016, Norway;

[3]Departament de Nutrició i Bromatologia-XaRTA-INSA, Facultat de Farmàcia, Universitat de Barcelona, 08028 Barcelona, Spain.

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Abstract

The present study aimed at evaluating the effects of dietary acid and re-esterified rapeseed oils as alternatives to native vegetable oils (VO) on growth performance and feed utilization in rainbow trout. Acid oils are a free fatty acid (FFA)-rich by-product from the refining of VO and re-esterified oils are the final product of a chemical esterification process between acid oils and glycerol. Because re-esterified oils have a high content of mono- and diacylglycerols (MAG and DAG), known for being good emulsifiers, a higher nutritive value than that of the native and the acid oils might be expected. A 72-day feeding trial where triplicate groups of rainbow trout were fed eight experimental diets formulated to contain a 15% of a native, a re-esterified and an acid rapeseed oil, in addition to a 5% of fish oil (FO), was carried out. Diets with the native or the re-esterified oils blended with the acid oil were also studied. A commercial fish oil was used for the control diet. Fish fed rapeseed acid and re-esterified oils diets (RA and RE, respectively) showed high fat and total fatty acid apparent digestibility coefficients (ADC) (RA: 90.5±0.3%, RE: 92.5±1.0% for total fat and RA: 95.7±0.1%, RE: 95.8±0.2% for total fatty acids). However, the lowest total fatty acid ADC was that obtained in animals fed RA, which was significantly lower ($P<0.05$) than that of fish fed the rapeseed native oil diet (RN: 96.7±0.1%). No significant differences in final weight were obtained between fish fed RA (375.9±2.9g) and RE (381.5±11.1g) and those fed RN (393.7±6.1g), even though both values were significantly lower ($P<0.05$) than that of fish fed the control diet (411.1±3.3g). Nonetheless, fish fed diets including blends of the rapeseed acid and the re-esterified oils (RE/RA and RA/RE) had higher final weights (392.8±4.4 and 394.6±1.6, respectively) than those of RA and RE, although differences were not statistically significant. Furthermore, RA and RE diets did not produce relevant changes in plasma parameters or in the morphology of liver and intestine of fish. Therefore, the inclusion of rapeseed acid and re-esterified oils along with a 5% of FO in aqua feeds does not seem to have negative effects on fat and fatty acid digestibility, growth, plasma parameter or morphology of liver and intestine in rainbow trout. However, before recommending their use, further studies regarding their effects on the final composition and quality of fillets should be carried out.

5.1. Introduction

There are many studies reporting the suitability of vegetable oils (VO) as an alternative to fish oil (FO) in fish feeds (Fonseca-Madrigal et al., 2005; Sun et al., 2011; Tocher et al.,

2003a; Turchini et al., 2009), as they are sustainable and economically advantageous sources. VO are mainly used in both the food and the feed industries, although their use by the biofuel industry has been rising notably since the early 2000s (Gunstone, 2011). In Europe, this is especially remarkable for rapeseed, which is the predominant feedstock for biodiesel production (Haas, 2005). Thus, the competition among industries has caused an increase of grains and oilseed prices (Behr and Pérez Gomes, 2010), which in turn has led to the need of finding suitable and economically interesting alternatives to the commonly VO used in fish nutrition. In this regard, the interest of the feed industry for the by- and co-products generated during the crude VO processing has also been growing. Indeed, a significant amount of by-products is generated from crude oil refining processes and can be valuable feedstocks for animal feeds (Dumont and Narine, 2007). Of these products, acid oils from the chemical refining of VO, a free fatty acid (FFA)-rich by-product, were found to be quite promising for feeding uses (Nuchi et al., 2009). In rainbow trout, an apparent digestibility coefficient (ADC) of total fatty acids above 95% was obtained for a diet including rapeseed acid oil, which did not differ from that of the native oil diet, the latter referring to the unrefined and unprocessed oil produced from vegetables (Trullàs et al., 2015).

Vegetable acid oils can be chemically re-esterified with glycerol to produce the so-called re-esterified VO. These oils can have a high final content of partial acyglycerols (monoacylglyerols, MAG and diacylglyerols, DAG), amphiphilic molecules that could exert a beneficial effect on digestibility (Fregolente et al., 2009; Martin et al., 2014). Good results in fat absorption and growth performance in broiler chickens and fattening pigs have been obtained when including re-esterified VO in diets (Vilarrasa et al., 2014, 2015b,d). Although the digestibility of rapeseed re-esterified oil has been investigated in rainbow trout (Trullàs et al., 2015), growth performance has not yet been assessed. Fatty acid digestibility coefficients of rainbow trout fed re-esterified oils from an unsaturated vegetable source such as rapeseed did not present differences compared to those of fish fed the native oil (Trullàs et al., 2015). Even so, from the economical point of view, acid oils seem to be a more interesting alternative than re-esterified oils since the latter are approximately 100 €/t more expensive due to the added cost of the chemical esterification (Parini, personal communication). The economic viability of re-esterified oils in relation to native oils is variable since it depends on the price differential between native and acid oils, which is in turn subjected to fluctuation.

While the digestibility of acid and re-esterified oils is acceptable in rainbow trout (Ng et al., 2010; Trullàs et al., 2015), growth performance and productive parameters have not been investigated (Aliyu-Paiko and Hashim, 2012).

Thus, one of the objectives of the present study was to assess the growth performance and the feed utilization of rainbow trout fed acid and re-esterified rapeseed oils in comparison with those of fish fed the native oil. We also aimed at evaluating the partial substitution of the native and the re-esterified oils by graded levels of the more economical acid oil in order to optimize their use.

Because diet composition could induce changes in specific plasma haematological and biochemical parameters (Peres et al., 1999), the evaluation of the plasma biochemical parameters and also the morphology of liver and intestine could provide additional information on the effects of the inclusion of these alternative oils.

5.2. Materials and methods

5.2.1. Experimental diets

Experimental diets (45% protein and 21% lipid) contained the same ingredient composition except for the added lipid source (Table 5.1.). Three different types of rapeseed oil – native (RNO), re-esterified (REO) and acid (RAO) – were included in the diets alone (single oil diets: RN, RE or RA) or blended in graded levels (diet RE/RA: 66% RE-33% RA; diet RA/RE: 66% RA-33% RE; diet RN/RA: 66% RN-33% RA and diet RA/RN: 66% RA-33% RN) in a proportion of 15%. A 5% of commercial fish oil (FO) was included in all experimental diets. A diet including only commercial fish oil (20% of the diet) was used as a control (F). Experimental oils were provided by SILO S.p.a. (Firenze, Italy) (RNO and REO) and Cargill (Schiphol, The Netherlands) (RA). The re-esterified oil (REO) was produced by SILO S.p.a. as described in Trullàs et al. (2015). Feeds were produced at the Skretting Feed Technology Plant (Aquaculture Research Center; Stavanger, Norway) as extruded pellets. Yttrium oxide (Y_2O_3) was added to the diets as an inert marker for the apparent digestibility of fatty acids determination. Nutrient composition of experimental diets was determined by standard procedures (AOAC, 2005): moisture (934.01), ash (942.05), crude protein (968.06) and crude lipid (920.39). Unsaponifiable matter was also calculated following AOAC (2005) (933.08) as a quality control. Gross energy of dried feed was determined using an adiabatic bomb calorimeter (IKA-Kalorimeter system C4000, Jankel-Kunkel, Staufen, Germany). Yttrium was

analysed in accordance to Austreng et al. (2000). The ingredient formulation and proximate composition of the diets are shown in Table 5.1.

Table 5.1. Ingredient formulation and proximate composition of the experimental diets.

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Ingredient composition (g kg^{-1})								
Wheat[a]	60	60	60	60	60	60	60	60
Wheat gluten[b]	232.8	232.8	232.8	232.8	232.8	232.8	232.8	232.8
Soya bean meal[c]	80	80	80	80	80.0	80.0	80.0	80.0
Soya protein concentrate[d]	150	150	150	150	150	150	150	150
Faba beans whole[e]	100	100	100	100	100	100	100	100
Fish meal[f]	150	150	150	150	150	150	150	150
Fish oil[g]	201.3	52	52	52	52	52	52	52
Experimental oils[h]	0	150	150	150	150	150	150	150
Yttrium premix[i]	1	1	1	1	1	1	1	1
Mineral and vitamin premix[i]	24.9	24.9	24.9	24.9	24.9	24.9	24.9	24.9
Proximate composition (g kg^{-1})								
Dry matter	925.7	925.9	927.9	929.9	931	928.9	926.8	927.3
Crude protein	472.2	466.1	485.1	468.2	468	466.2	471.7	474.3
Crude fat	204.1	215.7	187.7	210.4	219.5	214.3	191.9	201.4
Ash	64.2	63.3	65	70.6	67.6	65.6	65.2	68.1
Gross energy (kJ g^{-1})	22.8	22.5	22.8	22.4	22.3	22.7	22.4	22.4
Digestible energy (kJ g^{-1})	20.0	19.5	19.1	20.2	18.0	20.2	19.8	19.3

Experimental diets nomenclature: F: fish oil (control diet); RN: rapeseed native oil; REH: rapeseed re-esterified oil; RA: rapeseed acid oil; RE/RA: 66% rapeseed re-esterified oil - 33% rapeseed acid oil; RA/RE: 66% rapeseed acid oil - 33% rapeseed re-esterified oil; RN/RA: 66% rapeseed native oil - 33% rapeseed acid oil and RA/RN: 66% rapeseed acid oil - 33% rapeseed native oil.

[a]Statkorn, Norway.

[b]Cerestar Scandinavia AS, Denmark.

[c]IMCOPA, Brasil.

[d]Denofa, Norway.

[e]Ceremis, France.

[f]Welcon AS, Norway.

[g]Holtermann ANS, Norway.

[h]Experimental oils.

[i]Vitamin and mineral premix, according to requirement data from NRC (2011). Trow Nutrition, The Netherlands.

[j]Values were determined by calculating the apparent digestibility coefficient (ADC) of the gross energy of diets using the formula proposed by Maynard & Loosli (1979), prior to multiplying this value to the gross energy of the corresponding diet.

5.2.2. Fish husbandry and sampling

All the procedures were conducted in accordance with the Animal Protocol Review Committee of the Universitat Autònoma de Barcelona (UAB) and following the European Union Guidelines for the ethical care and handling of animals under experimental conditions (2010/63/EU). The trial was carried out at the Skretting Italia SPA (Mozzecane, Italy) facilities. A total of 576 rainbow trout with a mean initial body weight of 101.7±8.80 g were randomly distributed into 24 cylindro-conical tanks of 600 l of capacity (24 fish per tank) in an open freshwater system with a continuous water flow of 24 l min^{-1}. Water temperature (14.3ºC) and dissolved oxygen levels (7.4±0.37 mg/l) were maintained constant throughout all the experimental period. Tanks were subjected to a 24h light photoperiod. Each diet was randomly assigned to three replicate tanks and was fed twice a day by automatic feeders, adjusted to provide the 2.5% of biomass daily. Uneaten feed was collected by filtering effluent water from each tank and collectors were emptied after each meal and feed intake was recorded daily. At day 60, all the fish from each tank were weighed and measured individually before being anaesthetized with clove oil (Phytosynthese, Za de Mozac-Volvic, France; 0.04 ml/l). Faecal samples were collected from the hindgut by manual stripping, after which fish were put into tanks supplied with freshwater to recover from anaesthesia. Samples were pooled by tank and stored at -20°C prior to analysis of yttrium oxide, total fat, fatty acid composition and gross energy. At day 72, five fish from each tank were anaesthetized with clove oil (Phytosynthese, Za de Mozac-Volvic, France), having been previously fasted for 48 hours. Blood samples were then taken from the caudal vein by puncture with a heparinized syringe and collected in 2 ml tubes with heparin (Hospira Inc., CA, U.S.) for further plasma biochemical analyses. Once the blood sampling was finished, five fish from each tank were euthanized in excess anaesthetic and weighed. Liver and viscera were taken and weighed for biometrical measurements. Samples of liver and intestine were also taken and fixed in 10% buffered formalin for histological examination under a light microscopy.

5.2.3. Total fat and fatty acid composition

Total fat of diets and faeces was determined by Nuclear Magnetic Ressonance (NMR). Fatty acid composition was determined by gas chromatography-flame ionization detector (GC-FID). Fatty acid methyl esters (FAME) were obtained by direct methylation, according to Meier et al. (2006) and analysed using an HP 5890A gas chromatograph.

They were identified by comparison of their retention times with those of known standards, and quantified by internal normalization (FAME peak area/total FAME area, in %).

5.2.4. Lipid class composition

Lipid class composition (TAG, DAG, MAG and FFA) of FO, RNO, REO and RAO, as well as that of all experimental diets, were determined by size-exclusion chromatography on an Agilent 1100 series HPLC chromatograph equipped with a Refractive Index Detector (RID) set at 35Cº. Oils were melted at 55ºC prior to analysis, and a solution of approximately 10 mg of oil/ml of tetrahydrofurane was prepared. The solution was filtered through a Nylon filter (0.45 µm) and injected (20 µl loop) to the chromatograph equipped with two Styragel columns (StyragelHR 1 and Styragel HR 0.5) of 30 cm x 0.78 cm i.d., filled with a spherical styrenedivinylbenzene copolymer of 5µm particle size (Water Associates, Milford, MA, USA), connected in series and placed in an oven set at 35°C. The mobile phase consisted of tetrahydrofuran at 1 ml/min. For diets, fat was previously extracted with diethyl ether following the method 2003.05 from AOAC (2005). Data was expressed as peak area normalitzation (in %), considering the area of the peaks corresponding to TAG, DAG, MAG and FFA.

5.2.5. Calculations

Apparent digestibility coefficient (ADC) of fat, fatty acids and gross energy (GE) was calculated as: ADC (%) = 100 − [100 × (Y in feed/Y in faeces) · (F in faeces/F in feed)] (Maynard and Loosli, 1979), where F = fat ($mg \cdot kg^{-1}$), fatty acid ($mg \cdot kg^{-1}$) or gross energy ($kJ\ g^{-1}$) and Y = yttrium ($mg \cdot kg^{-1}$). ADC of GE was used to calculate the digestible energy (DE) of the diets.

Growth performance, feed utilization and biometrical parameters were calculated according to standard formulae. Weight gain was calculated from WG (g) = final weight-initial weight, feed intake was determined from [total dry matter intake / (number of fish x number of days fed)], feed conversion ratio from FCR = (dry feed fed) / (wet weight gain), specific growth rate (SGR) from [ln(final weight)-ln(initial weight)] / (number of days) x 100 and average daily growth from AVG = (gain %) / (number of days). Furthermore, condition factor (CF) = 100 x [final weight (g)] / [fork length (cm)]^3, hepatosomatic index (HSI) = (weight of liver) / (total fish weight) x 100 and viscerosomatic index (VSI) = (weight of viscera) / (total fish weight) x 100 were also calculated.

5.2.6. Plasma analyses

Plasma was obtained after immediate centrifugation at 11337 *g* for 2 minutes of the blood samples, pooled per tank and stored at -20 ºC for further analyses. Glucose, protein, triglycerides, cholesterol, free fatty acids, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) were analysed using standard clinical methods with an Olympus AU400 – 3112676 chemistry analyser, (Germany).

5.2.7. Liver and intestine histology

Samples of liver and intestine fixed in 10% buffered formalin were dehydrated in a graded ethanol series and embedded in paraffin. Sections of 4 mm were stained with haematoxylin and eosin (H&E) (Martoja and Martoja-Pierson, 1970) for morphological observations using a Leica DM5000B microscope (Jenoptik, Germany). Images were taken with ProgRes® CapturePro software (Jenoptik, Germany).

5.2.8. Statistical analysis

Data were subjected to a one-way analysis of variance (ANOVA) and the significance of the differences between means was tested by Tukey's test. Digestibility values are given as means±standard error of the mean of triplicate values, each being a pooled sample from 24 fish. Values of growth performance, feed utilization and biometrical parameters are given as means±standard error mean of triplicate values, each containing information from 24 fish. Differences were considered significant when $P<0.05$. All statistics were performed by means of the General Lineal Model (Proc GLM) of SAS® software version 9.2 (SAS Institute Inc., Cary, NC, USA).

5.3. Results

Characterization of experimental oils and diets

Results of fatty acid composition and unsaponifiable matter of experimental diets are shown in Table 5.2. Lipid class composition of experimental oils and diets are shown in Table 5.3.

Although differences among rapeseed oils were minor with respect to their fatty acid composition, as seen in diets, they were notable in terms of lipid class composition. FO and RNO were constituted by TAG in more than a 90%, while the re-esterified oil (REO) had a considerable amount of partial acylglycerols (35.4% MAG and 34% DAG) and the acid oil (RAO) was rich in FFA (64.3%). No presence of TAG polymers was observed.

As in the oils, minor differences in the fatty acid composition were found among diets. Although their lipid class composition mirrored those of the oils in the case of the natives (F and RN; TAG>90 %), differences were observed in RE and RA. Both in the acid oil diet (RA) and in the re-esterified oil diet (RE) higher percentages of TAG but lower of FFA and partial acylglycerols than in their corresponding oils were obtained.

In the blended oils diets, an increase in FFA was observed as more RAO was included. Similarly, an increase of MAG and DAG was observed as a higher level of REO was present.

Table 5.2. Fatty acid composition of the experimental diets.

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Fatty acid (%)								
C14:0	8.1	3.0	2.8	2.9	2.7	2.7	2.9	2.8
C16:0	18.6	10.3	11.0	13.5	10.4	10.6	12.4	11.5
C16:1n-7	8.7	3.3	3.1	3.3	3.0	3.1	3.2	3.0
C16:2n-6	1.0	0.4	0.3	0.3	0.3	0.3	0.3	0.3
C18:0	3.2	2.4	2.8	4.7	2.5	2.6	4.0	3.4
C18:1n-9	10.8	35.7	35.5	32.2	36.2	36.3	34.2	35.4
C18:1n-7	3.2	3.5	4.3	4.1	3.5	3.9	4.0	4.0
C18:2n-6	5.9	16.7	18.5	17.0	17.7	18.1	17.5	17.9
C18:3n-3	1.1	6.2	5.1	3.9	5.9	5.6	4.4	4.9
C18:4n-3	2.4	0.9	0.8	0.8	0.8	0.8	0.8	0.8
C20:1[a]	2.5	2.5	2.2	2.2	2.5	2.3	2.1	2.2
C20:4n-6	1.0	0.4	0.3	0.4	0.3	0.3	0.3	0.3
C20:4n-3	0.7	0.2	0.2	0.2	0.2	0.2	0.2	0.2
C20:5n-3 (EPA)	14.7	5.2	4.8	5.0	4.9	4.7	4.8	4.8
C22:1[a]	2.1	1.6	1.4	1.4	1.6	1.5	1.4	1.3
C22:5n-3	1.7	0.6	0.6	0.6	0.6	0.6	0.6	0.6
C22:6n-3 (DHA)	10.8	4.2	3.9	4.1	4.1	3.8	3.9	3.9
C24:1n-9	0.7	0.4	0.6	0.6	0.6	0.5	0.6	0.6
ΣSFA[b]	30.8	16.8	17.5	22.7	16.8	17.0	20.6	18.9
ΣUFA[c]	69.2	83.2	82.5	77.3	83.2	83.0	79.4	80.4
ΣMUFA[d]	28.5	47.8	47.7	44.4	47.8	48.0	46.0	46.9
ΣPUFA[e]	40.6	35.3	34.8	32.8	35.4	35.0	33.3	34.1
Σn-6 PUFA[e]	8.8	17.8	19.3	18.0	18.7	19.1	18.4	18.9
Σn-3 PUFA[e]	31.8	17.5	15.5	14.8	16.7	15.9	14.9	15.2
SFA:UFA	0.4	0.2	0.2	0.3	0.2	0.2	0.3	0.2
n-3:n-6	3.6	1.0	0.8	0.8	0.9	0.8	0.8	0.8

Experimental diets nomenclature as in experimental diets (Table 5.1).
[a]Sum of isomers.
[b]SFA: saturated fatty acids. It includes other SFA of small quantity.
[c]UFA: unsaturated fatty acids. It includes other UFA of small quantity.
[d]MUFA: monounsaturated fatty acids. It includes other MUFA of small quantity.
[e]PUFA: polyunsaturated fatty acids. It includes other PUFA of small quantity; n-6 PUFA: omega 6 polyunsaturated fatty acids; n-3 PUFA: omega 3 polyunsaturated fatty acids.

Table 5.3. Lipid class composition of the experimental oils and diets.

	Oils			
	FO	RNO	RAO	REO
Lipid classes (%)				
ΣTAG[a]	93.8	95.6	20.5	26.6
ΣDAG[a]	2.9	2.5	12.5	34.0
ΣMAG[a]	0.7	0.2	2.7	35.4
ΣFFA[a]	2.6	1.7	64.3	2.0

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Lipid classes (%)								
ΣTAG[a]	92.9	93.4	46.0	54.0	77.16	62.2	49.0	46.6
ΣDAG[a]	3.2	3.1	9.4	21.9	5.28	6.9	19.0	14.8
ΣMAG[a]	0.8	0.7	2.1	22.3	1.05	1.6	14.8	7.9
ΣFFA[a]	3.1	2.8	42.4	1.8	16.51	29.2	17.2	30.7

Experimental oils and diets nomenclature as in Table 5.1.
[a]TAG (triacylglycerols), DAG (diacylglycerols), MAG (monoacylglycerols) and FFA (free fatty acids).

Apparent digestibility of fat and fatty acids of the diets

The eight experimental diets were well accepted and total mortality was about 1%.

ADC of total fat and total fatty acids of the diets were all above 90% and 96%, respectively (Table 5.4.), being similar or higher than that of F. Minor but significant differences ($P<0.05$) were found among rapeseed diets regarding total fat and total fatty acids digestibility, the latter being slighter than those of total fat.

When single rapeseed oil diets were compared, the lowest total fatty acid ADC was that obtained for RA (95.7±0.1%), which was significantly lower ($P<0.05$) than that of RN (96.7±0.1%). In relation to the different categories of fatty acids, it is worth mentioning that significantly higher ($P<0.05$) ADC values were obtained for SFA (especially palmitic acid, C16:0, and stearic acid, C18:0) in RE.

Regarding the replacement of RAO by RNO, no differences due to the level of inclusion of RA were obtained. ADC of total fatty acid of RN/RA and RA/RN resulted in values between those of RA and RN, with no significant differences.

Similarly, no differences in total fatty acid ADC were observed as REO was replaced by RAO (RE/RA, RA/RE).

Table 5.4. Apparent digestibility coefficient (ADC %) of selected fatty acids in rainbow trout fed the experimental diets.

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Fatty acid				*ADC %*				
C14:0	95.5±0.2^{c}	97.7±0.1^{a}	96.6±0.2^{b}	97.1±0.2^{ab}	96.5±0.2^{b}	97.1±0.2^{ab}	97.6±0.2^{a}	97.1±0.1^{ab}
C16:0	91.8±0.3^{d}	95.4±0.1^{ab}	93.8±0.3^{c}	95.7±0.2^{a}	94.2±0.5^{bc}	94.8±0.4^{abc}	96.0±0.4^{a}	95.6±0.1^{ab}
C16:1n-7	98.8±0.2	99.0±0.2	98.1±0.5	98.8±0.2	98.8±0.1	99.0±0.0	98.9±0.2	98.8±0.1
C18:0	86.9±0.3^{bc}	83.7±0.2^{d}	88.9±0.3^{b}	93.5±0.4^{a}	84.1±1.0^{cd}	88.1±1.0^{b}	94.6±0.2^{a}	93.3±0.2^{a}
C18:1n-9	97.4±0.1^{b}	98.9±0.2^{a}	98.1±0.3^{ab}	98.3±0.1 ^{a}	98.8±0.1^{a}	98.9±0.1^{a}	98.5±0.2^{a}	98.6±0.1^{a}
C18:1n-7	97.7±0.2^{bc}	98.6±0.1^{a}	96.9±0.2^{c}	97.4±0.1^{bc}	97.8±0.2^{ab}	97.6±0.2^{bc}	97.4±0.2^{bc}	97.4±0.1^{bc}
C18:2n-6	95.4±0.1^{b}	98.1±0.1^{a}	97.4±0.2^{a}	97.6±0.1^{a}	98.0±0.1^{a}	98.0±0.2^{a}	97.6±0.2^{a}	97.7±0.1^{a}
C18:3n-3	96.9±0.2^{b}	99.3±0.1^{a}	98.6±0.2^{a}	98.5±0.1^{a}	99.2±0.1^{a}	99.1±0.1^{a}	98.8±0.2^{a}	99.0±0.1^{a}
C18:4n-3	99.4±0.1^{a}	92.8±3.1^{ab}	97.3±0.0^{ab}	77.9±3.7^{cd}	86.7±0.2^{abc}	87.6±0.4^{abc}	86.1±0.7^{bc}	73.1±0.5^{d}
C20:1	96.4±0.2^{b}	97.5±0.2^{a}	97.2±0.3^{ab}	97.6±0.3^{a}	97.6±0.1^{a}	97.8±0.2^{a}	97.6±0.2^{a}	97.8±0.2^{a}
C20:4n-6	80.6±0.3^{a}	67.2±0.6^{ab}	35.6±0.8^{c}	38.1±3.0^{c}	67.9±0.4^{ab}	67.9±0.8^{ab}	65.7±1.3^{b}	67.3±0.6^{ab}
C20:5n-3 (EPA)	99.6±0.1	99.5±0.1	99.1±0.1	99.4±0.0	99.5±0.1	99.5±0.1	99.4±0.1	99.5±0.1
C22:1	95.1±0.3^{b}	96.1±0.1^{ab}	96.5±0.2^{a}	97.1±0.1^{a}	96.2±0.2^{ab}	96.9±0.3^{a}	96.9±0.2^{a}	97.0±0.2^{a}
C22:5n-3	98.8±0.3^{a}	84.1±5.1^{ab}	84.3±0.7^{ab}	60.6±1.9^{bc}	82.3±0.2^{abc}	62.5±1.0^{bc}	60±1.5^{c}	61.9±0.6^{bc}
C22:6n-3 (DHA)	98.8±0.2^{a}	98.0±0.3^{ab}	96.6±0.0^{c}	96.9±0.1^{bc}	97.2±0.5^{bc}	97.3±0.4^{bc}	96.9±0.1^{bc}	97.0±0.1^{bc}
ΣSFA	92.1±0.3^{b}	92.0±0.1^{b}	92.8±0.3^{b}	95.0±0.2^{a}	91.2±0.6^{b}	92.9±0.5^{b}	95.5±0.4^{a}	94.8±0.1^{a}
ΣMUFA	96.9±0.2^{c}	98.3±0.2^{a}	97.4±0.2^{bc}	97.4±0.1^{bc}	98.0±0.2^{ab}	98±0.2^{ab}	97.6±0.2^{abc}	97.8±0.1^{ab}
ΣPUFA	97.2±0.2	97.0±0.2	96.0±0.3	95.8±0.7	96.7±0.5	96.5±0.0	96.1±0.2	95.9±0.5
Σn-6 PUFA	91.9±1.2^{b}	96.8±0.3^{a}	95.1±0.4^{a}	95±0.2^{a}	96.3±0.1^{a}	96.4±0.2^{a}	95.9±0.2^{a}	96.3±0.3^{a}
Σn-3 PUFA	98.6±0.0^{a}	96.9±0.1^{b}	95.7±0.6^{bcd}	94.2±0.1^{d}	96.1±0.4^{bc}	95.4±0.3^{bcd}	94.5±0.5^{cd}	94.4±0.1^{d}
Total FA[a]	95.4±0.1^{b}	96.7±0.1^{a}	95.7±0.1^{b}	95.8±0.2^{ab}	96.2±0.3^{ab}	96.4±0.3^{ab}	96.3±0.3^{ab}	96.3±0.1^{ab}
Total fat	93.0±0.4^{ab}	93.9±0.4^{a}	90.5±0.3^{b}	92.5±1.0^{ab}	94.2±0.6^{a}	93.7±0.2^{a}	91.7±0.6^{ab}	90.8±0.2^{b}

Experimental diets nomenclature as in Table 5.1.

Values represent mean±SEM of values of the three experimental groups (each group corresponding to a pooled sample from the 24 animals in each tank) fed the same diet. Values in the same row with different letters are significantly different ($P<0.05$), according to ANOVA. Lack of letters means no statistical significance obtained.

[a]Total FA: total fatty acids.

Growth performance, feed utilization and biometrical parameters

Results obtained for the performance parameters (Table 5.5.) followed the trend of those of total fatty acid digestibility. As observed, no significant differences ($P>0.05$) were obtained among the final weights of fish fed RN (393.7±6.1 g), RA (375.9±2.9 g) and RE (381.5±11.1 g). Those of RA and RE were, in turn, significantly lower ($P<0.05$) than that of F (411.1±3.3 g). Similar results were observed for WG and CF, while no statistical differences were obtained for the rest of the performance parameters studied.

As obtained in total fatty acid ADC, final weights of fish fed with RN/RA (380.7±20.6 g) and RA/RN (381.2±4.8 g) were in between those of RN and RA. The numerically highest was that of fish fed RN, although this was not statistically higher ($P>0.05$) than those of animals fed RN/RA or RA/RN. RE/RA and RA/RE diets obtained higher final weights (392.8±4.4 g and 394.6±1.6 g, respectively) than RE and RA, although differences were not significant ($P>0.05$). Very similar results were observed in WG in all cases. It is noteworthy that, although final weights and WG of fish fed diets including RE/RA and RA/RE did not result statistically higher than those of animals fed diets RN/RA and RA/RN, they were numerically higher.

In spite of the differences in final weights observed among diets, these were not reflected in SGR or in FCR.

Table 5.5. Growth performance, feed utilization and biometrical parameters of rainbow trout fed the different experimental diets.

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Initial weight (g)	101.6±0.2	101.5±0.2	101.6±0.0	101.7±0.1	101.8±0.5	101.8±0.2	101.5±0.1	101.8±0.2
Final weight (g)	411.1±3.3^{a}	393.7±6.1^{ab}	375.9±2.9^{b}	381.5±11.1^{b}	380.7±20.6^{b}	381.2±4.8^{b}	392.8±4.4^{ab}	394.6±1.6^{ab}
WG (g)[a]	309.4±3.1^{a}	292.1±5.9^{ab}	274.3±2.8^{b}	279.8±11.2^{ab}	278.8±21.1^{ab}	279.4±4.9^{ab}	291.3±4.5^{ab}	292.8±1.5^{ab}
Feed intake (gDM fish^{-1} d^{-1})[b]	4.48±0.28	4.16±0.06	4.18±0.16	4.73±0.19	4.34±0.12	4.19±0.08	4.70±0.28	4.41±0.19
FCR[c]	0.87±0.05	0.86±0.03	0.93±0.04	1.08±0.04	0.94±0.07	0.90±0.03	0.97±0.06	0.94±0.08
SGR (% day^{-1})[d]	2.36±0.01	2.29±0.03	2.21±0.01	2.22±0.07	2.21±0.1	2.23±0.02	2.28±0.02	2.29±0.02
ADG (% day^{-1})[e]	5.07±0.04	4.79±0.09	4.50±0.04	4.59±0.19	4.57±0.37	4.57±0.09	4.78±0.13	4.79±0.03
CF[f]	1.83±0.02^{a}	1.79±0.01^{ab}	1.75±0.01^{b}	1.78±0.01^{ab}	1.79±0.01^{ab}	1.75±0.01^{b}	1.78±0.01^{ab}	1.77±0.03^{ab}
HSI (%)[g]	1.09±0.02	1.00±0.04	1.07±0.03	1.08±0.09	1.10±0.01	1.06±0.06	1.11±0.04	1.07±0.09
VSI (%)[h]	12.29±0.59	11.0±0.55	11.71±1.06	12.26±0.45	12.08±0.92	11.12±0.74	12.21±0.74	10.92±0.43

Experimental diets nomenclature as in Table 5.1.

Values represent mean±SEM of values of the three experimental groups (each group corresponding to a pooled sample from the 24 animals in each tank) fed the same diet. Values in the same row with different letters are significantly different ($P<0.05$), according to ANOVA. Lack of letters means no statistical significance obtained.

[a]Weight gain: (final weight-initial weight).

[b]Feed intake: total dry matter intake / (number of fish x number of days fed).

[c]Feed conversion ratio: (dry feed fed) / (wet weight gain).

[d]Specific growth rate: [ln(final weight)-ln(initial weight)] / (number of days) x 100.

[e]Average daily growth: (gain %) / (number of days).

[f]Condition factor (K): 100 x [final weight (g)] / [fork length (cm)]^{3}.

[g]Hepatosomatic index: (weight of liver) / (total fish weight) x 100.

[h]Viscerosomatic index: (weight of viscera) / (total fish weight) x 100.

Plasma biochemical parameters

Values of the analysed plasma biochemical parameters of fish fed the experimental diets are shown in Table 5.6. Statistically significant differences in glucose, TAG, LDL-cholesterol, AST and ALT were found among the experimental rapeseed diets. Although fish fed diet RE/RA had significantly higher ($P<0.05$) level of TAG in plasma (565.03±39.52 mg dl^{-1}) than those fed F (384.17±8.09 mg dl^{-1}), RN (431.35±6.25 mg dl^{-1}) and RA (431.60±9.90 mg dl^{-1}), differences did not follow a clear trend related to the type of oil or to their level of inclusion. Similarly, in glucose, animals fed RA (67.27±2.36 mg dl^{-1}) and RA/RN (87.45±3.45 mg dl^{-1}) had a significantly higher ($P<0.05$) glucose plasmatic level than those fed diet RA (67.27±2.36 mg dl^{-1}).

Fish fed diets RN and RE had significantly lower ($P<0.05$) LDL-cholesterol levels (114.67±4.30 mg dl^{-1} and 125.48±10.98 mg dl^{-1}, respectively) than those fed F (201.67±16.36 mg dl^{-1}). No differences were found in blended oils diets when compared among themselves or among their corresponding single oil diets. However, RE/RA (125.48±10.98 mg dl^{-1}) and RA/RE (116.22±11.78 mg dl^{-1}) were significantly lower ($P<0.05$) than F. In fact, all diets resulted numerically lower than F.

For ALT and AST, animals fed RE (ALT: 3.67±1.20 IU l^{-1}; AST: 5.33±0.67 IU l^{-1}) showed significantly lower ($P<0.05$) values than those fed F (ALT: 20.50±0.50 IU l^{-1}; AST: 20.50±2.50 IU l^{-1}) and RN (ALT: 13.50±2.50 IU l^{-1}; ALT: 11.67±2.40 IU l^{-1}). In relation to RN/RA, RA/RN and their corresponding single oil diets (RN and RA), no differences were observed. Even so, AST and ALT plasmatic levels of fish fed RN/RA (ALT: 10.00±1.73 IU l^{-1}; AST: 9.33±1.45 IU l^{-1}) were significantly lower ($P<0.05$) than those of fish fed F. For diets with blends of RE and RA, the only significant difference ($P<0.05$) was found in ALT between RA (10.33±1.67 IU l^{-1}) and RE/RA (2.50±0.50 IU l^{-1}), being RA the highest. Indeed, RA obtained the numerically highest values, although they were not statistically higher, in both parameters. For plasmatic LDL-cholesterol, ALT, and AST, fish fed diet F had the highest values when comparing all treatments.

Histology of intestine and liver

No differences were observed in the morphology of liver or intestine among fish fed the different experimental diets, including F. Normal histology patterns were observed under a light microscope, as presented in Figure 5.1.

Table 5.6. Plasma biochemical analysis of rainbow trout fed the experimental diets.

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Parameters								
Glucose (mg dl^{-1})	73.20±1.10^{ab}	87.80±0.40^{a}	67.27±2.36^{b}	74.60±3.13^{ab}	71.85±4.85^{ab}	87.45±3.45^{a}	74.57±3.04^{ab}	77.33±4.77^{ab}
TAG (mg dl^{-1}) [a]	384.17±8.09^{b}	431.35±6.25^{b}	431.60±9.90^{b}	468.20±36.14^{ab}	501.05±24.65^{ab}	456.77±38.06^{ab}	565.03±39.52^{a}	439.05±32.35^{ab}
Total cholesterol (mg dl^{-1})	406.87±30.20	292.10±25.14	373.60±34.01	327.95±31.68	296.73±34.68	337.90±34.30	360.13±11.00	327.47±15.19
HDL-cholesterol[a] (mg dl^{-1})	126.38±16.53	111.97±16.48	130.24±6.27	155.34±9.21	109.01±6.53	127.67±8.44	162.93±15.52	153.02±9.32
LDL-cholesterol[a] (mg dl^{-1})	201.67±16.36^{a}	114.67±4.30^{b}	167.18±20.84^{ab}	125.48±10.98^{b}	127.28±15.44^{ab}	166.92±18.84^{ab}	125.48±10.98^{b}	116.22±11.78^{b}
VLDL-cholesterol[a] (mg dl^{-1})	78.12±7.82	64.99±4.99	75.55±7.86	80.50±0.58	61.26±11.85	67.76±16.80	71.17±11.85	57.66±12.86
FFA (mg dl^{-1}) [a]	2.33±0.11	2.48±0.11	2.45±0.13	2.79±0.09	2.39±0.21	2.82±0.49	2.86±0.12	2.58±0.19
Protein (g dl^{-1})	4.29±0.21	4.47±0.24	3.87±0.21	4.56±0.21	4.06±0.30	4.17±0.47	4.75±0.39	4.14±0.15
ALT (IU l^{-1}) [a]	20.50±0.50^{a}	13.50±2.50^{ab}	10.33±1.67^{abc}	3.67±1.20^{cd}	10.00±1.73^{bcd}	16.50±0.50^{ab}	2.50±0.50^{d}	3.67±1.20^{cd}
AST (IU l^{-1}) [a]	20.50±2.50^{a}	11.67±2.40^{ab}	11.67±2.33^{ab}	5.33±0.67^{b}	9.33±1.45^{b}	14.33±2.03^{ab}	7.00±2.31^{b}	5.00±1.53^{b}
GGT (IU l^{-1}) [a]	ND	ND	ND	ND	ND	ND	ND	ND

Experimental diets nomenclature as in Table 5.1.

Values are means±SEM of pooled plasma samples from the three experimental groups (n=5 per group) fed the same diet. Values within the same row with different letters are significantly ($P<0.05$) different, according to ANOVA.

[a]TAG (triacylglycerols), HDL (high density lipoproteins), LDL (low density lipoproteins), VLDL (very low density lipoproteins), FFA (free fatty acids), ALT (alanine aminotransferase), AST (aspartate aminotransferase) and GGT (gamma-glutamyl transferase).

ND means not detectable.

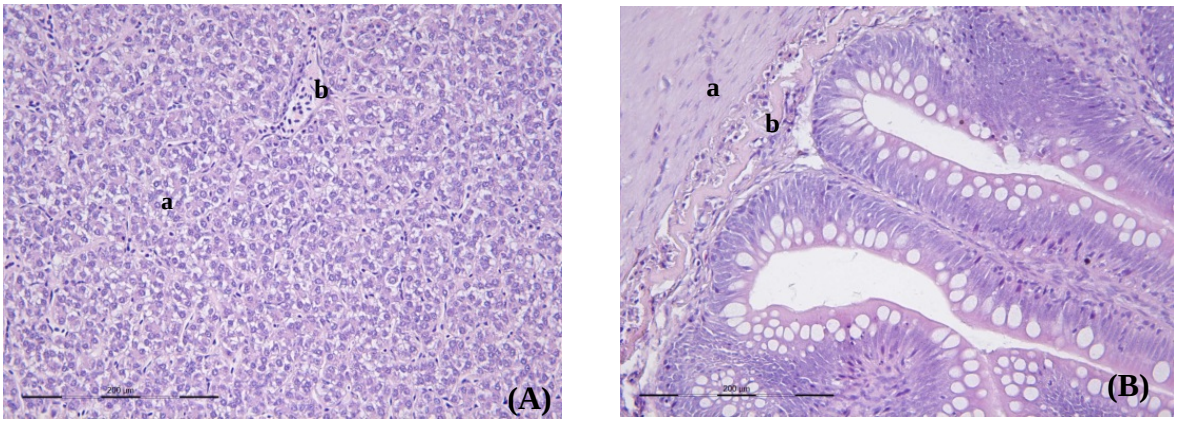

Figure 5.1. (A) Liver section from rainbow trout fed RA diet; **a** hepatocyte, **b** blood vessel. **(B)** Intestine cross-section from rainbow trout fed RA diet; **a** submucosa, **b** lamina *propria*, **c** enterocytes with basal nucleus. Hematoxylin and eosin stain; 20X magnification; scale bar = 200µm. Both tissues exhibited a normal morphology, the liver having regular-shaped hepatocytes and no lipid vacuolization and the intestine having no evidence of lipid vacuolization in the enterocytes.

5.4. Discussion

Minor differences in the fatty acid composition among rapeseed oils were observed. Similarly as it has been described in previous studies (Trullàs et al., 2015; Vilarrasa et al., 2014), the chemical esterification reaction did not have an effect on their fatty acid composition.

Regarding lipid classes, both native and acid oils showed the standard composition described for these types of oils. TAG was the predominant molecule (N95%) in RNO (Flickinger and Matsuo, 2003) and FFA represented a 64.3% in the acid oil (RAO) (Nuchi et al., 2009). On the other hand, a high content of partial acylglycerols (69.4%) was present in REO. When the lipid class composition of diets was compared to that of their corresponding oils, differences were observed in diets RA and RE. These differences were mostly related to the 5% of FO added to all the experimental rapeseed diets, which was mainly composed of TAG.

Both total fat and total fatty acid ADC of the different experimental diets were high (90.5–96.7%), which is in accordance with authors reporting similar results with diets including rapeseed as a FO replacer in rainbow trout (Caballero et al., 2002; Martins et al., 2006;

Turchini et al., 2013). This replacement could even increase lipid digestibility at low water temperatures in salmonid species (Caballero et al., 2002; Karalazos et al., 2007).

As found in a previous study in rainbow trout (Trullàs et al., 2015), a few differences in fatty acid ADC were obtained among rapeseed diets. For the single oil diets, the numerically lowest ADC of RA could be a consequence of its richness in FFA. As it is widely known in mammals, the main products of the hydrolysis by pancreatic lipase during lipid digestion are FFA and 2-MAG. Taking into account that a bile salt-dependent pancreatic lipase with sn-1,3-specific hydrolytic activity has been pointed out as the main lipolytic enzyme in rainbow trout (Bogevik et al., 2007; Gjellesvik et al., 1992; Tocher, 2003b), we would assume that a similar digestion process as in mammals would take place in this species. Then, the main hydrolytic products would be solubilized or emulsified in bile salt micelles, followed by diffusion to the intestinal mucosa (Tocher, 2003b). The large amount of FFA in RA could produce a "saturation effect" at the time of their incorporation into the mixed micelles during digestion, since the amount of FFA would greatly exceed that of MAG and DAG, responsible of expanding the micelle in order to allow the solubilisation of other products. However, to our best knowledge, there is a paucity of information regarding this phenomenon in fish. On the other hand, if present, this effect would possibly be more noticeable if the amount of FFA was mainly constituted of SFA, since high levels of SFA have been reported to negatively affect the formation of micelles in the intestinal lumen of Atlantic salmon (Menoyo et al., 2003).

While free MUFA and PUFA are easily absorbed, free long-chain SFA have a poorer absorption as a consequence of their hydrophobicity and high melting points. In native VO, SFA are mainly found in the external positions of TAG (Grundy and Denke, 1990; Karupaiah and Sundram, 2007) and thus are easily converted to FFA during digestion, part of which will form insoluble soaps in the gut to end up excreted in faeces. The fact that the reduction of ADC in RA was slight could be related to the low amount of SFA present in rapeseed.

Compared to RN and RA, RE had the significantly highest ADC of SFA, which could be a consequence of the emulsifying effect that the partial acylglycerols exert during the digestion process. As amphiphilic intermediate products of TAG digestion, DAG and especially MAG would facilitate the incorporation of hydrophobic FFA in the core of micelles during fat digestion, as described in humans, mammals and poultry (Da Costa, 2003; Krogdahl, 1985; Mattson et al., 1979).

Another factor that could have a beneficial effect on the ADC of SFA is that the chemical esterification reaction increases the amount of SFA located at the sn-2 position of acyglycerols, which would imply SFA being directly absorbed as 2-MAG, improving fatty acid digestibility of VO. This rise was of up to 10 points (as % on the total SFA content) in the rapeseed re-esterified oil in comparison to its corresponding native oil in the study by Trullàs et al. (2015). It is possible, though, that the low content of SFA in rapeseed did not exert a clear effect on the total fatty acid ADC. Related to this, Trullàs et al. (2015) concluded that the lipid class composition of the oil seemed to be of less importance as an influential factor on fatty acid digestibility than its degree of saturation. Certainly, the importance of the degree of saturation and the chain length on digestibility as the major factors affecting fatty acid digestibility in fish had been previously pointed out (Francis et al., 2007; Hua and Bureau, 2009). Thus, the slight differences observed in the present work could be due to the predominance of the degree of unsaturation of rapeseed over other factors.

When RNO or REO were substituted by graded levels of RAO (RN–RN/RA–RA/RN–RA or RE–RE/RA–RA/RE–RA), no significant effect of the level of inclusion of RAO (100%, 33% or 66%) in diets on total fatty acid ADC was observed. However, in diets with substitution of RN by RAO, there was a slight but progressive decrease of ADC as more RAO was present, which suggests that the ADC of a diet could be in direct relation to the richness of FFA of RA. The detrimental effect on digestibility appears when the level of FFA in diets is of around 30%, regardless of the rest of the lipid classes.

In diets with substitution of REO by RAO, total fatty acid ADC of RE/ RA and RA/RE were higher than that of RA, indicating a possible effect of the partial acylgycerols and the higher amount of SFA at sn-2 than the rest of diets.

Final weights of fish fed the experimental single rapeseed oil diets were all high, which is in agreement with many studies in salmonids (Bell et al., 2003; Huang et al., 2008; Pettersson et al., 2009; Turchini et al., 2013). Also, values of FCR and SGR were similar to those obtained in studies including different levels of rapeseed oil in salmonid diets (Caballero et al., 2002; Turchini et al., 2013). As reported for Atlantic salmon, rapeseed oil is an effective substitute of FO in terms of growth rates and feed efficiency, since it provides sufficient energy in the form of monoenoic fatty acids to maintain high growth rates Bell et al. (2001).

As has long been reported, rainbow trout requires solely linolenic (C18:3n-3) acid as essential fatty acid (Castell et al., 1972) for maximal growth (Watanabe, 1982). Regarding this, it is important to highlight that all our rapeseed diets included 5% of FO in order to ensure a minimum dietary content of n-3 long-chain PUFA. However, fish fed rapeseed diets had lower final weights than those fed diet F, although these differences were significantly lower only in fish fed RA and RE. Final weights of fish fed RN/RA and RA/RN were not different from each other but numerically lower than those of fish fed RN. As observed, detrimental effects in growth appeared in diet RA, but not when RAO was blended with RNO.

It is important to remind that the experimental period lasted 72 days, and so a longer period of time could have shown noticeable differences among diets regarding final weight and especially SGR and FCR. The higher final weights obtained in fish fed RE/RA and RA/RE compared to those fed RE and RA seemed to be caused by a synergism between REO and RAO, the causes for this being probably those previously described for digestibility.

Haematological and biochemical parameters reflect the physiological processes undertaken in an animal and give information about its physiological status (Peres et al., 2012). Moreover, fatty acid structure and also its position on the glycerol backbone have an influence on plasma lipids in both humans (Dubois et al., 2007) and fish (Denstadli et al., 2011).

In the present study, no clear relation was obtained between glucose and TAG among diets regarding the type of oil (native, re-esterified and acid) or their level of inclusion (100%, 66% or 33%). Therefore, the dietary fatty acid composition could have had a greater effect on plasma parameters than these two factors, because oleic acid, the main fatty acid in rapeseed, had been shown to be neutral with regard to plasma lipids in studies in humans (Clarke et al., 1997; Grundy, 1986).

For the lipoprotein cholesterol, LDL-cholesterol was the only one to show significant differences among diets, although they were not clearly related to the different types of oils. This is in accordance with studies reporting a decrease in plasma and LDL-cholesterol in salmonid species fed VO-based diets when compared to fish fed F (Jordal et al. 2007; Richard et al. 2006). In a study with rainbow trout fed a diet with a high proportion of RO, Richard et al. (2006) suggested the high levels of oleic and linoleic acids in the diet, as well as the presence of phytosterols, as possible causes for the decreased plasma total cholesterol and LDL-cholesterol. Similarly, oleic acid and PUFA had been found to reduce

levels of plasma LDL-cholesterol in mammals (Fernandez and West, 2005; Grundy and Denke, 1990).

A similar tendency was observed in total cholesterol, for which the lack of significant differences among diets seemed to be a consequence of the high variability of the data. In fact, Kimet al. (2012) reported that a decrease in the total cholesterol of fish fed a diet containing VO has not been well established.

Values of the two hepatic transaminases (ALT and AST) presented similarities. It is difficult to classify values of hepatic enzymes as normal or pathological, since they vary largely among studies and species. Also, reference values for clinical-normal and non-stressed animals are lacking for most fish species (Peres et al., 2012).

Nevertheless, an increase in the levels of plasma and serum transaminases has been associated with liver damage in marine (Lemaire et al., 1991) and freshwater species (Babalola et al., 2009), which was directly related to histopathological findings. In the present study, the normal morphology of livers of fish fed the different diets might indicate that differences in ALT and AST found among diets were possibly not relevant.

Díaz-López et al. (2009) observed a significant decrease in several hepatic enzymes in sea bream after 4 months of feeding with rapeseed diets in relation to fish fed a control diet FO as the main fat source. Then, the higher values obtained for F in comparison with the rest of the diets would be in accordance with results found in the aforementioned study, although our trial had half the duration of the trial performed by Díaz-López et al. (2009).

In contrast to the results obtained in the present study, lipid vacuole accumulation in the intestine and/or in the liver have been reported when VO are the main fat source in fish diets (Caballero et al., 2002, 2004; Lie and Lambertsen, 1987; Olsen et al., 1999, 2000, Ruyter and Moya-Falcón, 2006).

In the intestine, the enterocytic supranuclear lipid droplet accumulation observed in fish fed VO (Olsen et al., 1999, 2000) has been considered a temporary physiological state, due to the presence of a high amount of PUFA and an insufficient lipoprotein synthesis. Certain SFA (mainly C16:0) are required to maintain the cellular synthesis of phosphatidylcholine, necessary for the lipoprotein synthesis. Then, diets containing VO poor in SFA and rich in 18:2n-6 and 18:3n-3, would promote the accumulation of lipid droplets due to the insufficient formation of phospholipids and subsequently of lipoproteins. Nonetheless, in sea bream fed a rapeseed oil diet, poor in SFA and rich in MUFA (mainly C18:1n-9), accumulation of lipid droplets in enterocytes was suggested to

be caused by the lower enterocytic reacylation of the oleic acid observed in the polar lipid fraction in comparison with other fatty acids. This fact would be reducing lipoprotein synthesis rates (Caballero et al., 2003). In the present study, no lipid droplet accumulation was observed, but it has to be considered that a different microscopy technique than in Caballero et al. (2003) was used. In addition, the different times of sampling (i.e. 4 h after feeding in Caballero et al. (2003) and after 48 h of fasting in our case) should also be taken into account.

For liver, several studies in gilthead sea bream found a low or non-existent percentage of lipid vacuoles in fish fed rapeseed oil diets compared to those fed diets with only FO (Caballero et al., 2004; Fountoulaki et al., 2009). These studies suggested that this low degree of vacuolation could be due to the reduced activity of the fatty acid synthase enzyme found in these livers in comparison of those of fish fed the F diet, which was consequence of the high 18:1n-9 content in this diet. In relation to this, Caballero et al. (2004) established an order among the characteristic fatty acids in VO and its relationship with the appearance of steatosis in the liver: linoleic acid>linolenic acid>oleic acid. Considering that lipid vacuolation has been related to the nutritional imbalance due to the high content of n-6 fatty acids present in many VO (Montero and Izquierdo, 2011; Tacon, 1996), the fact that rapeseed contains limited n-6 PUFA could also be a possible explanation for our results.

In conclusion, results from the present study indicate that rainbow trout fed diets including RAO and REO showed acceptable fat and fatty acid digestibility, with no relevant changes in plasma parameters or in the morphology of liver and intestine. However, growth of fish fed these two diets did not reach that obtained in fish fed F, while growth of fish fed diets including a blend of RAO and REO improved when combined with REO at both 33% and 66% levels of inclusion.

Therefore, the rapeseed acid oil, which is the most economically advantageous, yields better growth results when blended with the reesterified oil. It has to be taken into account that the inclusion of these oils should be done with a minimum proportion of 5% of FO in diets.

However, before recommending their use, further studies regarding the inclusion of these oils in aqua feeds should be carried out in order to study their effect on the fat content and the fatty acid composition of tissues, as well as on the final product quality parameters in rainbow trout.

CHAPTER 6

QUALITY CHARACTERISTICS OF FILLETS OF RAINBOW TROUT FED ACID OR RE-ESTERIFIED RAPESEED OILS AS DIETARY FAT SOURCES

Quality characteristics of fillets of rainbow trout fed acid or re-esterified rapeseed oils as dietary fat sources

Trullàs, C.[1*], Tres, A.[2], Saldo, J.[3], Fontanillas, R.[4], Sala, R.[1]

[1]Departament de Ciència Animal i dels Aliments, Servei de Nutrició i Benestar Animal (SNiBA), Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain;

[2]Departament de Nutrició i Bromatologia-XaRTA-INSA, Facultat de Farmàcia, Universitat de Barcelona, 08028 Barcelona, Spain.

[3]Centre Especial de Recerca Planta de Tecnologia dels Aliments (CERPTA), Department of Animal and Food Sciences, Universitat Autònoma de Barcelona, Bellaterra 08193, Spain;

[4]Skretting Aquaculture Research Center (Skretting ARC), Sjøhagen 3, Stavanger 4016, Norway;

Abstract

Acid oils are a free fatty acid (FFA)-rich by-product from the refining of VO and re-esterified oils are the final product of a chemical esterification reaction between acid oils and glycerol. Re-esterified oils have less FFA and more mono- and diacylglycerols (MAG and DAG), known for being good emulsifiers, and they could therefore have a higher nutritive value than that of the native and the acid oils. Indeed, in two earlier studies in rainbow trout (Trullàs et al., 2015; 2016), diets including the aforementioned oils resulted in total fatty acid apparent digestibility coefficients above 95%. Moreover, no negative effects on growth, plasma biochemical parameters and morphology of tissues were observed when compared to the native oil diet. For all these reasons, the present study aimed at assessing their effects on the final quality of fillets of rainbow trout. Triplicate groups of rainbow trout were fed eight experimental diets containing 15% of different types of experimental rapeseed oil in addition to 5% of fish oil (FO) during 72 days. Dietary experimental rapeseed oils were native (RNO), re-esterified (REO), acid (RAO) or blends (66% REO-33% RAO / 33% REO-66% RAO or 66% RN-33% RAO / 33% RN-66% RAO). A commercial fish oil was used for the control diet (F). Fillets of fish fed the re-esterified oil diet (RE) deposited significantly more ($P<0.05$) saturated fatty acids (SFA) than those of fish fed the native (RN) and the acid oil diets (RA). The colorimetric analysis resulted in significant differences ($P<0.05$) only in b* and C* in both fresh and thawed fillets, as well as in significant correlations between parameters among diets. For the total fat content, fillets of fish fed the control diet obtained the highest values, higher than those of fish fed diets containing RNO and the blend 66% REO-33% RAO. Regarding tocopherol concentrations, diet RE resulted in a significantly higher concentration of α-tocopherol while F had a significantly lower concentration of β+γ-tocopherol than the rest of diets. No differences in texture, liquid holding capacity and TBARS were found among diets. Therefore, even though the aforementioned differences were found among fillets of fish fed the different diets, they did not seem to be relevant concerning the fillets final quality.

6.1. Introduction

The rise in the use of vegetable oils (VO) as a feedstock in the biofuel industry, which started in the early 2000s, generated a subsequent increment in their prices that peaked in 2008 (Gunstone, 2011). This shift in the use of VO towards non-food uses created

competition between the feed and the biofuel industries, since both oilseeds and feed grains used as ingredients in diets suffered the increase in their prices, placing the animal feed industry in a difficult situation. This also had a remarkable impact on the aquaculture industry, which had already made a great effort in research on the use of VO to replace fish oil (FO) from fish diets. Consequently, studies focused on finding alternatives to the use of native VO as FO replacers in aqua feeds have been carried out, all of them using palm derivatives (Ng et al., 2006, 2010; Aliyu-Paiko and Hashim, 2012). Among them, those using by- and co-products generated during the crude VO processing are of particular interest. VO need to be refined to be edible (Vaisali et al., 2015) so by- and co-products from their refining, generated in large amounts, represent an industrial waste and are cheaper than their original sources (Haas et al., 2003). Vegetable acid oils, a free fatty acid (FFA)-rich by-product from the refining of VO, were pointed out as a promising fat source for feeding uses (Nuchi et al., 2009). Acid oils can be subjected to a chemical esterification process with glycerol to generate the so-called re-esterified vegetable oils, which differ from acid oils in the lipid class composition. Re-esterified oils have less FFA and more mono- and diacylglycerols (MAG and DAG) (Vilarrasa et al., 2014; Trullàs et al., 2015). These partial acyglycerols have emulsifying properties (Redgrave et al., 1988) and so beneficial effects on digestibility and feed utilization in humans and monogastric animals (Cruz-Hernandez et al., 2012; Garrett and Young, 1975; Martin et al., 2014).

In light of this, in addition to these oils as sole lipid sources, blends of the acid oil with both the native or the re-esterified oils could also be an interesting lipid source. It is important to mention that the dietary inclusion of re-esterified oils implies the previous cost of the esterification process and so acid oils seem to be more interesting from the economic point of view. In fact, the economic viability in relation to native oils depends on the price differential between native and acid oils, which is in turn subjected to fluctuation.

Indeed, in two earlier studies in rainbow trout (Trullàs et al., 2015; in press), diets including rapeseed acid and re-esterified oils as a sole fat source or blended, together with a 5% of FO, resulted in total fatty acid apparent digestibility coefficients (ADC) above 95%. Furthermore, no negative effects on growth and health status indicators such as plasma biochemical parameters and morphology of tissues were observed when compared to the native oil diet.

In spite of the good results obtained in fatty acid digestibility and growth performance, the inclusion of these two types of oil could have a repercussion on the final product quality.

As is well known, the inclusion of VO in fish diets as well as its type can significantly affect the characteristics of the flesh. Replacing FO with VO will increase the presence of fatty acids 18:2n-6, 18:1n-9 and 18:3n-3 in diets and thus decrease eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:5n-6), which will cause an imbalance in the n-6:n-3 ratio and a negative effect on the overall health (Simopoulos, 2008). In addition to the effects on the nutritional quality of fish fillets, the dietary presence of native VO could also affect their sensorial and physico-chemical properties and therefore many studies focused on these aspects have been carried out (Rosenlund et al., 2001; Rosenlund et al., 2011; Izquierdo et al., 2003; Regost et al., 2003; Mørkøre et al., 2007). Even though results of flesh quality parameters when fish are fed VO diets are somewhat contradictory (Rørå et al., 2005; Ng and Bahurmiz, 2009), changes in physico-chemical parameters such as texture, colour, liquid holding capacity (LHC) and lipid peroxidation have been reported in salmonid species (Bjerken et al., 1997; Ng and Bahurmiz, 2009; Regost et al., 2004). No studies on the effects of by- and co-products of the VO processing on flesh quality have been found in salmonids.

Given the fact that rapeseed acid and re-esterified oils are by-products with a potential interest as fat sources in aqua feeds, the aim of the present work was to assess their effects on the final quality of fillets of rainbow trout.

6.2. Materials and methods

6.2.1. Experimental diets

Experimental diets (45% protein and 21% lipid) had the same ingredient composition except for the added lipid source (Table 6.1.). Three different types of rapeseed oil – native oil (RNO), re-esterified rich in MAG and DAG (REO) and acid (RAO) – were included in the diets alone (diets RN, RE or RA) or in blends (diet RE/RA: 66% REO-33% RAO; diet RA/RE: 66% RAO-33% REO; diet RN/RA: 66% RNO-33% RAO and diet RA/RN: 66% RAO-33% RNO) in a proportion of 15%. 5% of commercial fish oil was included in all experimental diets. A diet including only commercial fish oil (20%) was used for the control diet (F). Experimental oils were provided by SILO S.p.a. (Firenze, Italy) (RN and RE) and Cargill (Schiphol, The Netherlands) (RA). The re-esterified oil (REO) was obtained by SILO S.p.a. (Firenze, Italy) as described in Trullàs et al. (2015). Feeds were produced at the Skretting Feed Technology Plant (Aquaculture Research Center; Stavanger, Norway) as extruded pellets. The ingredient formulation and proximate

composition of the diets are shown in Table 6.1. Nutrient composition of experimental diets was determined by standard procedures (AOAC, 2005): moisture (934.01), ash (942.05), crude protein (968.06) and crude lipid (920.39). Gross energy of dried feed was determined using an adiabatic bomb calorimeter (IKA-Kalorimeter system C4000, Jankel-Kunkel, Staufen, Germany).

Table 6.1. Ingredient formulation and proximate composition of the experimental diets.

	Diets[a]							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Ingredient composition (g kg^{-1})								
Wheat[b]	60	60	60	60	60	60	60	60
Wheat gluten[c]	232.8	232.8	232.8	232.8	232.8	232.8	232.8	232.8
Soya bean meal[d]	80	80	80	80	80.0	80.0	80.0	80.0
Soya protein concentrate[e]	150	150	150	150	150	150	150	150
Faba beans whole[f]	100	100	100	100	100	100	100	100
Fish meal[g]	150	150	150	150	150	150	150	150
Fish oil[h]	201.3	52	52	52	52	52	52	52
Experimental oils[i]	0	150	150	150	150	150	150	150
Yttrium premix[i]	1	1	1	1	1	1	1	1
Mineral and vitamin premix[i]	24.9	24.9	24.9	24.9	24.9	24.9	24.9	24.9
Proximate composition (g kg^{-1})								
Dry matter	925.7	925.9	927.9	929.9	931	928.9	926.8	927.3
Crude protein	472.2	466.1	485.1	468.2	468	466.2	471.7	474.3
Crude fat	204.1	215.7	187.7	210.4	219.5	214.3	191.9	201.4
Ash	64.2	63.3	65	70.6	67.6	65.6	65.2	68.1
Gross energy (kJ g^{-1})	22.8	22.5	22.8	22.4	22.3	22.7	22.4	22.4

[a]Experimental diets nomenclature: F: fish oil (control diet); RN: rapeseed native oil; RE: rapeseed re-esterified oil; RA: rapeseed acid oil; RE/RA: 66% rapeseed re-esterified oil - 33% rapeseed acid oil; RA/RE: 66% rapeseed acid oil - 33% rapeseed re-esterified oil; RN/RA: 66% rapeseed native oil - 33% rapeseed acid oil and RA/RN: 66% rapeseed acid oil - 33% rapeseed native oil.
[b]Statkorn, Norway.
[c]Cerestar Scandinavia AS, Denmark.
[d]IMCOPA, Brasil.
[e]Denofa, Norway.
[f]Ceremis, France.
[g]Welcon AS, Norway.
[h]Holtermann ANS, Norway.
[i]Vitamin and mineral premix, according to requirement data from NRC (2011). Trow Nutrition, The Netherlands.

6.2.2. Fish husbandry and sampling

All the procedures were conducted in accordance with the Animal Protocol Review Committee of the Universitat Autònoma de Barcelona (UAB) and following the European Union Guidelines for the ethical care and handling of animals under experimental conditions (2010/63/EU). The trial was carried out at the Skretting Italia Aquaculture Research Center in Mozzecane, Italy. A total of 576 rainbow trout with a mean initial body weight of 101.7 g ± 8.8 g were randomly distributed into 24 cylindro-conical tanks of 600 l of capacity (24 fish per tank) in an open freshwater system with a continuous water flow of 24 l min^{-1}. Water temperature (14.3ºC) and dissolved oxygen levels (7.4 mg/l ± 0.37 mg/l) were maintained constant throughout all the experimental period. Tanks were subjected to a 24 h light photoperiod. Fish were fed the experimental diets for 72 days. Each diet was randomly assigned to three replicate tanks and was fed twice a day by automatic feeders, adjusted to provide 2.5% of the biomass daily. Uneaten feed was collected by filtering effluent water from each tank. Collectors were emptied after each meal and feed intake was recorded daily. At day 72 five fish from each tank were sacrificed by an over-dose of anaesthetic and individually gutted and filleted. A left fillet colorimetric determination was performed immediately after filleting. The left fillet was then skinned, bagged and frozen at -20 ºC until the corresponding quality analyses were performed (colour, liquid holding capacity -LHC- and texture). The right fillet was cut in two different specific portions, frontal and central, and also bagged and frozen at -20 ºC for carrying out the rest of the analyses (fatty acid composition, pH, total fat, moisture, thiobarbituric acid-reactive substance -TBARS- and tocopherol).

6.2.3. Fatty acid composition of diets

Fatty acid composition of diets and faeces was determined by gas chromatography-flame ionization detector (GC-FID). Fatty acid methyl esters (FAME) were obtained by direct methylation, according to Meier et al. (2006) and analysed using an HP 5890A gas chromatograph. They were identified by comparison to their retention times with those of known standards, and quantified by internal normalization (FAME peak area/total FAME area, in %).

6.2.4. Lipid class composition of oils and diets

Lipid class composition (TAG, DAG, MAG and FFA) of FOO, RNO, REO and RAO oils, as well as that of experimental diets, were determined by size-exclusion chromatography

on an Agilent 1100 series HPLC chromatograph equipped with a Refractive Index Detector (RID) set at 35 ºC, as described in Trullàs et al. (2015).

6.2.5. Fatty acid composition of fillets

Fat was extracted from fillets by using chloroform: methanol (2:1, v/v). Briefly, 30 mL of chloroform:methanol (2:1, v/v) were added to 2 g of fillet and stirred. The mixture was filtered through a filter paper (Whatman nº1) and the residue was re-extracted twice more with 8 mL of chloroform:methanol (2:1, v/v), stirring the mixture each time. The extracts were filtered and pooled and 8 mL of NaCl (1%, w/v) were added. The mixture was shaken and centrifuged at 540 g for 20 minutes. The chloroformic phase was separated and filtered through anhydrous sodium sulphate. Solvent was then evaporated by using a rotatory vacuum evaporator. Fatty acid methyl esters (FAME) were obtained following the method described by Guardiola et al. (1994), identified by comparison to their retention times with those of known standards and quantified by internal normalization (FAME peak area/total FAME area, in %).

6.2.6. Colour evaluation of fillets

Colorimetric determinations were made on fresh fillet (immediately after filleting) and on thawed fillet (after three months of storage at -20 ºC) on the Norwegian Quality Cut (NQC) section (NS9401, 1994). Defrosting of fillets was carried out by letting it thaw overnight in the refrigerator (5 ºC). Measurements were performed in the colorimetric space L^*, a^*, b^* (CIE, 1976) in the area between the dorsal and the adipose fins, above the lateral line (Figure 6.1.), using a Minolta Chroma meter (Model CR-410, Minolta Co., Ltd, Osaka, Japan); L^* represents the colour lightness that goes from 0 (black) to 100 (diffuse white), a^* is the position between red/magenta and green and b^* is the position between yellow and blue. Later, values obtained were transformed in the colour appearance parameters L^*, C^*, $H(°)_{ab}$ (Wyszecki and Stiles, 1967); C^* (chroma) expresses the colour intensity and $H(°)_{ab}$ (hue) is the attribute of a visual sensation according to which an area appears to be similar to one of the perceived colours, red, yellow, green and blue, or a combination of two of them (Fairchild, 2005). Three measurements were performed on each of the five fillets per tank (15 fillets/diet; n = 120), and the mean value was used for the statistical analysis of the data.

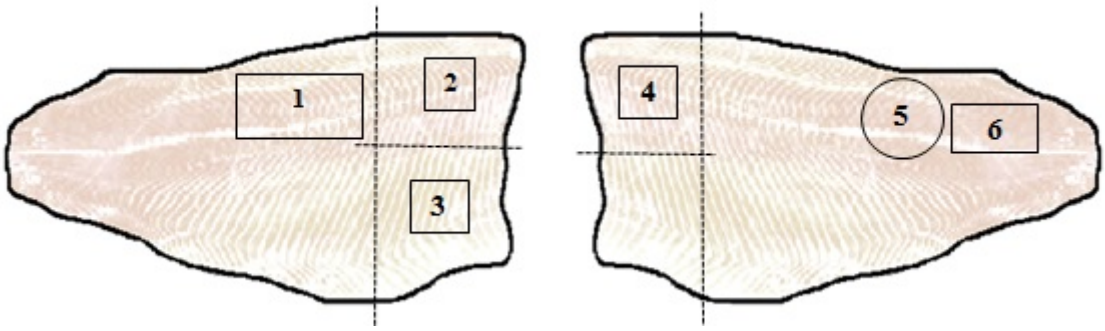

Figure 6.1. Diagram of the distribution of fillets of rainbow trout regarding the physicochemical analyses performed. Numbers indicate the part of the fillet used for the different determinations: 1) fatty acid composition; 2) total fat and moisture; 3) TBA; 4) liquid holding capacity (LHC); 5) color and 6) texture.

6.2.7. Texture evaluation of fillets

The texture of the fillet was measured using a TA-TX2 Texture Analyzer (Stable Micro Systems, Surrey, England) texturometer equipped with a 5 kg load cell and the texture data analysis software Exponent 6.1.5.0 (Stable Micro Systems, Surrey, England). Frozen fillet portions were thawed overnight in the refrigerator (5 ºC) and were then cut in two standardised pieces (2x2 cm length x width) from the area located under the dorsal fin, about 1.5 cm above the lateral line (Figure 6.1.). Each sample was subjected to a texture profile analyses (TPA), followed by a uniaxial compression test. The TPA test was performed using a 100 mm compression plate (type P/100) and the testing conditions were two consecutive cycles at 25% compression (10 mm depth), cross-head movement at a pre-test constant speed of 5 mm/s and a test and post-test constant speed of 1 mm/s. The rest period between cycles was of 15 seconds and the probe always returned to its initial position after the second cycle. Texture variables (hardness, adhesiveness, springiness, cohesiveness and chewiness) were calculated as described by Bourne (1978). The compression test was performed using the same probe and the same pre-test, test and post-test speeds as for the TPA analysis. Both the work required for the compression of the thickness of the fillet to 90% (5 mm depth) and the force needed to reach the breaking point were measured. Two measurements were performed on each of the five fillets per tank (n = 120), and the mean value was used for statistical analyses of the data.

6.2.8. Determination of total fat, protein, moisture, pH and liquid holding capacity of fillets

Total fat was extracted from fillets (Figure 6.1.) and determined gravimetrically by homogenising them in chloroform/methanol (2:1, v/v) according to the method of Folch et al. (1957). Water was extracted from fillets by standard procedure for moisture (934.01) (AOAC, 2005).

A pH meter (micropH 2001, Crison, Spain) was used to measure pH after pooling the fillet region situated below the point where the dorsal fin begins (Figure 6.1.) of five fish of each tank and homogenising them in distilled water (ratio 1:10, v/v).

For the LHC evaluation, triplicate muscle samples from about 3‑4 cm in front of the dorsal fin, from the lateral line towards the belly (Figure 6.1.) were weighed (S) and placed in a tube with a weighted filter paper (Filter-Lab Filtros Anoia, Spain) (V1). The tubes were placed in a centrifuge (Sigma 4K15) at 500g for 10 min at 10 ºC and the wet

was calculated as 100% · (V1-V2) · S^{-1}, where S is the weight of muscle sample, water loss as 100%· (V2-V3) · S^{-1} and fat loss as 100% · (V3-V1) · S^{-1}. All losses were expressed as a percentage of muscle wet weight.

6.2.9. Determination of TBARS and tocopherol concentrations

Fillet TBARS were analysed as a measure of lipid peroxidation by determining equivalents of malondialdehyde (MDA), a secondary product in the oxidation of polyunsaturated fatty acids (PUFA), by spectrophotometry following an adaptation of Sørensen and Jørgensen (1996).

Tocopherol of diets and fillets was determined by high-performance liquid chromatography (HPLC) according to Bou et al. (2004). Both analyses were performed on the same region of the fillet as the LHC.

6.2.10. Statistical analysis

Data were subjected to a one-way analysis of variance (ANOVA) and the significance of the differences between means was tested by Tukey's test. Values are given as means±standard error of the mean (SEM) of triplicate groups of five fish fillets in the case of texture and colour, of triplicate groups of three fish fillets in total fat, LHC and tocopherol and of triplicate groups of pooled fillet samples from five fish in pH and TBARS. Differences were considered significant when $P<0.05$. All statistics were performed by means of the General Lineal Model (Proc GLM) of SAS® software version 9.2 (SAS Institute Inc., Cary, NC, USA). Data were also subjected to a correlation analysis (Pearson's correlation coefficient) in order to study the relationship between the different parameters. The significance level was also set at 5% ($P<0.05$) (SAS® software version 9.2; SAS Institute Inc., Cary, NC, USA).

6.3. Results

Characterization of experimental oils and diets

Results of the fatty acid composition of experimental diets and results of the lipid class composition of experimental oils and diets are shown in Tables 6.2. and 6.3., respectively. Differences between fatty acid composition of rapeseed oils and diets were minor. However, they were more evident with regard to their lipid class composition. Native oils (FO and RNO) were mainly constituted of TAG, the re-esterified oil had a high amount of partial acylglycerols (35.4% MAG and 34% DAG) and the acid oil was rich in FFA (64.3%). In diets, RE and RA resulted in higher percentages of TAG but lower of FFA and

MAG-DAG than their corresponding oils, mainly due the effect of the additional amount of TAG provided by the dietary presence of 5% of FO.

In addition, an increase in FFA was observed as more RAO was included in the diets with blended oils, together with a higher amount of partial acylglycerols as the presence of REO increased.

Table 6.2. Fatty acid composition of the experimental diets.

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Fatty acid (%)								
C14:0	8.1	3.0	2.8	2.9	2.7	2.7	2.9	2.8
C16:0	18.6	10.3	11.0	13.5	10.4	10.6	12.4	11.5
C16:1n-7	8.7	3.3	3.1	3.3	3.0	3.1	3.2	3.0
C16:2n-6	1.0	0.4	0.3	0.3	0.3	0.3	0.3	0.3
C18:0	3.2	2.4	2.8	4.7	2.5	2.6	4.0	3.4
C18:1n-9	10.8	35.7	35.5	32.2	36.2	36.3	34.2	35.4
C18:1n-7	3.2	3.5	4.3	4.1	3.5	3.9	4.0	4.0
C18:2n-6	5.9	16.7	18.5	17.0	17.7	18.1	17.5	17.9
C18:3n-3	1.1	6.2	5.1	3.9	5.9	5.6	4.4	4.9
C18:4n-3	2.4	0.9	0.8	0.8	0.8	0.8	0.8	0.8
C20:1[a]	2.5	2.5	2.2	2.2	2.5	2.3	2.1	2.2
C20:4n-6	1.0	0.4	0.3	0.4	0.3	0.3	0.3	0.3
C20:4n-3	0.7	0.2	0.2	0.2	0.2	0.2	0.2	0.2
C20:5n-3 (EPA)	14.7	5.2	4.8	5.0	4.9	4.7	4.8	4.8
C22:1[a]	2.1	1.6	1.4	1.4	1.6	1.5	1.4	1.3
C22:5n-3	1.7	0.6	0.6	0.6	0.6	0.6	0.6	0.6
C22:6n-3 (DHA)	10.8	4.2	3.9	4.1	4.1	3.8	3.9	3.9
C24:1n-9	0.7	0.4	0.6	0.6	0.6	0.5	0.6	0.6
ΣSFA[b]	30.8	16.8	17.5	22.7	16.8	17.0	20.6	18.9
ΣUFA[c]	69.2	83.2	82.5	77.3	83.2	83.0	79.4	80.4
ΣMUFA[d]	28.5	47.8	47.7	44.4	47.8	48.0	46.0	46.9
ΣPUFA[e]	40.6	35.3	34.8	32.8	35.4	35.0	33.3	34.1
Σn-6 PUFA[e]	8.8	17.8	19.3	18.0	18.7	19.1	18.4	18.9
Σn-3 PUFA[e]	31.8	17.5	15.5	14.8	16.7	15.9	14.9	15.2
SFA:UFA	0.4	0.2	0.2	0.3	0.2	0.2	0.3	0.2
n-3:n-6	3.6	1.0	0.8	0.8	0.9	0.8	0.8	0.8

Experimental diets nomenclature as in experimental diets (Table 6.1.).
[a]Sum of isomers.
[b]SFA: saturated fatty acids. It includes other SFA of small quantity.
[c]UFA: unsaturated fatty acids. It includes other UFA of small quantity.
[d]MUFA: monounsaturated fatty acids. It includes other MUFA of small quantity.
[e]PUFA: polyunsaturated fatty acids. It includes other PUFA of small quantity; n-6 PUFA: omega 6 polyunsaturated fatty acids; n-3 PUFA: omega 3 polyunsaturated fatty acids.

Table 6.3. Lipid class composition of the experimental oils and diets.

	Oils			
	FO	RNO	RAO	REO
Lipid classes (%)				
ΣTAG[a]	93.8	95.6	20.5	26.6
ΣDAG[a]	2.9	2.5	12.5	34.0
ΣMAG[a]	0.7	0.2	2.7	35.4
ΣFFA[a]	2.6	1.7	64.3	2.0

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Lipid classes (%)								
ΣTAG[a]	92.9	93.4	46	54	77.16	62.2	49	46.6
ΣDAG[a]	3.2	3.1	9.4	21.9	5.28	6.9	19	14.8
ΣMAG[a]	0.8	0.7	2.1	22.3	1.05	1.7	14.8	7.9
ΣFFA[a]	3.1	2.8	42.4	1.8	16.51	29.2	17.2	30.7

Experimental oils nomenclature: FO: fish oil (control diet); RNO: rapeseed native oil; REO: rapeseed re-esterified oil and RAO: rapeseed acid oil. Experimental diets nomenclature as in Table 6.1.
[a]TAG (triacylglycerols), DAG (diacylglycerols), MAG (monoacylglycerols) and FFA (free fatty acids).

Fatty acid composition of fillets

The fatty acid compositions of fillets of rainbow trout fed the experimental diets are shown in Table 6.4. As observed, the fatty acid compositions of diets were mirrored in the fillets. Even though the proportion of each individual or category of fatty acids in the fillets did not present great differences among rapeseed diets, statistically significant differences were found.

Fillets of fish fed diet F had a considerable content of saturated fatty acids (SFA), which was 10 points higher (expressed in %) than in those of fish fed the experimental rapeseed diets. They also had a greater amount of n-3 PUFA, mainly because their content of highly unsaturated fatty acids (HUFA) EPA and DHA were around 5 and 6 points higher, respectively, than in fillets of fish fed rapeseed diets. Consequently, both SFA:unsaturated fatty acids (UFA) and n-3:n-6 PUFA ratios resulted superior in fillets of fish fed F than in the rest.

As observed, fatty acid composition of fillets of fish fed diets RN and RA did not differ between each other in almost any case while that of fish fed RE resulted in a higher amount of total SFA. As clearly observed, total SFA varied following the trend of C16:0, as this was the main SFA present in the experimental diets. Accordingly, total UFA, monounsaturated fatty acids (MUFA) and PUFA were lower in RE. Total MUFA varied as oleic acid (C18:1n-9), this being the major fatty acid in rapeseed.

In relation to fillets of fish fed the blended oils diets (RN/RA - RA/RN or RE/RA - RA/RE), no differences between the two diets with a different level of inclusion of RAO (33% or 66%) were present. For diets with graded levels of RE and RA, the two blended oils diets resulted in values of the different categories of fatty acids between those of RE and RA.

Colour

Colorimetric values of fresh and thawed fillets from fish fed the experimental diets are shown in Table 6.5.

In respect to the colorimetric space *L**, *a**, *b**, differences were present only in *b** in both fresh and thawed fillets. In fresh fillets, those of fish fed the control diet (F) showed the significantly lowest *b** value, along with the two diets with the highest content of REO (RE and RE/RA). On the other hand, diets with presence of RAO, especially as a sole source or when combined with RN, had the highest *b** values. In thawed fillets, parameters followed a similar trend as in fresh fillets.

According to the colorimetric space *L**, *C**, $H(°)_{ab}$, significant differences were only observed in *C** values in fresh and also in thawed fillets. Both in fresh and thawed fillets, *C** followed the trend observed in *b**.

All parameters increased from fresh to thawed, this being especially noticeable in *L**.

Significant correlations were observed between the different parameters (Table 6). All the parameters in fresh fillets were correlated in a positive way with their corresponding value in thawed fillets. In fresh fillets, *L** was positively correlated with *b** while *C** displayed a correlation with *b** and $H(°)_{ab}$. $H(°)_{ab}$ was, in turn, positively correlated with *b**. In thawed fillets, *L** had a significant positive correlation with $H(°)_{ab}$ while *C** and $H(°)_{ab}$ were positively correlated with *b**.

At the same time, *L**, *C** and $H(°)_{ab}$ from fresh fillets were negatively correlated in a significant way with *a**. In turn, *a** showed a significant negative correlation with *b**. In thawed fillets, *L**and $H(°)_{ab}$ showed a significant negative correlation with *a**.

Table 6.4. Composition of selected fatty acids of fillets of rainbow trout fed the experimental diets at the end of the trial (day 72).

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Fatty acid (%)								
C14:0	5.1±0.08a	2.2±0.05b	2.1±0.05b	2.3±0.01b	2.1±0.02b	2.1±0.05b	2.1±0.05b	2.1±0.01b
C16:0	19.3±0.12a	13.6±0.18e	14.1±0.14de	16.5±0.24b	13.6±0.19e	13.5±0.37e	15.7±0.40bc	15.0±0.09cd
C16:1n-7	7.3±0.13a	3.4±0.03c	3.5±0.15bc	3.9±0.08b	3.4±0.08c	3.3±0.01c	3.4±0.06c	3.5±0.04c
C18:0	4.6±0.16a	3.7±0.02a	4.0±0.02b	5.6±0.11b	3.7±0.04c	3.8±0.10c	5.3±0.15a	4.6±0.16b
C18:1n-9	17.4±0.48d	35.5±0.25a	35.2±0.06a	32.5±0.17c	35.4±0.40a	35.6±0.40a	33.7±0.14bc	34.3±0.11ab
C18:1n-7	3.2±0.01c	2.9±0.02d	3.5±0.04a	3.3±0.04bc	3.2±0.03a	3.3±0.04a	3.4±0.01bc	3.5±0.02ab
C18:2n-6	7.1±0.18b	14.1±0.07a	14.7±0.10a	14.2±0.27a	14.7±0.33a	15.0±0.22a	14.6±0.23a	14.8±0.08a
C18:3n-3	1.5±0.04e	4.4±0.03a	3.3±0.06c	2.8±0.05d	4.1±0.05a	3.8±0.08b	3.1±0.05c	3.3±0.06c
C20:1[a]	2.1±0.01b	2.5±0.04a	2.1±0.05b	2.3±0.03ab	2.5±0.06a	2.3±0.06ab	2.2±0.04b	2.3±0.08ab
C20:4n-6	0.8±0.02e	0.5±0.01abc	0.6±0.05bcd	0.4±0.02cd	0.5±0.03a	0.5±0.05abc	0.5±0.01d	0.4±0.03ab
C20:5n-3 (EPA)	7.3±0.10a	2.3±0.06b	2.1±0.07bc	1.8±0.05c	2.2±0.09bc	2.2±0.03bc	1.9±0.05c	1.8±0.09c
C22:6n-3 (DHA)	14.3±0.47a	8.3±0.21b	7.8±0.20b	7.9±0.10b	7.9±0.13b	7.8±0.35b	8.1±0.18b	7.7±0.16b
ΣSFA[b]	29.5±0.20a	20.0±0.15d	20.7±0.19d	25.2±0.31b	19.9±0.24d	19.9±0.43d	23.8±0.49bc	22.4±0.27c
ΣUFA[c]	70.5±0.20d	80.0±0.15a	79.4±0.19a	74.8±0.31c	80.1±0.24a	80.1±0.43a	76.2±0.50b	77.6±0.28bc
ΣMUFA[d]	31.1±0.55d	45.1±0.31ab	45.4±0.18a	42.8±0.26c	45.2±0.53a	45.4±0.44a	43.4±0.18bc	44.4±0.15abc
ΣPUFA[e]	37.8±0.48a	34.4±0.11b	33.4±0.32bc	31.3±0.46d	34.3±0.63b	34.2±0.17b	32.1±0.33cd	32.5±0.33bcd
Σn-6 PUFA[e]	8.4±0.19c	16.1±0.08b	17.1±0.13ab	16.1±0.30b	16.9±0.33ab	17.3±0.23a	16.4±0.26ab	16.9±0.10a
Σn-3 PUFA[e]	29.5±0.52a	18.3±0.19b	16.3±0.25cde	15.2±0.19e	17.4±0.32bc	16.9±0.28bcd	15.8±0.08de	15.6±0.32de
SFA:UFA	0.4±0.00a	0.2±0.00d	0.3±0.00d	0.3±0.00b	0.2±0.00d	0.2±0.00d	0.3±0.00bc	0.3±0.00c
n-3:n-6	3.5±0.12a	1.1±0.02b	1.0±0.01bc	0.9±0.01bc	1.0±0.01bc	1.0±0.03bc	1.0±0.01bc	0.9±0.02c

Experimental diets nomenclature as in experimental diets (Table 6.1).

Values are means±SEM of triplicate groups of five fish fillets. Values within the same row with different letters (a, b, c, d, e) are significantly ($P<0.05$) different, according to ANOVA and Tukey's post-hoc test.

[a]Sum of isomers.

[b]SFA: saturated fatty acids. It includes other SFA of small quantity.

[c]UFA: unsaturated fatty acids. It includes other UFA of small quantity.

[d]MUFA: monounsaturated fatty acids. It includes other MUFA of small quantity.

[e]PUFA: polyunsaturated fatty acids. It includes other PUFA of small quantity; n-6 PUFA: omega 6 polyunsaturated fatty acids; n-3 PUFA: omega 3 polyunsaturated fatty acids.

Table 6.5. Colour measurements of fresh and thawed fillets of rainbow trout fed the experimental diets.

	Diets							
Colorimetric values	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Fresh fillets[a]								
*L**	44.07±0.83	43.67±0.23	44.10±0.21	42.17±0.72	43.25±0.67	43.54±0.36	42.50±0.27	42.92±1.02
*C**	5.97±0.27$_{e}$	7.75±0.34$_{bcd}$	8.91±0.18$_{abc}$	5.90±0.17$_{e}$	8.21±0.12$_{abc}$	9.20±0.54$_{a}$	6.34±0.18$_{de}$	7.70±0.28$_{cd}$
$H(°)_{ab}$	61.92±5.29	76.45±1.38	76.63±4.08	58.81±5.42	74.05±2.41	76.76±4.58	65.12±3.20	70.72±1.39
*a**	2.72±0.37	1.77±0.14	2.03±0.64	3.08±0.34	2.20±0.42	2.01±0.72	2.62±0.32	2.46±0.16
*b**	5.21±0.50$_{c}$	7.40±0.49$_{ab}$	8.45±0.05$_{a}$	5.17±0.51$_{c}$	7.60±0.34$_{a}$	8.78±0.34$_{a}$	5.65±0.20$_{bc}$	7.04±0.16$_{ab}$
Thawed fillets[a]								
*L**	63.40±1.24	63.58±1.47	62.50±1.13	62.04±0.54	62.13±1.18	62.74±0.52	61.23±0.43	60.25±0.87
*C**	16.24±0.35$_{ab}$	17.37±0.19$_{ab}$	17.18±0.31$_{ab}$	15.56±0.37$_{b}$	17.72±0.58$_{ab}$	18.01±0.64$_{a}$	16.32±0.45$_{ab}$	17.16±0.09$_{ab}$
$H(°)_{ab}$	65.79±0.10	71.55±1.48	71.83±2.26	63.98±1.90	68.84±3.33	71.83±1.10	67.90±0.34	64.20±1.91
*a**	6.59±0.20	5.48±0.36	5.33±0.62	6.83±0.63	7.09±0.60	6.44±0.07	7.09±0.60	6.44±0.07
*b**	14.82±0.31$_{bcd}$	16.45±0.33$_{ab}$	16.27±0.42$_{abc}$	13.94±0.11$_{d}$	16.42±0.21$_{ab}$	17.07±0.55$_{a}$	14.64±0.37$_{cd}$	15.86±0.14$_{abc}$

Experimental diets nomenclature as in Table 6.1.

Values are means±SEM of triplicate groups of five fish fillets. Values within the same row with different letters (a, b, c, d, e) are significantly ($P<0.05$) different, according to ANOVA and Tukey's post-hoc test.

[a]*L**, luminosity; *C** (chroma), saturation = $(a^{*2} + b^{*2})^{1/2}$ (Wyszecki and Stiles, 1967); $H(°)_{ab}$, hue value = arctan *b**/*a** (Wyszecki and Stiles, 1967); *a** = position between red/magenta and green and *b** = position between yellow and blue.

Table 6.6. Correlations (r) among the different colour parameters on fresh and thawed fillets of rainbow trout fed the experimental diets.

	Colorimetric values									
	Fresh fillets					*Thawed fillets*				
Colorimetric values	*L**	*C**	$H(°)_{ab}$	*a**	*b**	*L**	*C**	$H(°)_{ab}$	*a**	*b**
Fresh fillets										
*L**	-									
*C**	0.33	-								
$H(°)_{ab}$	0.40	0.734*	-							
*a**	-0.26	-0.45*	-0.91*	-						
*b**	0.42*	0.96*	0.84*	-0.58*	-					
Thawed fillets										
*L**	0.46*	0.04	0.26	-0.37	0.14	-				
*C**	0.18	0.72*	0.57*	-0.38	0.70*	-0.09	-			
$H(°)_{ab}$	0.36	0.64*	0.82*	-0.79	0.69*	0.54*	0.33	-		
*a**	-0.31	-0.42*	-0.67*	0.71*	-0.48*	-0.61*	0.00	-0.94*	-	
*b**	0.29	0.82*	0.78*	-0.62*	0.83*	0.13	0.93*	0.66 *	-0.37	-

Colour parameters nomenclature as in Table 6.5.
Correlated values are means±SEM of triplicate groups of five fish fillets.
* Indicates a significant correlation ($p < 0.05$) (Pearson's correlation coefficient).

Texture profile analysis and compression test

Results of texture parameters of fish thawed fillets from rainbow trout fed the experimental diets are shown in Table 7. No significant differences in any parameter were obtained among diets. Only the relationship between springiness and cohesiveness resulted significantly correlated in a positive way ($r = 0.66$, P-value<0.05).

Total fat, protein, moisture, pH and liquid holding capacity of fillets of thawed fillets

Total fat, protein, moisture, pH and LHC values of thawed fillets from rainbow trout fed the experimental diets are shown in Table 7. Of these parameters, only differences in the total fat content of fillets were found, for which fish fed the control diet (F) had significantly higher values than those of fish fed diets with presence of RNO (RN, RN/RA and RA/RN), together with RE/RA.

No statistically significant differences were found in the LHC of fillets among diets, but it is worth mentioning that fillets of fish fed the control diet had the numerically highest result.

With regard to the correlations among parameters, the percentage of total fat in the fillets showed a significant positive correlation with the percentage of the fat retained in fillets ($r = 0.52$, P-value<0.05). In turn, the percentage of fat retained in the fillets resulted in a significant negative correlation with hardness and compression ($r = -0.43$, P-value<0.05 and $r = -0.54$, P-value<0.05, respectively). Moreover, moisture and protein of the fillets were significantly correlated ($r = 0.52$, P-value<0.05).

Table 6.7. Instrumental texture, total fat, protein, moisture, pH and liquid holding capacity measurements of thawed fillets of rainbow trout fed the experimental diets.

	Diets							
Texture profile analysis	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Hardness (N)	4.04±0.46	3.81±0.51	4.69±0.34	4.24±0.19	4.45±0.21	3.95±0.28	4.75±0.44	4.64±0.37
Gumminess (N)	2.60±0.23	2.62±0.34	3.06±0.26	2.74±0.14	3.04±0.08	2.63±0.17	3.04±0.34	3.03±0.13
Adhesiveness (N s)	-0.14±0.01	-0.14±0.04	-0.16±0.02	-0.15±0.02	-0.15±0.02	-0.14±0.03	-0.16±0.01	-0.15±0.03
Cohesiveness	0.77±0.01	0.79±0.01	0.77±0.00	0.77±0.01	0.77±0.02	0.79±0.02	0.76±0.01	0.77±0.02
Springiness	0.83±0.00	0.87±0.01	0.86±0.01	0.84±0.01	0.87±0.02	0.86±0.02	0.82±0.02	0.85±0.02
Compression test								
Force (N)	31.51±1.86	35.43±1.37	36.58±1.07	36.74±7.00	34.75±1.64	34.61±1.78	39.82±2.86	41.75±4.73
Total fat (%)[a]	7.53±0.11$_{a}$	5.36±0.34$_{bcd}$	6.02±0.31$_{abc}$	6.03±0.34$_{abc}$	4.41±0.28$_{d}$	5.64±0.25$_{bcd}$	4.72±0.07$_{cd}$	6.31±0.38$_{ab}$
Protein (%)[a]	66.70±0.54	63.74±2.13	63.81±2.81	63.40±4.71	66.27±1.90	65.56±0.69	64.42±1.86	65.54±1.53
Moisture (%)[a]	65.93±2.56	66.88±1.22	67.50±1.30	66.57±2.19	69.86±1.52	68.09±0.32	67.30±0.83	67.79±0.57
pH[b]	6.28±0.01	6.22±0.03	6.30±0.04	6.28±0.04	6.33±0.03	6.26±0.01	6.26±0.03	6.32±0.04
Liquid holding capacity (as % retained)[a]								
Water retained	78.02±0.02	79.24±0.01	76.59±0.00	75.64±0.01	76.13±0.02	77.41±0.01	78.17±0.01	78.00±0.01
Fat retained	38.99±0.09	23.02±0.00	26.28±0.02	30.99±0.08	12.47±0.06	36.51±0.03	19.90±0.08	32.93±0.04

Experimental diets nomenclature as in Table 6.1.

Values are means±SEM of triplicate groups of five fish fillets. Lack of letters means no statistical significance obtained ($P<0.05$).

[a]Values are means±SEM of triplicate groups of three fish fillets. Values within the same row with different letters (a, b, c, d, e) are significantly ($P<0.05$) different, according to ANOVA and Tukey's post-hoc test.

[b]Values are means±SEM of triplicate pooled fillet samples from five fish. Lack of letters means no statistical significance obtained ($P>0.05$), according to ANOVA.

Tocopherol and TBARS concentrations of thawed fillets

Concentrations of tocopherol in feeds and fillets (expressed as μg tocopherol/g fillet) are shown in Figure 6.2. In feeds, concentrations of α-tocopherol resulted higher than those of β+γ-tocopherol and differences in their concentrations were observed among diets (Figure 6.2.A). RE resulted in a significantly higher concentration of α-tocopherol (157.84±11.50 μg tocopherol/g fillet) compared to the rest of diets while F had a significantly lower concentration of β+γ-tocopherol (96.24±7.29 μg tocopherol/g fillet) than the rest. In contrast, no significant differences were present in the concentrations of tocopherol in fillets among diets (Figure 6.2.B) although this fact was probably due to the higher variability of the data. Levels of tocopherol in fillets seemed to follow the opposite trend than those in feeds, even though this was clearer for α- than for β+γ-tocopherol. Fillets of fish fed RE had the numerically lowest concentration of α-tocopherol (3.40±1.74 μg tocopherol/g fillet) while fillets of fish fed F (6.94±0.42 μg tocopherol/g fillet), along with those of RA/RE (6.62±0.91 μg tocopherol/g fillet), showed the highest values. The concentration in animals fed F was the numerically lowest (0.61±0.12 μg tocopherol/g fillet) for β+γ-tocopherol, which in the fillet also had lower values than α-tocopherol while those of RN/RA and RA/RN were the highest (1.09±0.71 and 1.08±0.98 μg tocopherol/g fillet, respectively).

Lipid oxidation measured as TBARS concentration (expressed as μg MDA/g fillet) of rainbow trout fed the experimental diets is shown in Figure 6.3. No significant differences were observed among diets but values of fillets of fish fed F were the highest numerically, the lack of significance probably being due to the higher variability of the data. TBARS fillets concentrations seemed to follow an opposite trend to the concentration of tocopherol in diets. With regard to correlations, the concentration of α-tocopherol in feeds was positively correlated with that of β+γ-tocopherol in feeds ($r = 0.50$, P-value<0.05). Regarding the relationship with other parameters, α-tocopherol in feeds resulted in significant negative correlations with TBARS of fillets ($r = -0.71$, P-value<0.05). The concentrations of α- and β+γ-tocopherol in feeds were negatively correlated with the content of PUFA in diets ($r = -0.53$, P-value<0.05 for α-tocopherol and $r = -0.70$, P-value<0.05 for β+γ-tocopherol).

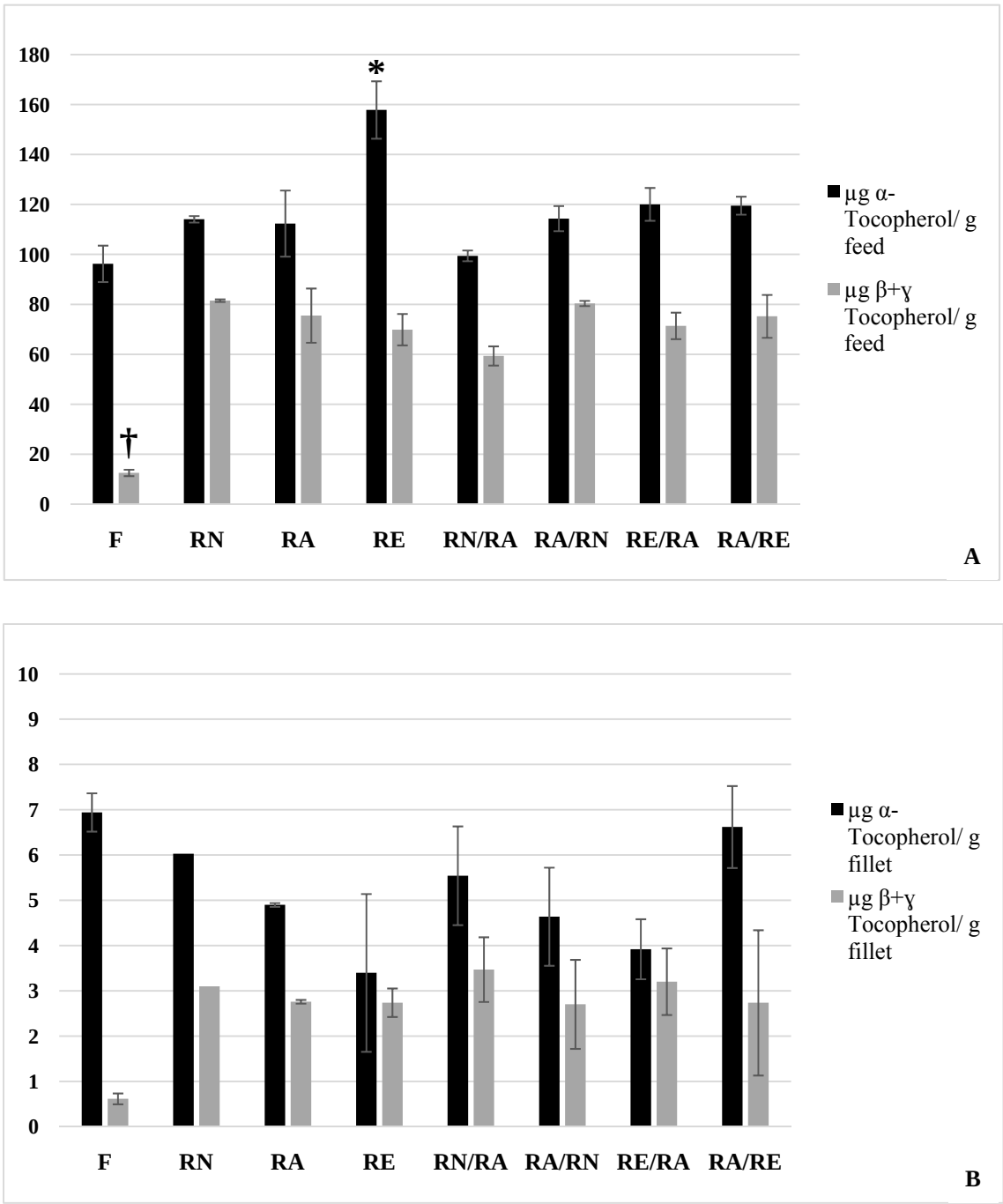

Figure 6.2. (**A)** α- and β+γ-tocopherol concentrations in experimental diets (nomenclature as in Figure 6.2.) expressed as µg of tocopherol per gram of feed and (**B)** α- and β+γ-tocopherol concentrations in fillets from rainbow trout fed the experimental diets expressed as µg of tocopherol per gram of fillet. Bars with a sign (*****/†) are significantly different ($P<0.05$) from the rest, according to ANOVA.

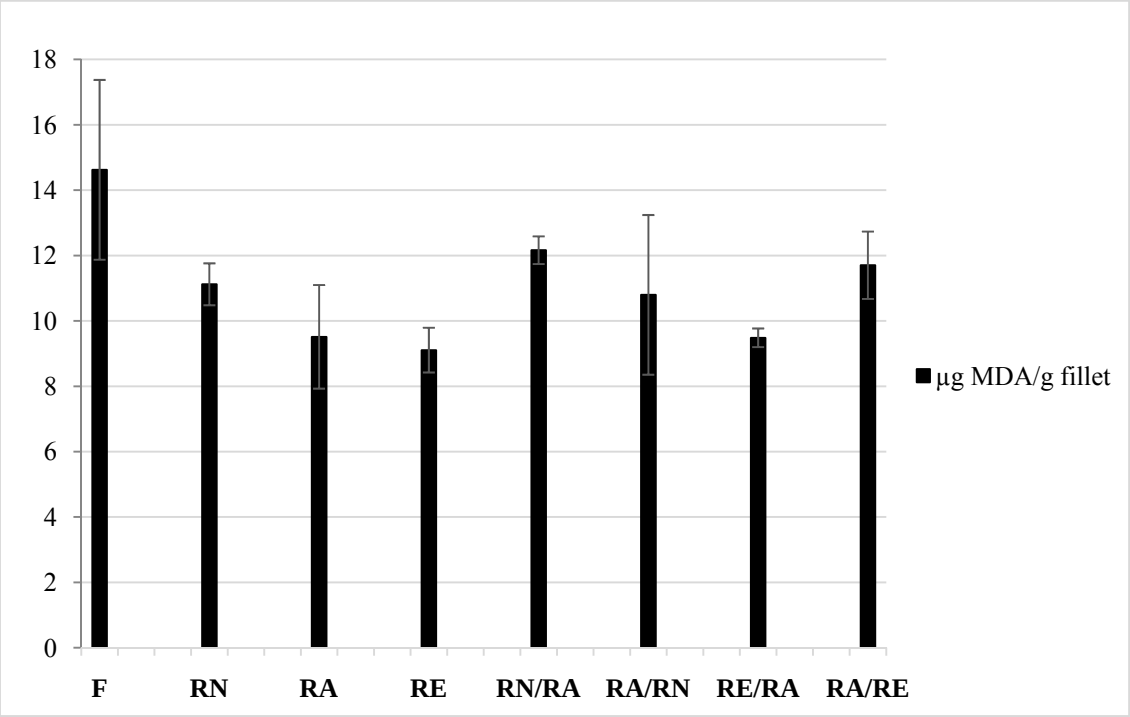

Figure 6.3. Thiobarbituric acid-reactive substances in fillets from rainbow trout fed the experimental diets [F: fish oil (control diet); RN: rapeseed native oil; RE: rapeseed re-esterified oil; RA: rapeseed acid oil; RE/RA: 66% rapeseed re-esterified oil - 33% rapeseed acid oil; RA/RE: 66% rapeseed acid oil - 33% rapeseed re-esterified oil; RN/RA: 66% rapeseed native oil - 33% rapeseed acid oil and RA/RN: 66% rapeseed acid oil - 33% rapeseed native oil] expressed as micrograms of MDA per gram of fillet. The lack of different letters or signs in bars mean no presence of significant differences ($P<0.05$), according to ANOVA.

6.3. Discussion

Characterization of experimental oils and diets

The fact that no differences in the fatty acid composition were present among the experimental diets showed that esterification does not change the fatty acid composition of oils, as previously reported (Trullàs et al., 2015; Vilarrasa et al., 2014). Lipid class compositions obtained in the different oils were similar to those previously described for each type of oil. Native oils were formed of TAG (Flickinger and Matsuo, 2003), the acid oil was composed mainly of FFA (Nuchi et al., 2009) and the re-esterified oil had a high content of MAG and DAG (Trullàs et al., 2015; Vilarrasa et al., 2015b).

Fatty acid composition of fillets

The fatty acid composition of the fillets was clearly influenced by dietary fatty acid as has been reported in many studies in salmonids (Bell et al., 2003; Turchini et al., 2009, 2013) and in other species (Francis et al., 2006; Turchini et al., 2003).

The higher contents of SFA and n-3 PUFA (mainly EPA and DHA) in fillets of fish fed diet F in comparison with those of fish fed rapeseed diets has been observed in studies in which FO was replaced by VO in diets for different species (Bell et al., 2003; Francis et al., 2006; Fountoulaki et al., 2009; Huang et al., 2008). SFA:UFA and n-3:n-6 PUFA ratios in fish fed F were thus also higher than in the rest. In human nutrition, these ratios, and particularly the n-3:n-6 PUFA ratio, are considered useful indexes in comparing the relative nutritional quality of fish flesh (Steffens and Wirt, 2005). Fish with a high concentration of EPA and DHA together with a low n-6:n-3 ratio are a fundamental part of a healthy human diet (Turchini et al., 2009). Indeed, balancing this ratio is very important for homeostasis and normal development and is an essential factor in decreasing the risk of numerous chronic diseases of high prevalence in both Western and developing countries (Simopoulos, 2008). As suggested by Simopoulos (2002), appropriate amounts of dietary omega-6 and omega-3 fatty acids at a ratio of about 1-2:1 need to be considered when making dietary recommendations.

Rapeseed is richer in MUFA (C18:1n-9) than in n-6 PUFA (C18:2n-6) and so fillets of fish fed rapeseed diets resulted in n-3:n-6 PUFA ratios close to 1 in all cases. When deposited in the flesh, rapeseed compromise less the nutritional characteristics of the final product compared to other dietary VO that lead to an increased deposition of SFA or n-6 PUFA (Turchini and Mailer, 2011b). Regarding differences among fillets of fish fed the experimental diets, the higher proportion of SFA in fillets of animals fed RE compared to the rest were possibly due to the higher content of SFA located at the central position (sn-2) of the acylglycerols in this oil (Trullàs et al., 2015). In fact, the chemical esterification process generates oils with a high content of MAG and DAG but it also increases the amount of SFA located at the sn-2 position of acyglycerols. Having more SFA in the central position would imply their direct absorption as 2-MAG after digestion, therefore improving their digestibility as observed in our previous study in rainbow trout (Trullàs et al., 2015). This is also valid for fillets of fish fed blended oils diets in which those fed diets with the presence of REO had more SFA in their flesh. In spite of this, the amount of SFA in fillets of fish fed REO diets did not reach that of fillets of fish fed F as rapeseed is poor in SFA.

The fatty acid composition of the flesh was independent of the level of inclusion of RAO in diets, since no variation was observed between fillets of fish fed RN and of those fed RA or between fish fed blended oils diets and those fed RA. This suggested that the lipid class composition of dietary oils did not exert an important effect on fatty acid metabolism.

Colour

Colour is one of the most important attributes in the perception of flesh quality in salmonids (Bell et al., 1998; Torrissen et al., 2001), being in direct association with the product acceptance or rejection by the consumer (Izquierdo et al., 2005). In rainbow trout the typical red to pink muscle colour of salmonids is due to astaxanthin, the natural pigment for salmonids flesh and the most efficient carotenoid used in aqua feeds to obtain fillet pigmentation (Torrissen et al. 1989; Storebakken and No, 1992). However, preferences in meat colour vary globally with the USA preferring white meat, but Europe and other parts of the world preferring pink meat (FAO, 2005).

In the present study the lack of astaxanthin in diets probably led to a reduction of a^* values, as this parameter represents the red-greenness. Differences were mainly due to variations in b^*. Indeed, b^* was lower in fillets of fish fed F and RE while it tended to be higher as more RAO was present in diets. However, no information on the relationship between fillet colour and lipid class composition of dietary oils is available in the bibliography to the best of our knowledge. Differences observed in C^* (saturation) were basically given by differences in b^* as it was one of the parameters present in the formula to obtain C^*.

In the present work differences in fillets b^* and C^* values could be due to variable proportions of unsaponifiable matter in the experimental oils, which consists of phospholipids, tocopherols, sterols, resins, carbohydrates, pesticides, proteins, trace metals, and pigments (O'Brien, 2008). Therefore, possible different concentrations of pigments among diets could explain differences in colour. Higher b^* and C^* were obtained with diets containing RAO while fillets from fish fed diets with REO had the lowest values. This could be explained by the fact that acid oils contain FFA, acylglycerols, pigments, and other lipophilic materials from the chemical refining (Haas et al., 2003). Pigments present in acid oils could partially or totally end up destroyed by the high temperatures used during the chemical esterification process as happens during the bleaching stage in the refining process (Brooks et al., 2013). The fact that fillets of fish fed F had the lowest

*b** and *C** values is in accordance with the study by Regost et al. (2004), that reported a decrease in *b** in fillets of salmon fed a fish oil diet than in those fed a rapeseed oil diet.

The rise in the values of all parameters in thawed fillet when compared to fresh fillets is in agreement with authors reporting an evident influence of freezing and thawing processes on the flesh colour (Alizadeh, 2012; Bjerken and Johnsen, 1995; Jensen et al., 1998; Regost et al., 2004). Alizadeh (2012) reported that changes can be seen mainly by a strong increase in lightness as was our case, even though he obtained a decrease in both *a** and *b**. In a similar way, No and Storebakken (1991) and Regost et al. (2004) reported an increase in *L**, *a** and *b** in rainbow trout and Atlantic salmon fillets as a direct consequence of freezing.

Correlations among the different colour parameters were mainly expected, because *a** and *b** were used to calculate *C** and $H(°)_{ab}$. In thawed fillets, fewer correlations than in fresh fillets were observed in spite of the similar trend parameters followed in comparison to fresh fillets.

Texture profile analysis and compression test

Texture is an important attribute regarding flesh quality in fish (Ayala et al., 2010) and one of the criteria involved in estimating freshness.

Fish fillet texture can be directly affected by diet, although it has been reported that it can be influenced by many other factors, both external (feeding regimes, slaughtering procedures, storage conditions, freezing, thawing) and internal (fat and water content, lipid oxidation, pH) (Andersen et al., 1997; Carbonell et al., 2003; Mørkøre et al., 2002).

In the present study the different types of dietary experimental oils did not seem to exert an effect on the texture of thawed fillets, as no significant differences in the TPA or in the compression test were obtained. Indeed, Rosenlund et al. (2011) suggested that the effect of dietary oils on raw fillet texture seems to be very limited, regardless of the species studied. Accordingly, many studies have reported a lack of effects of the partial or total inclusion of different VO on fillet texture in various fish species (Bell et al., 2004; Castro et al., 2015; Morkore et al., 2007; Ng and Bahurmiz, 2009; Richard et al., 2006; Regost et al., 2004; Rørå, 2003, 2005; Torstensen et al., 2004).

Even though a correlation between springiness and cohesiveness was obtained in the present study, no information on a positive correlation between these two parameters when determined instrumentally has been found in the literature.

The negative correlations obtained between the fat retained in fillets and both hardness and compression are in agreement with what other studies reported for different species (Andersen et al., 1997; Ginés et al., 2004; Mørkøre et al., 2002), all of them suggesting that increasing the fillet fat content leads to a softening of the flesh.

Total fat, protein, moisture, pH and liquid holding capacity

Total fat, protein, moisture, pH and LHC are important quality attributes of salmonid fillets (Hernández et al., 2009; Mørkøre et al., 2002, Rosenlund et al., 2011).

Effects of the presence of VO in diets on total fat are not clear. Several authors reported no changes when fish were fed either native VO or FO diets (Bell et al., 2003; Nanton et al., 2007; Ng et al., 2004; Pettersson et al., 2009; Richard et al., 2006; Torstensen et al., 2004, 2005) while others obtained even lower values in fish fed FO than in those fed VO (Turchini et al., 2003). In our case the higher values obtained in fish fed F compared to those fed diets including RNO was the most remarkable fact, coinciding with results reported in the study by Yildiz et al. (2015), carried out under very similar experimental conditions to those of the present work. With regard to fillets of animals fed the experimental diets differences found among them cannot be related to diets, as dietary lipid contents did not follow the same variation trend as the fat content in fillets.

Fillet drip-formation losses, which include total liquid, water or fat, could result in a drier and tougher cooked product with a decreased nutritive value, bearing the consequence this would have on the processing industry, on the consumer acceptance and on the economy (Elvevoll et al., 1996; Oyelese et al., 2007; Rørå et al., 2003). Losses vary with factors such as size of fish, muscle pH, the amount of fat and handling conditions among others (Johnssen, 2011; Oyelese et al., 2007) and have also been reported to be a direct consequence of frozen storage and thawing due to cell damage and denaturation of proteins (Alizadeh, 2012; Mørkøre et al., 2002). These two factors would, in turn, result in an increasing fillet hardness (Ng and Bahurmiz, 2009), as observed in our case. As reported, dietary oils can also have an effect on fillets LHC (Regost et al., 2004). According to Rosenlund et al. (2011) data on the effects of dietary oil on the liquid-holding capacity of fish are too limited and often contradictory to be able to speculate on how dietary oils affect fillet LHC. There are many studies reporting no differences in LHC in fillets of fish fed different dietary VO compared to fish fed FO (Bell et al., 2004; Ng and Bahurmiz, 2009; Richard et al., 2006; Rørå et al., 2003).

With regard to the fat retained some authors observed a higher retention in fish fed VO than in those fed FO (Regost et al., 2004; Rørå et al., 2005; Torstensen et al., 2004). In the present study fat losses were higher in the experimental diets than in the F diet, which was probably related to the higher total fat content in fillets of fish fed F. Indeed, a significantly positive correlation was obtained between fat loss and total fat.

Tocopherol and TBARS concentrations of thawed fillets

As widely reported, α-, ß-, γ- and δ-tocopherols together with tocotrienols are fat-soluble vitamin E isomers and the major antioxidants naturally present in VO (Brannan and Erickson, 1996; Kalyana et al., 2003). Vitamin E inhibits lipid peroxidation in biomembranes, lipoproteins and body lipids (Turchini et al., 2009) and this would translate to an extension of the shelf-life of seafood products (Ng et al., 2004).

In the present study, the higher concentration of α-tocopherol in experimental diets in comparison to that of β+γ-tocopherol was in accordance with values reported by Gunstone (1994) and Pettersson et al. (2009) for crude rapeseed oil.

No apparent cause for the significantly higher concentration of α-tocopherol in diet RE in comparison with the rest was found, as REO was obtained from RAO and so similar concentrations of α-tocopherol should be expected between each other. Moreover, Park et al. (1983) showed a negative influence of the chemical interesterification reaction on the oxidative stability of soybean oil due to the loss of tocopherol. The lower β+γ-tocopherol levels of diet F were probably due to the high concentration of this isomer in VO (Chu and Kung, 1998) and especially in rapeseed oil (Kamal-Eldin, 2005).

Fillets of animals fed RE presented the lowest concentration of α-tocopherol while those of fish fed F showed the highest value, even though no statistical differences were obtained between them. As observed, the concentration of tocopherol in fillets, and especially α-tocopherol, seemed to follow an opposite trend than that in feeds. This was not so clear for the concentration of β+γ-tocopherol, although they also varied in a different way in respect to diets. This is in contrast to the linear model for dietary α-tocopherol retention in rainbow trout muscle reported by Hamre (2011) and supported by many studies (Hung et al., 1980; Frigg et al. 1990; Puangkaew et al., 2005). A conclusive trend about the effect of dietary tocopherol on its deposition on fillets could not be elucidated.

Lipid oxidation is a major concern during processing and storage of fish products because it contributes to their quality deterioration (Kamireddy et al. 2011). The higher oxidation

level obtained in fillets of fish fed diet F was due to FO being richer in PUFA, highly susceptible to oxidation, than rapeseed diets. Similar results have indeed been obtained in various studies carried out in different species fed either FO or VO (Menoyo et al., 2004; Regost et al., 2004; Røra et al., 2005; Ng and Bahurmiz, 2009; Qingyuan et al., 2014). Moreover, according to this result, Baron et al (2013) reported that the use of a rapeseed oil in diets for rainbow trout resulted in a more oxidatively stable product when compared to other VO.

The opposite trend observed between the concentration of TBARS in fillets and that of α-tocopherol in diets could be explained by the well-known and already mentioned fact that tocopherol reduces the negative impact of lipid oxidation, enhancing oxidative stability. In addition, α-tocopherol has a more efficient antioxidant activity than β+γ-tocopherol (Burton and Traber, 1990).

Similar negative correlations such as those found in the present study between dietary α-tocopherol and fillets TBARS, as well as between dietary tocopherol and dietary PUFA, have been previously reported (Waagbø et al., 1991; Craig et al., 1999). They are possibly a consequence of the fact that dietary requirements for α-tocopherol in fish increase with the increasing amount of HUFA (Watanabe et al. 1981; Cowey et al. 1981; Roem et al. 1990).

In conclusion, results obtained in the present study indicate that differences in some of the final quality physico-chemical parameters in rainbow trout fed the different experimental diets were found. However, diets including 15% of rapeseed acid or re-esterified oil as a sole fat source or blended do not seem to produce relevant changes in the flesh quality of fish. Further studies regarding their effects on the composition of other tissues would be of interest in order to have more information on the metabolism of acid and re-esterified oils in fish.

CHAPTER 7

EFFECTS OF DIETARY ACID AND RE-ESTERIFIED RAPESEED OILS ON TISSUE FATTY ACID COMPOSITION IN RAINBOW TROUT

Effects of dietary acid and re-esterified rapeseed oils on tissue fatty acid composition in rainbow trout

Trullàs, C.[1*], Sala, R.[1], Fontanillas, R. [2], Codony, R. [3], Guardiola, F. [3], Tres, A.[3]

[1]Animal Nutrition and Welfare Service (SNiBA), Department of Animal and Food Sciences, Universitat Autònoma de Barcelona, Bellaterra 08193, Spain;

[2]Skretting Aquaculture Research Center (Skretting ARC), Sjøhagen 3, Stavanger 4016, Norway.

[3]Departament de Nutrició i Bromatologia-XaRTA-INSA, Facultat de Farmàcia, Universitat de Barcelona, 08028 Barcelona, Spain;

Abstract

Acid oils are a free fatty acid (FFA)-rich by-product from the refining of crude VO that can be re-esterified with glycerol to produce re-esterified VO. Re-esterified oils have less FFA and different proportions of triacylgycerols – TAG, diacylgycerols – DAG and monoacylgycerols – MAG. They also incorporate a higher proportion of SFA in the central position (sn-2) than their corresponding native VO (Vilarrasa et al., 2014; Trullàs et al., 2015). In our previous studies in fish, no negative effects on digestibility, growth, health status indicators and physico-chemical quality of fillets were observed in fish fed acid or re-esterified VO from an unsaturated source such as rapeseed compared to those fed diets with native VO (Trullàs et al., 2015, 2016). Therefore, the aim of the present study was to evaluate the effects of the dietary inclusion of acid and re-esterified rapeseed oils as alternatives to native vegetable oils (VO) on the total fatty acid composition of liver, fillet and abdominal fat in rainbow trout. Although rapeseed is poor in SFA, the sn-2 fatty acid composition of these tissues was also determined, as it could provide information on the possible conservation of the fatty acids located in this position and thus on the absorption and metabolism of the experimental oils. Given that the use of re-esterified VO implies the previous cost of the esterification process, acid oils are economically advantageous. Thus, we also included diets with partial substitution of the native and the re-esterified rapeseed oils by graded levels of the acid oil. Triplicate groups of rainbow trout were fed eight experimental diets containing 15% of different types of experimental rapeseed oil in addition to 5% of fish oil (FO) during 72 days. Dietary experimental rapeseed oils were native (RNO), re-esterified (REO), acid (RAO) or blends (66% REO-33% RAO / 33% REO-66% RAO or 66% RN-33% RAO / 33% RN-66% RAO). A commercial fish oil was used for the control diet (F).

The type of rapeseed oil used (native, acid or re-esterified, alone or blended) did not result in differences in fatty acid composition in the liver. In fillets, those of fish fed REO diet were richer in saturated fatty acids (SFA) and poorer in mono- (MUFA) and polyunsaturated (PUFA) fatty acids than those of animals fed RNO or RAO diets. When considering only the fillets from fish fed the rapeseed diets the main factor affecting the fatty acid composition was the dietary gradation depending on the type of oil. The increase in SFA and decrease in PUFA in fillets of fish fed REO diet was downsized when REO in diet was blended with RA. Total fatty acid composition of the abdominal fat showed similar results than those observed in fillets. For the sn-2 fatty acid composition,

differences between fish fed the different diets were obtained in fillet but not in liver or abdominal fat (except for EPA in abdominal fat). Although fillets of fish fed REO diet showed the higher SFA contents, this did not imply a higher fraction of SFA esterified to sn-2. In conclusion, re-esterified and acid rapeseed oils could be incorporated, along with 5% of fish oil, as fat sources in diets for rainbow trout without causing relevant effects on tissues total and sn-2 fatty acid composition compared to diets including native oil.

7.1. Introduction

The competition between the feed and the biofuels industries caused by their growing use of vegetable oils (VO) as feedstock resulted in a peak price of these VO in 2008 (Gunstone, 2011). As a consequence, the need for alternatives to VO as ingredients in animal diets aroused, this including feeds for both aquatic and monogastric land animals. As Turchini et al. (2009) reported for aqua feeds, one cheaper option available to aquafeed millers was to use by-products and waste products from VO processing. Indeed, refining of crude VO generates an important amount of by- and co-products that are an industrial waste, some of which have been increasingly studied as ingredients in diets for monogastric animals (Ng et al., 2000; 2004; 2010; Tres et al., 2015; Vilarrasa et al., 2014). This is the case of vegetable acid oils, a free fatty acid (FFA)-rich by-product that is cheaper than its original VO and promising as a fat source for feeding uses (Nuchi et al., 2009). However, these oils had shown low digestibility values in some animal species when included in diets, especially when produced from oils rich in saturated fatty acids (SFA) (Wiseman and Salvador, 1991; Vilà and Esteve-Garcia, 1996). In fish, our previous studies showed that acid oils do not affect early growth when they originate from unsaturated sources such as rapeseed. In addition, acid oils can be valorised by subjecting them to a chemical esterification process with glycerol to generate the so-called re-esterified VO, constituted of variable proportions of acylglycerols (triacylgycerols – TAG, diacylgycerols – DAG and monoacylgycerols – MAG), together with a small amount of FFA (Parini and Cantini, 2009, Trullàs et al., 2015; Vilarrasa et al., 2014). First, the evident reduction of FFA might increase their digestibility. Second, this esterification generates partial acylglycerols that have emulsifying properties and that might help FFA to solubilize and be absorbed. Finally, during the esterification process a rearrangement of the fatty acids in the glycerol molecule occurs, the re-esterified oil having a different fatty acid

positional distribution within its acylglycerol molecules than that of its corresponding native oil. In native VO, SFA are mainly located in the external positions of the TAG molecules (sn-1 and sn-3) (Hunter, 2001; Karupaiah and Sundram, 2007) while in re-esterified oils part of these SFA are located in sn-2 (Vilarrasa et al., 2014; Trullàs et al., 2015). This increase of SFA in sn-2 could improve SFA and the overall lipid digestibility in both humans and monogastric animals (Innis et al., 1995; Kennedy et al., 1999; Smink et al., 2008) because fatty acids located in sn-2 would remain bound to the glycerol molecule and would be directly absorbed as 2-MAG (Schulthess et al., 1994). As reported for humans and other monogastric animal species, variations of the fatty acid positional distribution could have important effects not only in digestibility but also in absorption and metabolism (Bracco, 1994; Mu and Porsgaard, 2005; 2006; Karupaiah and Sundram, 2007; Berry, 2009; Innis, 2011; Michalski et al., 2013). Regarding this, Akesson et al. (1978) and Kubow (1996) reported 75% of conservation of the fatty acids located in sn-2 through digestion and absorption processes in rat and in humans, respectively. However, in rainbow trout fed diets including palm or rapeseed re-esterified oils, a digestibility improvement was not observed (Trullàs et al., 2015). Results on the productive parameters showed that the dietary inclusion of rapeseed acid and re-esterified oils alone or in blends did not have negative effects on digestibility, growth or health status indicators. In fact, a slightly higher final weight was obtained in fish fed diets with blended acid and re-esterified oils (Trullàs et al., 2016). Moreover, it did not seem to have relevant effects on the final quality of the fillets (Trullàs et al., unpublished). The aim of the present study was to evaluate the effects of the dietary inclusion of acid and re-esterified rapeseed oils on the fatty acid composition of different tissues in rainbow trout. Because the use of re-esterified VO implies the previous cost of the esterification process, acid oils seem to be more economically advantageous. Therefore, for our purpose, we also included diets with partial substitution of the native and the re-esterified rapeseed oils by graded levels of the acid oil. Here, we report the effects of acid and re-esterified rapeseed oils, along with blends of the acid oil with the native or the re-esterified oils, on fatty acid composition of liver, fillet and abdominal fat in rainbow trout. Even though rapeseed is poor in SFA, the sn-2 fatty acid composition of these three tissues was also evaluated as it could provide information on the possible conservation of the fatty acids located in this position and thus on the absorption and metabolism of the experimental oils.

7.2. Materials and methods

7.2.1. Experimental diets

Experimental diets (45% protein and 21% lipid) had the same ingredient composition except for the added lipid source (Table 7.1). Three different types of rapeseed oil – native oil (RNO), re-esterified (REO) and acid (RAO) – were included in the diets alone (diets RN, RE or RA) or in blends (diet RE/RA: 66% REO-33% RAO; diet RA/RE: 66% RAO-33% REO; diet RN/RA: 66% RNO-33% RAO and diet RA/RN: 66% RAO-33% RNO) in a proportion of 15%. Also, 5% of commercial fish oil was included in all experimental diets. A diet including only commercial fish oil (20% FO) was used for the control diet (F). Experimental oils were provided by SILO S.p.a. (Firenze, Italy) (RNO and REO) and Cargill (Schiphol, The Netherlands) (RAO). The re-esterified oil (REO) was obtained as described in Trullàs et al. (2015). Feeds were produced at the Skretting Feed Technology Plant (Aquaculture Research Center; Stavanger, Norway) as extruded pellets. The ingredient formulation and proximate composition of the diets are shown in Table 7.1. Nutrient composition of experimental diets was determined by standard procedures (AOAC, 2005): moisture (934.01), ash (942.05), crude protein (968.06) and crude lipid (920.39). Gross energy of dried feed was determined using an adiabatic bomb calorimeter (IKA-Kalorimeter system C4000, Jankel-Kunkel, Staufen, Germany).

7.2.2. Fish, experimental conditions and sampling

All the procedures were conducted in accordance with the Animal Protocol Review Committee of the Universitat Autònoma de Barcelona (UAB) and following the European Union Guidelines for the ethical care and handling of animals under experimental conditions (2010/63/EU). The trial was carried out at the Skretting Italia Aquaculture Research Center in Mozzecane, Italy. A total of 576 rainbow trout with a mean initial body weight of 101.7 g±8.80 g were randomly distributed into 24 cylindro-conical tanks of 600 l of capacity (24 fish per tank) in an open freshwater system with a continuous water flow of 24 l min^{-1}. Water temperature (14.3ºC) and dissolved oxygen levels (7.4 mg/l±0.37 mg/l) were maintained constant throughout all the experimental period. Tanks were subjected to a 24h light photoperiod. Fish were fed the experimental diets for 72 days. Each diet was randomly assigned to three replicate tanks and was fed twice a day by automatic feeders, adjusted to provide 2.5% of the biomass daily. Uneaten feed was collected by filtering effluent water from each tank. Collectors were emptied after each meal and feed intake was recorded daily. At day 72, five fish from each tank were sacrificed by an over-dose of

anaesthetic and individually gutted and filleted. The right fillet was cut in two different portions that were bagged and stored frozen at -20 º C for carrying out analyses of total and sn-2 fatty acid composition. With the same purpose, the liver and the abdominal fat of each of the five animals were taken and stored frozen at -20 ºC.

Table 7.1. Ingredient formulation and proximate composition of the experimental diets.

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Ingredient composition (g kg^{-1})								
Wheat[a]	60	60	60	60	60	60	60	60
Wheat gluten[b]	232.8	232.8	232.8	232.8	232.8	232.8	232.8	232.8
Soy bean meal[c]	80	80	80	80	80.0	80.0	80.0	80.0
Soy protein concentrate[d]	150	150	150	150	150	150	150	150
Faba beans whole[e]	100	100	100	100	100	100	100	100
Fish meal[f]	150	150	150	150	150	150	150	150
Fish oil[g]	201.3	52	52	52	52	52	52	52
Experimental oils[h]	0	150	150	150	150	150	150	150
Yttrium premix[i]	1	1	1	1	1	1	1	1
Mineral and vitamin premix[i]	24.9	24.9	24.9	24.9	24.9	24.9	24.9	24.9
Proximate composition (g kg^{-1})								
Dry matter	925.7	925.9	927.9	929.9	931	928.9	926.8	927.3
Crude protein	472.2	466.1	485.1	468.2	468	466.2	471.7	474.3
Crude fat	204.1	215.7	187.7	210.4	219.5	214.3	191.9	201.4
Ash	64.2	63.3	65	70.6	67.6	65.6	65.2	68.1
Gross energy (kJ g^{-1})	22.8	22.5	22.8	22.4	22.3	22.7	22.4	22.4
Digestible energy (kJ g^{-1})[j]	20.0	19.5	19.1	20.2	18.0	20.2	19.8	19.3

Experimental diets nomenclature: F: fish oil (control diet); RN: rapeseed native oil; RE: rapeseed re-esterified oil; RA: rapeseed acid oil; RE/RA: 66% rapeseed re-esterified oil - 33% rapeseed acid oil; RA/RE: 66% rapeseed acid oil - 33% rapeseed re-esterified oil; RN/RA: 66% rapeseed native oil - 33% rapeseed acid oil and RA/RN: 66% rapeseed acid oil - 33% rapeseed native oil.

[a]Statkorn, Norway.

[b]Cerestar Scandinavia AS, Denmark.

[c]IMCOPA, Brasil.

[d]Denofa, Norway.

[e]Ceremis, France.

[f]Welcon AS, Norway.

[g]Holtermann ANS, Norway.

[h]Vitamin and mineral premix, according to requirement data from NRC (2011). Trow Nutrition, The Netherlands.

[i]Values were determined by calculating the apparent digestibility coefficient (ADC) of the gross energy of diets using the formula proposed by Maynard & Loosli (1979), prior to multiplying this value to the gross energy of the corresponding diet.

7.2.3. Lipid extraction

To determine the fatty acid composition of liver, fillet and abdominal fat, fat was extracted using chloroform: methanol (2:1, v/v). Briefly, 30 mL of chloroform:methanol (2:1, v/v) were added to 2 g of tissue and homogenised at 19000 rpm for 30 s using a Polytron PT3100 (Kinematica, Lucerne, Switzerland). Then, the mixture was filtered through a filter paper (Whatman nº1), and the residue was re-extracted twice more with 8 mL of chloroform:methanol (2:1, v/v), homogenizing the mixture each time. The extracts were filtered and pooled and 8 mL of NaCl (1%, w/v) were added. The mixture was shaken and centrifuged at 540 *g* for 20 minutes. The chloroformic phase was separated and filtered through anhydrous sodium sulphate. Solvent was then evaporated by using a rotatory vacuum evaporator. The extracted lipid fraction was transferred to a screw capped tube with diethyl ether that was then evaporated under N_2 at 35 C. Tubes were capped and frozen at -20C.

7.2.4 Fatty acid methyl esters and GC conditions

FAME were obtained from oils and extracted lipids of liver, fillets and abdominal fat following the method described by Guardiola et al. (1994). Then, they were determined by GC using an Agilent 4890D gas chromatograph (Agilent Technologies; Santa Clara, CA) equipped with a flame ionization detector and a polar capillary column (SP-2380 60m, 0.25mm i.d., 0.2 μm from Supelco; Bellefonte, PA). Hydrogen was used as the carrier gas. Injector and detector were set at 270 and 300ºC, respectively. Oven temperature was set at 150ºC for 2 min, increased at 1.5ºC/min to 180ºC, then increased at 7.5ºC/min to 220ºC, maintained for 6 min, and finally increased at 5ºC/min to 250ºC, maintained for 6 min. Sample volume injected was 1 μL. FAME were identified by comparison of their retention times with those of known standards (Supelco 37 component FAME Mix, Sigma–Aldrich Co., St. Louis, MO) and quantified by internal normalization (FAME peak area/total FAME area, in %).

For diets, FAME were obtained by direct methylation, according to Meier et al. (2006) and analysed using an HP 5890A gas chromatograph.

7.2.5. Sn-2 fatty acid composition

The determination of the composition of fatty acids located at the sn-2 position of the acylglycerides was performed following the same methodology as described by Tres et al., (2015). Briefly, oils or extracted lipid fractions were hydrolysed by pancreatic lipase and

2-MAG were separated from free fatty acids and other acylglyceride forms by thin layer chromatography. The fatty acid composition of scrapped 2-MAG was determined after obtaining the corresponding methyl esters as described in section 2.3. Results were expressed as internal normalization (sn-2 FAME peak area/total sn-2 FAME area, in %). Then, the distribution of each fatty acid within glycerol positions [sn-2 and sn-1 (3)] of the different acylglycerol molecules (TAG, DAG and MAG) in the oil was calculated as the fraction of each fatty acid located at the sn-2 position of these acylglycerols. Values in brackets correspond to fatty acid composition of the experimental oils breaking down only sn-2 fatty acids. For this calculation, results from the fatty acid composition and the composition of fatty acids at sn-2 that had been expressed in area% (FAME area% and sn-2 FAME area%) were calculated in mol% (FAME mol% and sn-2 FAME mol%). Then, an adaptation of the formula suggested by Mattson and Volpenhein (1961) was applied: fraction of each fatty acid located at the sn-2 position of these acylglycerols in the oil = (sn-2/Total) x *a* x 100, where sn-2 is the percentage of a specific fatty acid at the sn-2 position (sn-2 FAME mol%), Total is the percentage of that fatty acid in the oil (FAME mol%) and *a* is the ratio between the moles of fatty acid located at the sn-2 position and the moles of total fatty acid.

7.2.6. Statistical analysis

Principal component analyses (PCA) on the fatty acid composition of each tissue (liver, fillet and abdominal fat) were performed to visualize the main information in the variables by a lower number of variables, the so-called principal components (PC) by means of SIMCA software (v 13.0, Umetrics AB, Umea, Sweden). Then, data were subjected to a one-way analysis of variance (ANOVA) and the significance of the differences between means was tested by Tukey's test. Values are given as means±standard error of the mean (SEM) of triplicate groups of five fish fillets Differences were considered significant when $P<0.05$. All statistics were performed by means of the General Lineal Model (Proc GLM) of SAS® software version 9.2 (SAS Institute Inc., Cary, NC, USA).

7.3. Results

Characterization of experimental oils and diets

Results of the fatty acid composition of experimental oils and diets are shown in Table 7.2. and their lipid class composition in Table 7.3. The fatty acid composition of the sn-2 position of oils is shown in Table 7.4.

Differences in the fatty acid composition among rapeseed oils, as well as among rapeseed diets, were minor. Even though, the re-esterified oil (REO) and its corresponding diet (RE) were richer in SFA and poorer in UFA (Table 7.2.). As observed, n-3:n-6 ratios were close to 1 in all experimental rapeseed diets (range of 0.8-1) while that of the control diet (F) was 3.6.

With regard to the lipid classes, FO and RNO were constituted mainly by TAG (>90%), the re-esterified oil (REO) was rich in partial acylglycerols (35.4% MAG and 34% DAG) and the acid oil (RAO) had a high proportion of FFA (64.3%). No formation of TAG polymers was found. In diets, even though the lipid class composition mirrored those of the oils in the case of F and RN, differences were observed in RE and RA. These two diets had higher percentages of TAG but lower of FFA and partial acylglycerols than their corresponding oils. In the blended oil diets, the increase in FFA as more RAO was present was clearly observed. Similarly, a rise in the proportion of MAG and DAG was observed as more REO was present.

In relation to the fatty acid composition of the sn-2 position of oils (Table 7.4.), a slight increase in the proportion of SFA (2.4%) was observed in the re-esterified oil (REO) compared to the native oil (RNO, 2.0%) when calculated as the fatty acid in sn-2 on the total fatty acid composition of the experimental oils. REO had a slightly higher presence of SFA in sn-2 and it was poorer in MUFA (4.0%) and PUFA (4.9%) than RNO (MUFA: 14.2%, PUFA: 12.7%).

Table 7.2. Fatty acid composition (FAME area %) of the experimental oils and diets.

	Oils				Diets							
	FO	RNO	RAO	REO	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Fatty acid (%)												
C14:0	8.5	0.9	0.4	0.6	8.1	3.0	2.8	2.9	2.7	2.7	2.9	2.8
C16:0	19.7	5.8	5.5	8.5	18.6	10.3	11.0	13.5	10.4	10.6	12.4	11.5
C16:1n-7	9.4	1.0	0.4	0.8	8.7	3.3	3.1	3.3	3.0	3.1	3.2	3.0
C18:0	3.8	2.1	2.6	4.4	3.2	2.4	2.8	4.7	2.5	2.6	4.0	3.4
C18:1n-9	10.9	52.2	54.8	50.1	10.8	35.7	35.5	32.2	36.2	36.3	34.2	35.4
C18:1n-7	3.4	3.1	4.8	4.1	3.2	3.5	4.3	4.1	3.5	3.9	4.0	4.0
C18:2n-6	1.4	18.8	21.4	19.5	5.9	16.7	18.5	17.0	17.7	18.1	17.5	17.9
C18:3n-3	1.0	9.4	7.5	6.7	1.1	6.2	5.1	3.9	5.9	5.6	4.4	4.9
C18:4n-3	2.8	0.3	0.0	0.1	2.4	0.9	0.8	0.8	0.8	0.8	0.8	0.8
C20:1[a]	1.4	1.7	1.1	1.2	2.5	2.5	2.2	2.2	2.5	2.3	2.1	2.2
C20:4n-6	0.2	0.3	0.3	0.3	1.0	0.4	0.3	0.4	0.3	0.3	0.3	0.3
C20:5n-3 (EPA)	17.7	1.4	0.1	0.7	14.7	5.2	4.8	5.0	4.9	4.7	4.8	4.8
C22:1[a]	1.2	0.1	0.0	0.0	2.1	1.6	1.4	1.4	1.6	1.5	1.4	1.3
C22:5n-3	2.3	0.2	0.1	0.1	1.7	0.6	0.6	0.6	0.6	0.6	0.6	0.6
C22:6n-3 (DHA)	12.8	1.1	0.1	0.6	10.8	4.2	3.9	4.1	4.1	3.8	3.9	3.9
ΣSFA[b]	32.3	9.5	9.0	14.8	30.8	16.8	17.5	22.7	16.8	17.0	20.6	18.9
ΣUFA[c]	67.7	90.5	91.0	85.2	69.2	83.2	82.5	77.3	83.2	83.0	79.4	80.4
ΣMUFA[d]	27.9	58.2	61.5	56.5	28.5	47.8	47.7	44.4	47.8	48.0	46.0	46.9
ΣPUFA[e]	39.7	32.2	29.5	28.6	40.6	35.3	34.8	32.8	35.4	35.0	33.3	34.1
Σn-6PUFA[e]	1.7	19.0	21.7	19.8	8.8	17.8	19.3	18.0	18.7	19.1	18.4	18.9
Σn-3PUFA[e]	38.1	13.2	7.7	8.7	31.8	17.5	15.5	14.8	16.7	15.9	14.9	15.2
n-3:n-6	23.0	0.7	0.4	0.4	3.6	1.0	0.8	0.8	0.9	0.8	0.8	0.8

Experimental oils nomenclature: FO: fish oil; RNO: rapeseed native oil; REO: rapeseed re-esterified oil; RAO: rapeseed acid oil. Experimental diets nomenclature as in experimental Table 7.1.

[a]Sum of isomers.

[b]SFA: saturated fatty acids. It includes other SFA of small quantity.

[c]UFA: unsaturated fatty acids. It includes other UFA of small quantity.

[d]MUFA: monounsaturated fatty acids. It includes other MUFA of small quantity.

[e]PUFA: polyunsaturated fatty acids. It includes other PUFA of small quantity; n-6 PUFA: omega 6 polyunsaturated fatty acids; n-3 PUFA: omega 3 polyunsaturated fatty acid.

Table 7.3. Lipid class composition of the experimental oils and diets.

	Oils			
	FO	RNO	RAO	REO
Lipid classes (%)				
ΣTAG[a]	93.8	95.6	20.5	26.6
ΣDAG[a]	2.9	2.5	12.5	34.0
ΣMAG[a]	0.7	0.2	2.7	35.4
ΣFFA[a]	2.6	1.7	64.3	2.0

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Lipid classes (%)								
ΣTAG[a]	92.9	93.4	46.0	54.0	77.2	62.2	49.0	46.6
ΣDAG[a]	3.2	3.1	9.4	21.9	5.3	6.9	19.0	14.8
ΣMAG[a]	0.8	0.7	2.1	22.3	1.1	1.6	14.8	7.9
ΣFFA[a]	3.1	2.8	42.4	1.8	16.5	29.2	17.2	30.7

Experimental oils and diets nomenclature as in Table 7.1.
[a]TAG (triacylglycerols), DAG (diacylglycerols), MAG (monoacylglycerols) and FFA (free fatty acids).

Table 7.4. Fatty acid composition of the sn-2 position of the experimental oils.

	Oils							
	FO	RNO	RAO	REO	RNO/RAO	RAO/RNO	REO/RAO	RAO/REO
sn-2 (%)								
C16:0	13.7 (2.7)	23.4 (1.3)	12.1 (0.7)	15.6 (1.8)	19.4 (0.9)	15.7 (0.9)	14.3 (1.4)	13.1 (1.1)
C18:0	8.4 (0.3)	29.3 (0.6)	11.3 (0.3)	15.5 (0.7)	23.1 (0.4)	17.1 (0.4)	14.0 (0.6)	12.5 (0.4)
C18:1n-9	11.5 (1.2)	28.8 (15.0)	7.8 (4.3)	14.3 (1.6)	21.6 (9.9)	14.7 (7.8)	12.0 (2.5)	9.9 (3.4)
C18:2n-6	57.1 (0.8)	51.5 (9.7)	10.5 (2.2)	19.5 (2.1)	37.4 (6.4)	23.9 (4.7)	16.0 (3.0)	13.2 (2.6)
C18:3n-3	21.9 (0.2)	45.2 (4.2)	9.3 (0.7)	10.4 (0.7)	32.9 (2.8)	21.0 (1.7)	9.9 (0.7)	9.6 (0.2)
ΣSFA	12.2 (3.9)	21.5 (2.0)	19.9 (1.8)	13.6 (2.4)	20.7 (1.3)	20.2 (1.8)	15.5 (2.2)	17.6 (2.0)
ΣMUFA	12.2 (3.4)	24.5 (14.2)	10.1 (6.2)	10.7 (4.0)	19.5 (9.4)	14.7 (8.8)	10.4 (4.7)	10.2 (5.4)
ΣPUFA	22.4 (8.9)	39.4 (12.7)	13.4 (3.9)	12.3 (4.9)	30.4 (8.4)	21.8 (6.8)	12.5 (4.5)	12.9 (4.2)

Experimental oils nomenclature as in Table 7.2.

Fraction of each fatty acid located at the sn-2 position of acylglycerols (in %) in the oil. Values in brackets correspond to fatty acid composition of the experimental oils breaking down only sn-2 fatty acids.

Total fatty acid composition of livers

PCA of the fatty acid composition of the livers of fish fed the experimental rapeseed diets are shown in Figure 7.1. and the fatty acid composition of the livers of fish fed the experimental diets is shown in Table 7.5.

PCA conducted on liver fatty acid composition revealed that FO samples clustered separately from rapeseed samples (Figure 7.1.). This separation was mainly on PC1, in which fatty acids such as C14:0 and n-3 LC-PUFA had high positive loadings. Within livers from rapeseed diets, no clear separation was observed depending on the type of rapeseed used in feed.

Clustering observed in PCA agreed with ANOVA results. Livers from animals fed F were richer in C14:0, C16:1n-7 and C20:5n-3, and livers from animals fed rapeseed oil diets were richer in C18:1n-9 and C18:2n-6 (Table 7.5. and Supplementary table 7.2.). Regarding rapeseed diets, no significant differences ($p>0.05$) were observed in the proportion of individual or total SFA, MUFA or long chain n-3 PUFA of the livers among fish fed the experimental diets (Table 7.5.).

Total fatty acid composition of fillets

PCA of the fatty acid composition of the fillets of fish fed the experimental diets are shown in Figures 7.2. and 7.3. The fatty acid composition of the fillets of fish fed the experimental diets is shown in Table 7.5.

As observed in Figure 7.2.A, the first two PCA factors explained a 76.6% of variance. Fatty acid composition of the fillets showed a clear separation between fish fed the control diet and those fed the experimental rapeseed diets. This separation was mainly on PC1, which showed high loading values for fatty acids such as C14:0 , C16:0 and n-3 LC-PUFA (Figure 7.2.B). A separation of fatty acids coming from diets with graded levels of RN and RA (RN-RN/RA-RA/RN-RA) or with graded levels of RE and RA (RE-RE/RA-RA/RE-RA) was also observed (Figure 7.2.A). This separation was mainly along PC2. For a more detailed study of this grouping, fillets of fish fed F were excluded and PCA was recalculated (Figure 7.3.). Then, it was clearly observed that the separation agreed with RAO replacement by graded levels of REO (along PC1) or by graded levels of RNO (along PC2), resulting in a sort of two sequential clustering. Fillets of fish fed diets containing increasing levels of REO, agreed with high positive loading values in the first factor for SFA, while the separation of treatments with increasing RNO agreed with high

positive values in PC2 for fatty acids such as C20:3n-3, C20:4n-3 or C20:1n-9, among others

Clustering observed in PCA agreed with ANOVA results. The fillets of animals fed F had the highest and the lowest ($p<0.05$) proportions of SFA and MUFA, respectively (Table 7.5.). Within rapeseed diets, the fillets of fish fed RE and RE/RA were significantly richer ($p<0.05$) in total SFA than the rest, even though the total SFA values of the latter did not differ ($p>0.05$) from those of RA/RE. For MUFA, the fillets of rainbow trout fed RE and RE/RA had a lower value than the rest, although values of RA/RE did not differ ($p>0.05$) from those of RE/RA. As described for SFA, the fillets of rainbow trout fed the control diet obtained the highest ($p<0.05$) proportions of total PUFA while diets RE and RE/RA obtained the lowest ($p<0.05$) values, although they did not differ from those of the fillets from fish fed RA ($p>0.05$). SFA values varied mainly according to C16:0 values (Supplementary Table 7.3.). Furthermore, fish fed F had the lowest and highest ($p<0.05$) proportions of n-6 and n-3 PUFA in the fillets, respectively. Among rapeseed diets, there was a significant difference ($p<0.05$) in the proportion of n-6 PUFA, values obtained in fish fed RA/RN and RA/RE being higher than those of fish fed RN and RE. Fish fed RN had significantly higher proportions of n-3 PUFA in the fillets than those fed RA, RE, RE/RA and RA/RE. In relation to the n-3:n-6 ratios, differences were similar to those observed in diets, that obtained with diet F being significantly higher ($p<0.05$) than the rest. Among rapeseed diets, a significant difference ($p<0.05$) was present between RN and RA/RE, the latter being lower, even though all values were very similar numerically.

Total fatty acid composition of abdominal fat

PCA of the fatty acid composition of the abdominal fat of fish fed the experimental rapeseed diets are shown in Figure 7.4. The fatty acid composition of the abdominal fat of fish fed the experimental diets is shown in Table 7.5.

PCA of total fatty acid composition in the abdominal fat showed similar results than those observed in the fillets, showing a clear separation of FO samples from rapeseed samples. Similarly to fillets, a sort of sequential clustering was observed for RAO replaced by REO (along PC1) and by RNO (mostly along PC2). Abdominal fat from fish fed diets with the highest REO amounts showed high negative values for PC1, which agreed with high values for SFA. In turn, abdominal fat from fish fed diets with the highest RNO content showed high positive values in PC2, which agreed with high loading in PC2 for fatty acids

such as C20:3n-3, C20:4n-3 and C20:1n-9, among others. In both cases, these fatty acids were also the most important in the separation between RE and RN fillets.

As expected, results observed in PCA agreed with those from ANOVA. The abdominal fat of fish fed the control diet presented the highest and the lowest proportion of SFA and MUFA, respectively. Regarding rapeseed diets, those with a higher presence of REO (RE and RE/RA) resulted in the highest ($p<0.05$) presence of total SFA in the abdominal fat. In contrast, the abdominal fat of animals fed diets containing RNO (RN, RN/RA and RA/RN) had the significantly lowest ($p<0.05$) proportion of SFA, although that of fish fed RA did not differ ($p>0.05$) from them. No significant differences ($p>0.05$) in the amount of total MUFA in the abdominal fat were observed among fish fed rapeseed diets.

For total PUFA, the abdominal fat of fish fed F had the highest ($p<0.05$) total PUFA proportion, as well as the lowest and highest ($p<0.05$) proportions of n-6 and n-3 PUFA, respectively. Within rapeseed diets, total PUFA diminished as RAO was replaced by REO due to a reduction of both n-6 and n-3 PUFA. Although no differences were observed in total PUFA when RAO was replaced by RNO, n-6 PUFA diminished and n-3 increased.

Table 7.5. Categories of fatty acids in liver, fillet and abdominal fat of rainbow trout fed the experimental diets at the end of the trial (day 72).

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Category of fatty acid (%)								
Liver[a]								
ΣSFA[b]	34.8±2.61	25.1±0.42	29.3±1.26	27.6±2.25	28.3±4.33	29.1±2.35	26.8±1.85	30.2±1.44
ΣMUFA[c]	35.1±2.04b	45.2±1.19ab	43.6±2.17ab	45.4±2.85ab	45.8±1.04a	44.0±2.41ab	46.0±1.03a	39.9±1.75ab
ΣPUFA[d]	30.1±1.18	29.7±1.11	27.1±2.07	27.0±1.72	30.0±1.19	26.8±0.41	27.2±2.13	29.8±1.67
Σn-6 PUFA[d]	4.6±0.32b	9.9±0.10b	10.0±0.31b	9.7±0.62b	10.8±1.22a	9.7±0.68b	10.0±0.52b	9.7±0.57b
Σn-3 PUFA[d]	25.6±1.14a	19.8±1.01ab	17.1±1.81b	17.3±2.00b	19.2±0.04ab	17.2±0.96b	17.2±1.63b	20.1±1.40 ab
n-3:n-6	5.7±0.46a	2.0±0.08b	1.7±0.15b	1.8±0.27b	1.8±0.21b	1.8±0.22b	1.7±0.28b	2.1±0.17b
Fillet[a]								
ΣSFA[b]	29.5±0.20a	20.0±0.15d	20.7±0.19d	25.2±0.31b	19.9±0.24d	19.9±0.43d	23.8±0.49bc	22.4±0.27c
ΣMUFA[c]	31.1±0.55d	45.1±0.31ab	45.4±0.18a	42.8±0.26c	45.2±0.53a	45.4±0.44a	43.4±0.18bc	44.4±0.15abc
ΣPUFA[d]	37.8±0.48a	34.4±0.11b	33.4±0.32bc	31.3±0.46d	34.3±0.63b	34.2±0.17b	32.1±0.33cd	32.5±0.33bcd
Σn-6 PUFA[d]	8.4±0.19c	16.1±0.08b	17.1±0.13ab	16.1±0.30b	16.9±0.33ab	17.3±0.23a	16.4±0.26ab	16.9±0.10a
Σn-3 PUFA[d]	29.5±0.52a	18.3±0.19b	16.3±0.25cde	15.2±0.19e	17.4±0.32bc	16.9±0.28bcd	15.8±0.08de	15.6±0.32de
n-3:n-6	3.5±0.12a	1.1±0.02b	1.0±0.01bc	0.9±0.01bc	1.0±0.01bc	1.0±0.03bc	1.0±0.01bc	0.9±0.02c
Abdominal fat[a]								
ΣSFA[b]	27.4±0.38a	18.2±0.11d	18.5±0.31cd	22.4±0.33b	17.5±0.34d	17.8±0.32d	22.0±0.42b	19.7±0.04c
ΣMUFA[c]	33.8±0.37b	48.3±0.22a	48.6±0.52a	47.2±0.19a	48.6±0.25a	48.7±0.40a	47.6±0.42a	48.3±0.32a
ΣPUFA[d]	38.8±0.17a	33.5±0.23b	32.9±0.73b	29.9±0.42d	33.8±0.30b	33.4±0.31b	30.4±0.23cd	32.0±0.31bc
Σn-6 PUFA[d]	10.9±0.33d	17.7±0.13bc	19.0±0.45ab	17.5±0.3c	18.9±0.33abc	19.2±0.34a	17.8±0.07abc	18.6±0.18abc
Σn-3 PUFA[d]	27.9±0.42a	15.8±0.14b	13.9±0.29cd	12.4±0.11f	14.9±0.03bc	14.2±0.03cd	12.6±0.19ef	13.4±0.14de
n-3:n-6	2.6±0.11a	0.9±0.01b	0.7±0.01b	0.7±0.01b	0.8±0.02b	0.7±0.01b	0.7±0.01b	0.7±0.01b

Experimental diets nomenclature as in Table 7.1.

Values are means±SEM of triplicate pooled liver samples from five fish. Values in the same row with different letters are significantly different ($P<0.05$) according to ANOVA.

[a]A complete fatty acid composition of tissues can be found in Supplementary Tables (Tables 2, 3 and 4 for liver, fillet and abdominal fat, respectively).

[b]SFA: C14:0, C16:0, C18:0 and other SFA of small quantity.

[c]MUFA: C16:1n-7, C18:1n-9, C18:1n-7, C20:1 and other MUFA of small quantity.

[d]PUFA: C18:2n-6, C18:3n-3, C20:4n-6, C20:5n-3 (EPA), C22:6n-3 (DHA) and other PUFA of small quantity. n-6 PUFA: C18:2n-6, C20:4n-6 and other n-6 PUFA of small quantity. n-3 PUFA: C18:3n-3, C20:5n-3 (EPA), C22:6n-3 (DHA) and other n-3 PUFA of small quantity.

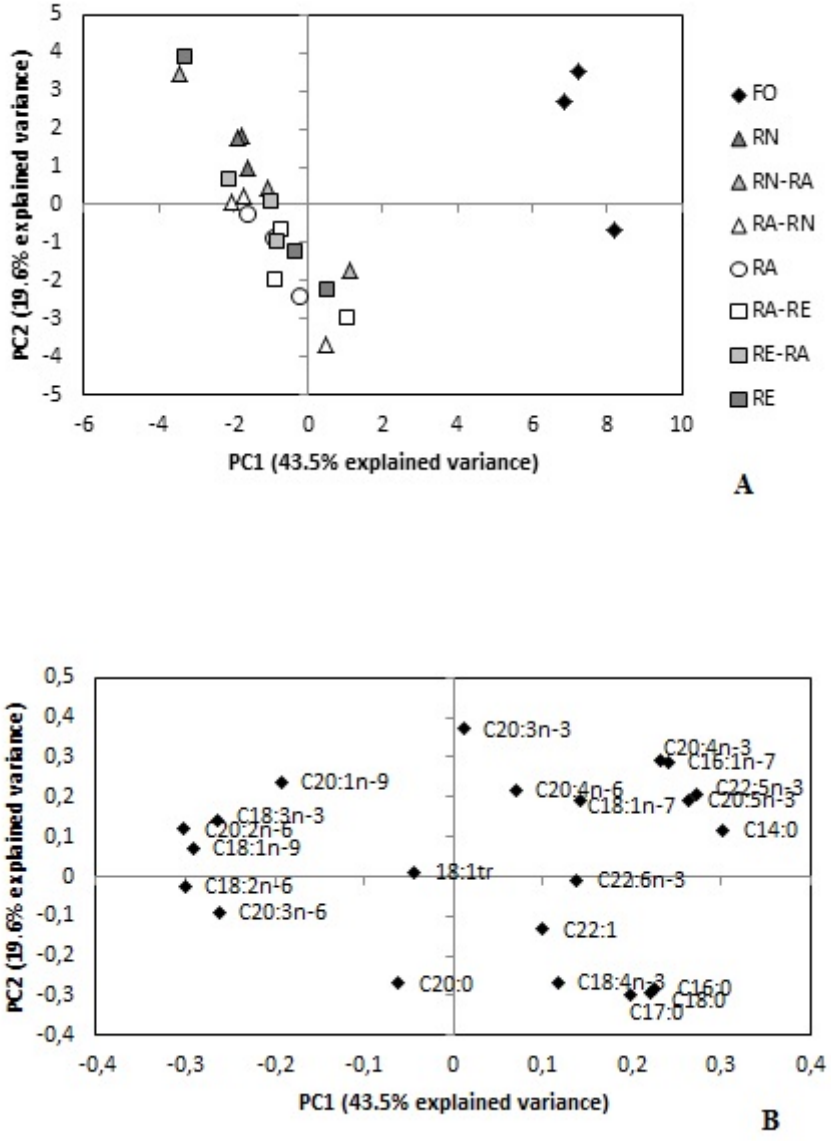

Figure 7.1. PCA on the fatty acid composition of livers from rainbow trout fed the experimental diets (n=21) (A) Score plot and (B) loadings plot. Diets nomenclature: RN, rapeseed native oil; RE, rapeseed re-esterified oil; RA, rapeseed acid oil; RE/RA, 66% rapeseed re-esterified oil: 33% rapeseed acid oil; RA/RE, 66% rapeseed acid oil: 33% rapeseed re-esterified oil; RN/RA, 66% rapeseed native oil: 33% rapeseed acid oil and RA/RN, 66% rapeseed acid oil: 33% rapeseed native oil.

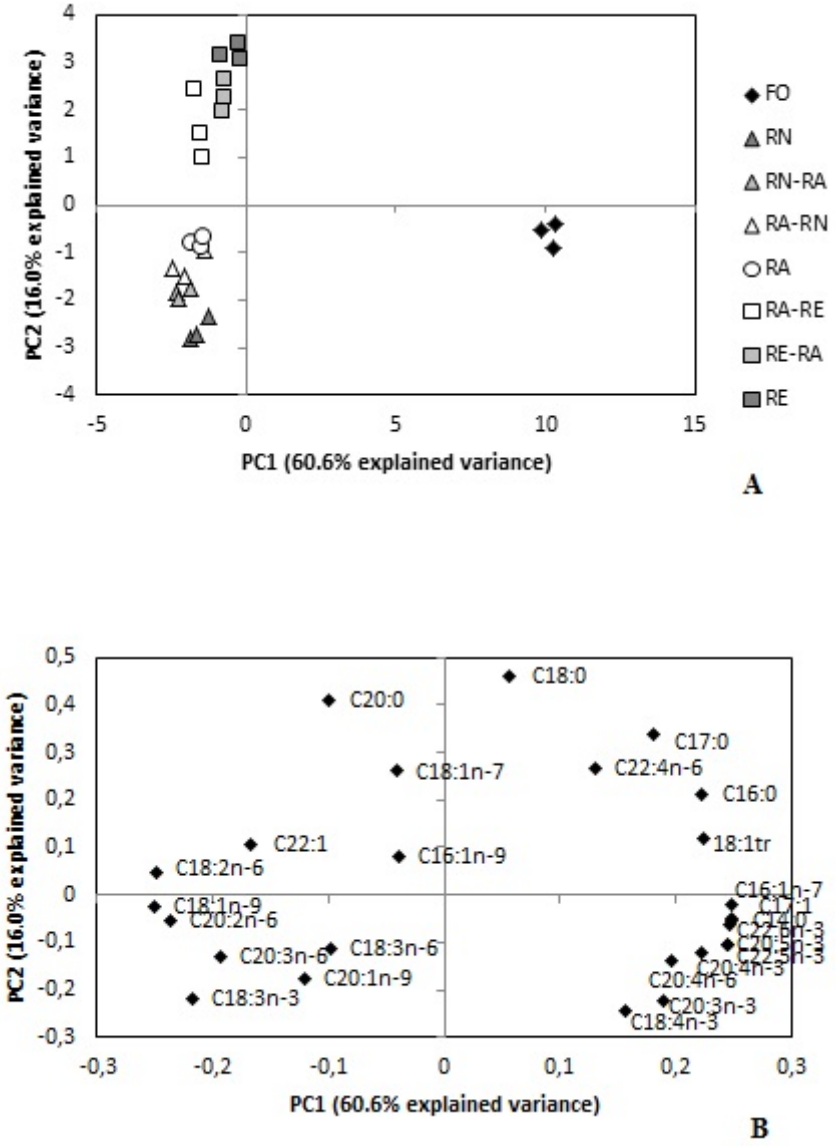

Figure 7.2. PCA on the fatty acid composition of fillets from rainbow trout fed the experimental rapeseed diets (n=24) (A) Score plot (B) and loadings plot. Diets nomenclature: F, fish oil (control diet); RN, rapeseed native oil; RE, rapeseed re-esterified oil; RA, rapeseed acid oil; RE/RA, 66% rapeseed re-esterified oil - 33% rapeseed acid oil; RA/RE, 66% rapeseed acid oil - 33% rapeseed re-esterified oil; RN/RA, 66% rapeseed native oil - 33% rapeseed acid oil and RA/RN, 66% rapeseed acid oil - 33% rapeseed native oil.

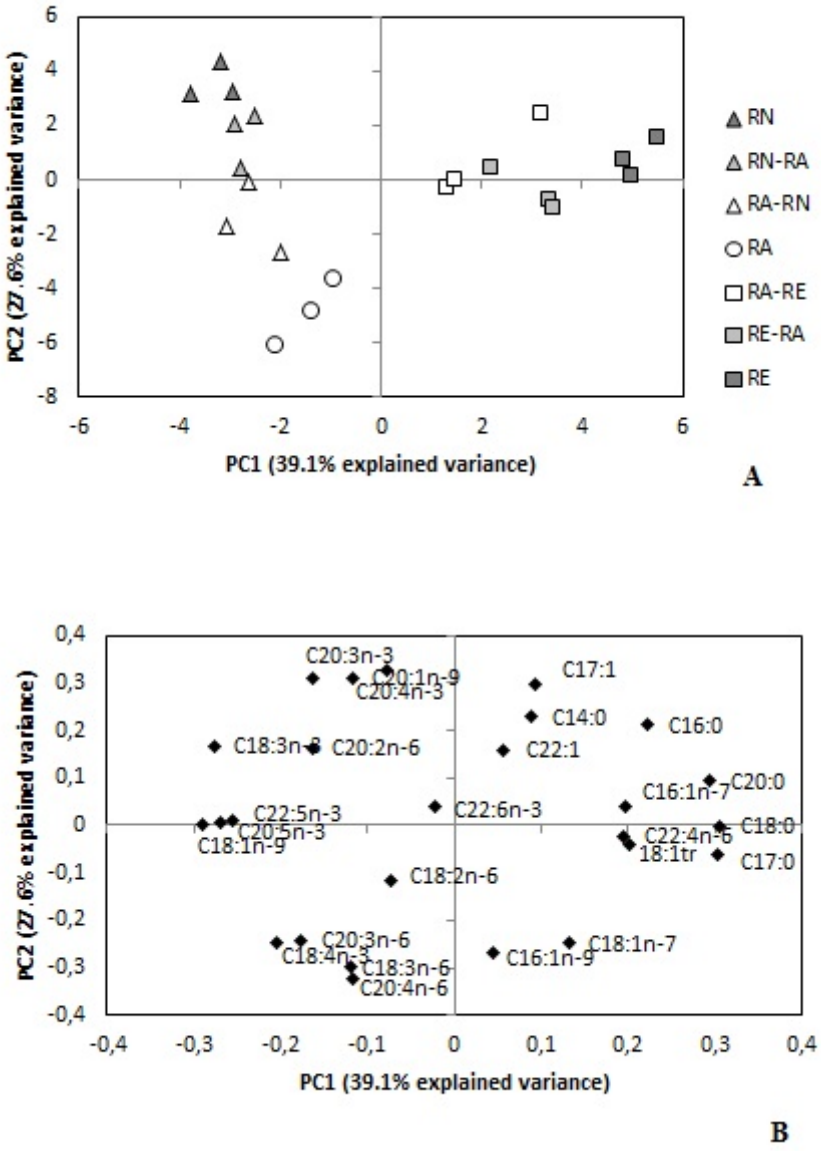

Figure 7.3. PCA on the fatty acid composition of fillets from rainbow trout fed the experimental rapeseed diets (n=21) (A) Score plot (B) and loadings plot. Diets nomenclature: RN, rapeseed native oil; RE, rapeseed re-esterified oil; RA, rapeseed acid oil; RE/RA, 66% rapeseed re-esterified oil - 33% rapeseed acid oil; RA/RE, 66% rapeseed acid oil - 33% rapeseed re-esterified oil; RN/RA, 66% rapeseed native oil - 33% rapeseed acid oil and RA/RN, 66% rapeseed acid oil - 33% rapeseed native oil.

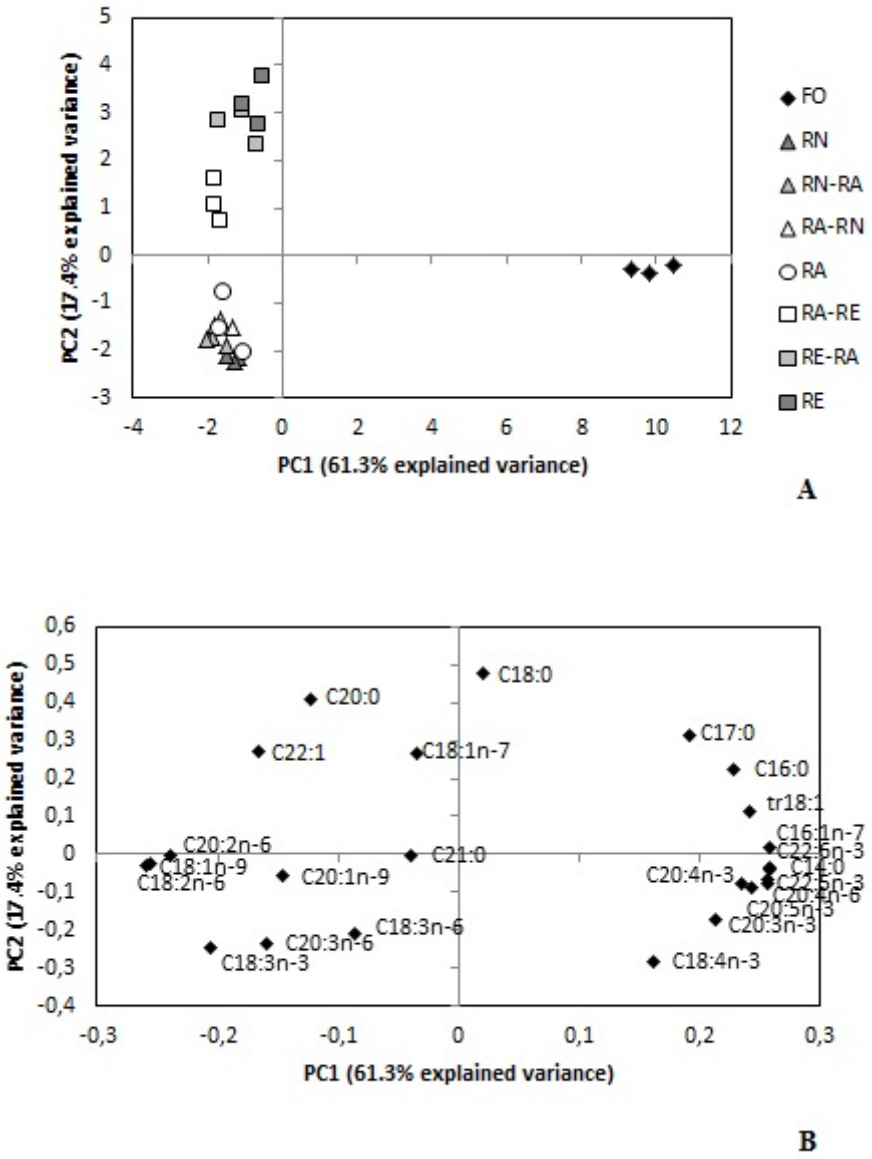

Figure 7.4. PCA on the fatty acid composition of adipose tissue from rainbow trout fed the experimental rapeseed diets (n=21) (A) Score plot (B) and loadings plot. Diets nomenclature: RN, rapeseed native oil; RE, rapeseed re-esterified oil; RA, rapeseed acid oil; RE/RA, 66% rapeseed re-esterified oil - 33% rapeseed acid oil; RA/RE, 66% rapeseed acid oil - 33% rapeseed re-esterified oil; RN/RA, 66% rapeseed native oil - 33% rapeseed acid oil and RA/RN, 66% rapeseed acid oil - 33% rapeseed native oil.

Sn-2 fatty acid composition of fillets, livers and abdominal fat

The fraction of each fatty acid located at the sn-2 position was calculated for fillet, liver and abdominal fat (Table 7.6.). Then, this value was used to multiply the corresponding values in total fatty acid composition to break it down by TAG positions. Values corresponding to the sn-2 are those reported between brackets in Table 7.6.

No significant differences in the sn-2 fatty acid composition of the livers were obtained among fish fed the experimental diets (Table 7.6.).

Regarding fillets, C16:0 had a higher ($p<0.05$) presence at sn-2 position in fish fed the control diet than in the rest, which implied that a 3.5% of all fatty acids corresponded to C16:0 esterified to sn-2 position (Table 7.6.). Among rapeseed diets, no significant differences ($p>0.05$) in its proportion were observed. Differently, for C18:0 in sn-2, the fillets of fish fed RE had a higher ($p<0.05$) value than those of fish fed RN and RN/RA. Also for C18:1n-9, the fillets of animals fed diets RE, RA/RN, RE/RA and RA/RE showed higher fraction of this fatty acid at the sn-2 ($p<0.05$) than those of fish fed RN and F. Regarding EPA and DHA in sn-2, only differences in EPA in the fillets were found among diets. Fish fed diets with higher contents of REO had higher EPA fractions located in sn-2 in their flesh than those fed RN, RA and RN/RA. In spite of all this, differences in the presence of C18:0, C18:1n-9 and EPA in sn-2 in fish flesh were only observed for the fraction of these fatty acids at the sn-2. Instead, when considering to which EPA amount in the total fatty acid composition corresponded this EPA fraction, differences were downsized. .

EPA was the only fatty acid showing differences in sn-2 in abdominal fat. Similarly to fillet, EPA fraction at sn-2 of abdominal fat was higher in fish fed RE/RA ($p<0.05$) than in those of animals fed RN, RN/RA, RA/RN and F. In addition, presence of EPA in sn-2 of TAG of the abdominal fat in animals fed RE was significantly higher ($p<0.05$) than in those fish fed RN and RA/RN. But also, when considering to which EPA amount corresponded to this EPA fraction in the total fatty acid composition, abdominal fat of fish fed F showed the highest value since its total EPA amount was the highest.

Furthermore, both in fillet and abdominal fat, samples showing lower total EPA amounts than the rest (Table 7.5. and Supplementary table 7.3.) led to higher fractions of EPA located at the sn-2, indicating that there was a preference for EPA to be located at the sn-2 position of these tissues.

Table 7.6. Fatty acid composition of the sn-2 position of the different tissues (liver, fillet and abdominal fat).

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Liver								
Fatty acid (%)								
C16:0	30.6±2.46 (6.4)	15.3±2.41 (2.4)	25.5±1.50 (4.6)	17.4±3.41 (2.9)	22.7±4.8 (4.1)	26.4±2.9 (4.8)	21.8±1.02 (3.5)	20.6±0.93 (3.9)
C18:0	26.3±1.74 (2.7)	18.7±3.67 (1.4)	33.9±6.53 (3.1)	23.4±7.66 (2.0)	24.1±4.7 (2.0)	34.0±1.8 (3.0)	26.3±2.24 (2.2)	26.3±2.46 (2.4)
C18:1n-9	42.0±1.71 (8.4)	52.0±1.02 (16.6)	34.3±3.82 (10.6)	50.1±8.00 (16.2)	43.0±4.3 (14.4)	38.7±3.4 (12.3)	44.1±4.13 (14.4)	42.9±0.49 (12.1)
C18:2n-6	32.1±3.45 (1.0)	27.0±1.16 (1.9)	28.1±6.46 (2.0)	30.4±3.34 (2.1)	29.0±4.6 (1.9)	22.2±2.9 (1.5)	36.0±8.65 (2.6)	29.6±3.05 (2.1)
C18:3n-3	42.7±9.52 (0.3)	27.7±3.30 (0.4)	27.1±15.31 (0.3)	33.2±6.94 (0.4)	31.1±5.6 (0.4)	37.9±8.6 (0.5)	33.9±2.36 (0.4)	32.9±3.33 (0.4)
C20:5n-3 (EPA)	11.0±1.44 (0.5)	9.3±2.00 (0.2)	20.6±2.13 (0.4)	10.8±2.80 (0.2)	19.1±5.8 (0.3)	11.7±1.4 (0.2)	8.6±2.58 (0.1)	13.0±0.45 (0.2)
C22:6n-3 (DHA)	12.9±1.99 (2.0)	10.6±0.33 (1.5)	19.7±3.04 (2.7)	11.2±3.59 (1.3)	16.2±4.2 (2.1)	12.8±2.1 (1.5)	13.5±3.67 (1.6)	12.3±0.35 (1.8)
Fillet								
Fatty acid (%)								
C16:0	18.1±1.69a (3.5)	12.6±0.20b (1.7)	12.3±0.47b (1.7)	11.6±0.40b (1.9)	11.6±0.59b (1.6)	12.8±0.77b (1.7)	10.8±0.06b (1.7)	11.3±0.38b (1.7)
C18:0	4.0±0.40a (0.2)	3.3±0.05c (0.1)	5.9±0.88abc (0.2)	6.4±0.66a (0.3)	3.5±0.14bc (0.1)	5.1±0.34abc (0.2)	5.6±0.06ab (0.3)	4.7±0.30abc (0.2)
C18:1n-9	34.0±1.72b (5.9)	36.3±0.78b (13.0)	37.2±0.69ab (13.1)	39.3±0.89a (12.8)	37.9±1.05ab (13.4)	38.5±0.22a (13.7)	40.5±0.18a (13.6)	38.5±0.58a (13.2)
C18:2n-6	48.8±1.31a (3.5)	45.8±0.59a (6.4)	43.9±0.50a (6.4)	48.5±1.14a (6.9)	44.6±1.88a (6.5)	39.0±0.56b (5.8)	44.7±0.21a (6.5)	45.7±1.29a (6.8)
C18:3n-3	56.5±2.85ab (0.8)	65.3±2.86a (2.9)	66.2±5.28a (2.2)	58.0±4.68ab (1.6)	60.0±4.18ab (2.5)	47.7±1.45b (1.8)	50.8±0.45ab (1.6)	60.0±3.36ab (2.0)
C20:5n-3 (EPA)	38.5±0.62ab (2.8)	29.9±0.81c (0.7)	35.5±2.21bc (0.7)	44.2±2.78a (0.8)	34.8±1.99bc (0.8)	42.9±1.60ab (0.9)	44.9±1.02a (0.8)	37.9.0±1.21abc (0.7)
C22:6n-3 (DHA)	50.4±1.45 (7.2)	43.6±2.28 (3.6)	48.7±3.11 (3.8)	47.7±4.05 (3.8)	48.3±3.72 (3.8)	58.6±1.01 (4.6)	51.7±1.21 (4.2)	50.6±3.50 (3.9)
Abdominal fat								
Fatty acid (%)								
C16:0	19.9±1.80 (3.5)	14.1±0.41 (1.7)	14.2±1.73 (1.7)	13.0±0.97 (1.9)	18.5±4.86 (2.1)	18.8±1.24 (2.2)	11.6±1.22 (1.6)	13.5±0.78 (1.7)
C18:0	8.4±1.40 (0.3)	6.0±0.36 (0.2)	11.4±6.14 (0.4)	7.8±2.29 (0.4)	10.2±4.38 (0.3)	16.4±3.86 (0.5)	6.2±0.08 (0.3)	7.7±0.35 (0.3)
C18:1n-9	36.5±2.65 (7.0)	35.6±0.12 (13.6)	34.9±0.06 (13.3)	36.8±0.55 (13.3)	36.0±0.54 (13.9)	36.4±0.76 (14.0)	35.9±1.78 (13.2)	36.5±0.58 (13.8)
C18:2n-6	45.4±2.14 (4.2)	45.5±0.23 (7.2)	43.9±1.32 (7.2)	43.2±0.27 (6.8)	37.3±5.24 (6.3)	40.0±1.29 (6.8)	47.2±1.82 (7.5)	44.7±0.48 (7.4)
C18:3n-3	43.8±0.46 (0.9)	52.4±0.73 (2.5)	44.1±7.77 (1.6)	49.1±9.63 (1.5)	55.4±4.37 (2.6)	41.7±1.00 (1.8)	50.0±5.21 (1.7)	51.7±0.68 (1.9)
C20:5n-3 (EPA)	32.0±3.98bc (2.3)	29.0±1.15c (0.6)	38.9±3.01abc (0.7)	44.4±3.01ab (0.6)	33.2±2.00bc (0.6)	29.4±3.87c (0.5)	49.4±2.82a (0.7)	40.5±1.84abc (0.6)
C22:6n-3 (DHA)	57.1±12.84 (6.8)	52.2±1.09 (2.8)	71.5±11.0 (3.6)	68.3±0.10 (3.4)	64.5±4.70 (3.2)	54.3±9.41 (2.6)	71.2±2.92 (3.6)	64.5±3.76 (3.2)

Experimental diets nomenclature as in Table 7.1.

Values are means±SEM of triplicate pooled fillet samples from five fish. Values in the same row with different letters are significantly different ($P<0.05$), according to ANOVA. Values are given as fraction of each fatty acid located at the sn-2 position of acylglycerols (in %) in the oil. Values in brackets correspond to fatty acid composition of the experimental oils breaking down only sn-2 fatty acids.

7.3. Discussion

Characterization of experimental oils and diets

For the lipid classes, both native and acid oils showed the standard composition described for these types of oils. RNO was constituted mainly of TAG and FFA accounted for more than 60% in the acid oil (RAO) (Nuchi et al., 2009). The re-esterified oil (REO) was richer in partial acylglycerols as obtained in previous studies in monogastric animals including re-esterified VO in diets (Trullàs et al., 2015; Vilarrasa et al., 2014, 2015). When the lipid class composition of diets was compared to that of their corresponding oils, differences were observed in diets RA and RE and were mostly caused by the inclusion of the 5% of FO (source of TAG) in the rapeseed diets.

As described in previous studies (Trullàs et al., 2015; Vilarrasa et al., 2014, 2015), the chemical esterification process did not have an effect on the fatty acid composition of oils, as also previously reported for interesterification reactions (Berry, 2009; Farfán, 2013). In spite of this, a slightly lower proportion of UFA, and thus higher of SFA, was observed in REO in comparison with the rest of rapeseed oils. This could be indicating a slight loss of UFA during the esterification reaction.

In terms of the sn-2 position of the acylglycerols, REO was also richer in sn-2 SFA (2.4%) than the rest of rapeseed oils (1.3-2.2%). This is similar to that observed in our previous study in rainbow trout (Trullàs et al., 2015) and was also possibly due to the fact that this oil had slightly fewer UFA and therefore the proportion of SFA became higher.

Total fatty acid composition of livers

The use of rapeseed oils instead of FO led to lower amounts of C14:0, C18:1n-9 and C18:2n-6 in livers (Table 7.2. from Supplementary Tables). These differences agree with the fact that diet F had around twice the proportion of C14:0 and almost three times those of C18:1n-9 and C18:2n-6 than rapeseed diets.

The type of rapeseed oil used (native, acid or re-esterified, alone or blended) did not result in differences in the hepatic total SFA, MUFA and PUFA, which was possibly caused by the minor differences in the fatty acid composition among rapeseed diets.

Compared to diets, livers of fish fed rapeseed diets had more SFA and slightly fewer MUFA and PUFA, which was mainly due to a loss of C18:2n-6 and C18:3n-3. This is in accordance with studies in Atlantic salmon reporting that C18:1n-9, C18:2n-6 and C18:3n-

3 are readily β-oxidised when present in high concentrations while n-3 PUFA (especially DHA) are retained and/or produced from their precursors C18:2n-6 and C18:3n-3 in the liver (Bell et al., 2003; Torstensen et al., 2004).

Total fatty acid composition of fillets

Similarly to what was found in the liver, the use of rapeseed oils instead of FO affected fillet fatty acid composition, leading to fillets richer in MUFA and n-6 PUFA, and with lower SFA and n-3 PUFA. This led to differences in the n-3:n-6 ratios among the fillets of fish fed F and those fed the rapeseed diet that were expected taking into account the higher and the lower amounts of n-3 PUFA (mainly EPA and DHA) and n-6 PUFA, respectively, present in F than in the experimental rapeseed diets. As previously reported in many studies in rainbow trout, the fillet fatty acid compositions mirrored those of the experimental diets (Skall Nielsen et al., 2005; Torstensen et al., 2004; Turchini et al., 2009; Rosenlund et al., 2011). The lower presence of MUFA and PUFA and higher of SFA in the fillets of fish fed the control diet than in the rest was probably due to the higher proportion of SFA in diet F compared to the rapeseed diets. In a similar way, in our previous study the predominance of the degree of unsaturation of dietary oils over their positional distribution or their lipid class composition was reported (Trullàs et al., 2015).

More interestingly, our present study revealed that the type of rapeseed oil used (native, acid or re-esterified) affected fillet FA composition. Fillets of fish fed RE were richer in SFA and poorer in MUFA and PUFA than those of animals fed to RN or RA. Actually, when taking into account only the fillets from fish fed the rapeseed diets the main factor affecting the fatty acid composition was the dietary gradation depending on the type of oil (native or re-esterified). The increase in SFA and decrease in PUFA in fillets of fish fed RE was downsized when REO in diet was blended with RA. This agreed with the lower %PUFA and higher %SFA in REO. Blends of RAO and RNO did not lead to major differences in fillet fatty acid composition, being the main cause for this the slight differences in the fatty acid composition among these two groups.

As reported in the introduction section, chemical esterification tends to increase SFA at the sn-2 position, which implies a risk of a higher SFA absorption (Trullàs et al., 2016). This could be translated to higher SFA in the flesh, which would not be nutritionally interesting. However, in this case, the esterification of a mainly unsaturated oil led to a certain reduction of its UFA content, and thus an increase in the %SFA, but not to an increase in

the fraction of SFA located at sn-2 (compared with RNO). Consequently, the fraction of SFA at sn-2 position in fillets was not affected by the use of REO.

As observed, the fillets incorporated slightly more SFA, similar or slightly lower MUFA and similar total n-6, n-3 and PUFA with respect to the diets. As Bell et al. (2003) reported, while dietary fatty acids are closely correlated to the fatty acids in the flesh, specific fatty acids are selectively retained or utilized. For instance, Turchini et al. (2003) reported a higher presence of SFA in the fillets of Murray cod than in diets when the latter had a relatively low proportion of this fatty acid class (Turchini et al., 2003). In the present study, moreover, it is important to highlight that the fillets had fewer EPA but more DHA than diets, as other studies in rainbow trout had previously reported (Bell et al., 2001; Caballero et al., 2002). This indicated both the selective catabolism of EPA over DHA (Torstensen et al., 2004) and the ability of rainbow trout to retain DHA (Stubhaug et al., 2007) and to synthesise it from C18:3n-3, as reported in salmonid species fed VO (Caballero et al., 2002; Torstensen et al., 2004; Tocher, 2010). The minor differences in the fatty acid composition between the diets and the fillets indicated that fatty acids such as oleic (C18:1n-9) or linoleic (C18:2n-6), present in high quantities in rapeseed oils, are not deposited in excess. This is desired from a nutritional point of view because the adequate dietary n-6:n-3 ratios are maintained in the fillets. As reported by Molendi-Coste et al. (2011), the optimal dietary intakes of the n-6:n-3 ratio in humans should be around 1–4:1.

Total fatty acid composition of abdominal fat

Total fatty acid composition of the abdominal fat showed similar results than those observed in fillets. The higher and lower proportions of SFA and MUFA, respectively, described for fillets of fish fed F were also present in the abdominal fat, the possible causes being those already suggested for fillets. As observed, the more REO in the diet the fewer PUFA found in the tissue, this following the trend of their presence in both the total content and the sn-2 position.

The main factor influencing the fatty acid composition in abdominal fat when taking into account only those fillets from fish fed the rapeseed diets was the dietary gradation depending on the type of oil. Again, abdominal fat of fish fed RE presented a higher %SFA than those fed RN or RA, which decreased as RAO was added, the cause for this being the higher SFA amounts in REO. These differences were parallel to increases in PUFA when RAO was blended with REO. For the RAO and RNO blends, no differences were observed

for SFA and PUFA, the cause for this being possibly the slight differences in the fatty acid composition among these two groups. However, increases in n-6 PUFA and decreases in n-3 PUFA were observed when RAO increased with respect to RNO. Thus, since abdominal fat is the primary site for long-term storage of TAG in many fish, most of the observed differences mirroring those encountered in diets.

Sn-2 fatty acid composition of livers, abdominal fat and fillets

The fraction of each fatty acid at sn-2 showed differences between fish fed the different diets in fillet, but not in liver or abdominal fat (except for EPA in abdominal fat). Thus, it seems that in fillets, the fraction of each fatty acid esterified at sn-2 is affected by the dietary fatty acid composition in a greater extent than that in liver. For instance, fillets of fish fed F positioned more C16:0 in sn-2 than those fed rapeseed diets. Within rapeseed diets, although fillets of fish fed RE showed the higher SFA contents, this did not imply a higher fraction of SFA esterified to sn-2. The lack of relevant changes in the SFA located at sn-2 of TAG in tissues were observed among fish fed rapeseed diets was probably caused by the few SFA present in rapeseed, as mentioned before. As Bracco (1994) reported, the role that the positional distribution plays in lipid metabolism and in physiological response is particularly critical for fats and oils containing relatively high amounts of LC-SFA. In the present work, the lack of relevant changes in diets was translated to tissues.

Values of SFA and C18:1n-9 in sn-2 obtained in fillets of fish fed RA deserve special attention. They tended to present higher fractions of these fatty acids (C18:0 and C18:1n-9) esterified to sn-2 compared to RN, although RAO was the richest in FFA and thus yielded the lower 2-MAG during digestion. Therefore, it seemed that the higher amount of dietary FFA, the higher the relocation of SFA and MUFA to sn-2 from diet to tissues. As described for humans (Small, 1991), the reacylation of 2-MAG in order to resynthesize TAG in the enterocytes of fish appears to be the predominant pathway utilized during fat absorption (Oxley et al., 2007). However, in cases of a lower arrival of MAG through the gut, as would happen in the case of acid oils, the resynthesis of TAG would mainly be carried out by means of the utilization of glycerol through the glycerol 3-phophate path. TAG generated by this mechanism does not generally maintain the original sn-2 position as it was in the diet (Small, 1991).

In conclusion, the results of the present study indicate that both the re-esterified and the acid oil from rapeseed could be incorporated, along with 5% of fish oil, as fat sources in diets for rainbow trout without causing relevant effects on tissues total and sn-2 fatty acid composition compared to diets including native oil. As the most economical source, the rapeseed acid oil can therefore be incorporated in diets at the three different levels evaluated without negatively affecting total and sn-2 fatty acid composition. Given that the rapeseed acid oil yielded better growth results when blended with the re-esterified oil (Trullàs et al., 2016), diets RA/RE and RE/RA would be of choice.

The minor differences in the sn-2 fatty acid composition found between diets and tissues suggest a high conservation of the fatty acid located in sn-2 in the acylglycerols of the experimental oils during digestion, absorption and metabolism.

Supplementary Tables

Supplementary Table 7.1. Estimated fatty acid composition of the sn-2 position of the experimental diets.

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
sn-2 (%)								
C16:0	13.9 (2.5)	21.0 (2.1)	12.5 (1.4)	15.2 (2.0)	18.0 (1.9)	15.2 (1.6)	14.2 (1.7)	13.3 (1.5)
C18:0	8.5 (0.3)	24.1 (0.5)	10.6 (0.3)	13.8 (0.6)	19.4 (0.5)	14.9 (0.4)	12.6 (0.5)	11.5 (0.4)
C18:1n-9	11.7 (1.2)	24.5 (8.7)	8.8 (3.1)	13.6 (4.3)	19.1 (6.8)	13.9 (5.0)	11.9 (4.0)	10.3 (3.6)
C18:2n-6	58.1 (3.4)	53.1 (8.8)	22.4 (4.1)	28.7 (4.8)	42.6 (7.5)	32.5 (5.8)	26.5 (4.6)	24.4 (4.3)
C18:3n-3								
ΣSFA	12.3 (3.7)	19.2 (3.2)	18.0 (3.1)	13.2 (3.0)	18.6 (3.1)	18.2 (3.0)	14.7 (3.0)	16.3 (3.1)
ΣMUFA	12.4 (3.4)	21.5 (10.2)	10.7 (5.1)	11.1 (4.9)	17.7 (8.4)	14.2 (6.7)	10.9 (5.0)	10.7 (5.0)
ΣPUFA	22.2 (8.8)	35.1 (12.3)	15.6 (5.4)	14.8 (4.8)	28.4 (10.0)	21.9 (7.6)	14.9 (4.9)	15.2 (5.1)

Experimental diets nomenclature as in Table 7.1.
Values are given as the estimated fraction of each fatty acid located at the sn-2 position of acylglycerols (in %) in the diet. Values in brackets correspond to the estimated fatty acid composition of the experimental diet breaking down only sn-2 fatty acids.

Supplementary Table 7.2. Fatty acid composition of livers of rainbow trout fed the experimental diets at the end of the trial (day 72).

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Fatty acid (%)								
C14:0	3.2±0.24$_{a}$	1.4±0.06$_{b}$	1.4±0.04$_{b}$	1.4±0.08$_{b}$	1.4±0.16$_{b}$	1.4±0.07$_{b}$	1.4±0.11$_{b}$	1.4±0.06$_{b}$
C16:0	20.8±1.77	15.5±0.33	18.2±0.92	16.9±1.39	18.1±2.69	18.2±1.75	16.1±1.24	18.9±1.11
C16:1n-7	4.9±0.24$_{a}$	2.7±0.32$_{b}$	2.5±0.10$_{b}$	2.9±0.28$_{b}$	2.7±0.13$_{b}$	2.4±0.22$_{b}$	2.8±0.08$_{b}$	2.2±0.02$_{b}$
C18:0	10.1±0.65	7.7±0.11	9.2±0.47	8.6±0.89	8.2±1.29	8.8±0.50	8.6±0.47	9.3±0.48
C18:1n-9	20.1±1.33$_{b}$	31.9±0.58$_{a}$	30.8±1.58$_{a}$	32.4±3.23$_{a}$	33.5±0.77$_{a}$	31.8±1.95$_{a}$	32.6±0.53$_{a}$	28.2±1.42$_{ab}$
C18:1n-7	4.4±0.31	3.6±0.20	3.9±0.24	3.8±0.12	3.8±0.07	3.7±0.10	4.1±0.21	3.6±0.14
C18:2n-6	3.1±0.20$_{b}$	6.9±0.05$_{a}$	7.0±0.29$_{a}$	7.1±0.52$_{a}$	6.7±1.12$_{a}$	6.9±0.51$_{a}$	7.2±0.44$_{a}$	7.0±0.37$_{a}$
C18:3n-3	0.6±0.05$_{b}$	1.4±0.19$_{ab}$	1.2±0.10$_{ab}$	1.3±0.25$_{ab}$	1.5±0.30$_{a}$	1.3±0.09$_{ab}$	1.2±0.07$_{ab}$	1.2±0.05$_{ab}$
C20:1[a]	2.9±0.21	4.3±0.21	3.4±0.45	3.9±0.62	3.8±0.17	3.5±0.32	4.0±0.30	3.1±0.34
C20:4n-6	0.3±0.02	0.3±0.01	0.2±0.01	0.3±0.01	0.3±0.02	0.3±0.01	0.3±0.01	0.2±0.02
C20:5n-3 (EPA)	4.5±0.27$_{a}$	2.2±0.23$_{b}$	1.9±0.21$_{b}$	1.7±0.13$_{b}$	1.7±0.39$_{b}$	2.0±0.11$_{b}$	1.7±0.17$_{b}$	2.0±0.13$_{b}$
C22:6n-3 (DHA)	15.9±0.69	13.8±0.63	12.1±1.60	12.1±2.22	12.9±0.39	11.6±0.83	12.2±1.36	14.6±1.40

Experimental diets nomenclature as in Table 7.1.

Values are means±SEM of triplicate pooled liver samples from five fish. Values in the same row with different letters are significantly different ($P<0.05$) according to ANOVA.

[a]Sum of isomers.

Supplementary Table 7.3. Fatty acid composition of fillets of rainbow trout fed the experimental diets at the end of the trial (day 72).

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Fatty acid (%)								
C14:0	5.1±0.08^{a}	2.2±0.05^{b}	2.1±0.05^{b}	2.3±0.01^{b}	2.1±0.02^{b}	2.1±0.05^{b}	2.1±0.05^{b}	2.1±0.01^{b}
C16:0	19.3±0.12^{a}	13.6±0.18^{e}	14.1±0.14^{de}	16.5±0.24^{b}	13.6±0.19^{e}	13.5±0.37^{e}	15.7±0.40^{bc}	15.0±0.09^{cd}
C16:1n-7	7.3±0.13^{a}	3.4±0.03^{c}	3.5±0.15^{bc}	3.9±0.08^{b}	3.4±0.08^{c}	3.3±0.01^{c}	3.4±0.06^{c}	3.5±0.04^{c}
C18:0	4.6±0.16^{a}	3.7±0.02^{a}	4.0±0.02^{b}	5.6±0.11^{b}	3.7±0.04^{c}	3.8±0.10^{c}	5.3±0.15^{a}	4.6±0.16^{b}
C18:1n-9	17.4±0.48^{d}	35.5±0.25^{a}	35.2±0.06^{a}	32.5±0.17^{c}	35.4±0.40^{a}	35.6±0.40^{a}	33.7±0.14^{bc}	34.3±0.11^{ab}
C18:1n-7	3.2±0.01^{d}	2.9±0.02^{e}	3.5±0.04^{a}	3.2±0.04^{cd}	3.1±0.03^{d}	3.3±0.04^{bc}	3.4±0.01^{b}	3.5±0.02^{a}
C18:2n-6	7.1±0.18^{b}	14.1±0.07^{a}	14.7±0.10^{a}	14.2±0.27^{a}	14.7±0.33^{a}	15.0±0.22^{a}	14.6±0.23^{a}	14.8±0.08^{a}
C18:3n-3	1.5±0.04^{e}	4.4±0.03^{a}	3.3±0.06^{c}	2.8±0.05^{d}	4.1±0.05^{a}	3.8±0.08^{b}	3.1±0.05^{c}	3.3±0.06^{c}
C20:1[a]	2.1±0.01^{b}	2.5±0.04^{a}	2.1±0.05^{b}	2.3±0.03^{ab}	2.5±0.06^{a}	2.3±0.06^{ab}	2.2±0.04^{b}	2.3±0.08^{ab}
C20:4n-6	0.8±0.02^{a}	0.5±0.01^{c}	0.6±0.05^{b}	0.4±0.02^{c}	0.5±0.03^{bc}	0.5±0.05^{bc}	0.5±0.01^{c}	0.4±0.03^{c}
C20:5n-3 (EPA)	7.3±0.10^{a}	2.3±0.06^{b}	2.1±0.07^{bc}	1.8±0.05^{c}	2.2±0.09^{bc}	2.2±0.03^{bc}	1.9±0.05^{c}	1.8±0.09^{c}
C22:6n-3 (DHA)	14.3±0.47^{a}	8.3±0.21^{b}	7.8±0.20^{b}	7.9±0.10^{b}	7.9±0.13^{b}	7.8±0.35^{b}	8.1±0.18^{b}	7.7±0.16^{b}

Experimental diets nomenclature as in Table 7.1.

Values are means±SEM of triplicate pooled fillet samples from five fish. Values in the same row with different letters are significantly different ($P<0.05$) according to ANOVA.

[a]Sum of isomers.

Supplementary Table 7.4. Fatty acid composition of abdominal fat of rainbow trout fed the experimental diets at the end of the trial (day 72).

	Diets							
	F	RN	RA	RE	RN/RA	RA/RN	RE/RA	RA/RE
Fatty acid (%)								
C14:0	5.2±0.15^{a}	2.4±0.04^{b}	2.2±0.04^{b}	2.2±0.04^{b}	2.2±0.09^{b}	2.2±0.05^{b}	2.2±0.00^{b}	2.2±0.03^{b}
C16:0	17.5±0.29^{a}	11.9±0.12^{c}	12.1±0.32^{c}	14.5±0.24^{b}	11.5±0.24^{c}	11.7±0.24^{c}	14.0±0.32^{b}	12.7±0.06^{c}
C16:1n-7	7.7±0.17^{a}	3.7±0.04^{bc}	3.6±0.22^{bc}	4.1±0.07^{b}	3.6±0.07^{c}	3.5±0.08^{c}	3.6±0.05^{bc}	3.6±0.04^{c}
C18:0	4.1±0.06^{cd}	3.3±0.05^{e}	3.7±0.11^{d}	5.4±0.11^{a}	3.3±0.07^{e}	3.4±0.05^{de}	5.0±0.10^{b}	4.2±0.04^{c}
C18:1n-9	19.1±0.58^{c}	38.2±0.22^{a}	38.2±0.42^{a}	36.2±0.18^{b}	38.5±0.17^{a}	38.5±0.33^{a}	36.9±0.39^{ab}	37.8±0.28^{ab}
C18:1n-7	3.4±0.04^{c}	3.1±0.01^{d}	3.7±0.04^{a}	3.6±0.01^{abc}	3.3±0.04^{c}	3.5±0.05^{bc}	3.6±0.03^{ab}	3.7±0.02^{a}
C18:2n-6	9.2±0.33^{b}	15.9±0.11^{a}	16.4±0.33^{a}	15.8±0.30^{a}	16.9±0.36^{a}	17.0±0.35^{a}	16.0±0.10^{a}	16.7±0.18^{a}
C18:3n-3	2.0±0.08^{f}	4.9±0.03^{a}	3.7±0.03^{cd}	3.1±0.06^{e}	4.7±0.06^{a}	4.2±0.05^{b}	3.4±0.01^{d}	3.8±0.09^{c}
C20:1[a]	2.2±0.02^{d}	2.6±0.02^{a}	2.3±0.02^{cd}	2.4±0.03^{bc}	2.5±0.05^{ab}	2.4±0.03^{bc}	2.4±0.03^{bc}	2.4±0.00^{bc}
C20:4n-6	0.7±0.02^{a}	0.3±0.0^{c}	0.4±0.03^{b}	0.3±0.01^{c}	0.3±0.01^{c}	0.3±0.01^{c}	0.3±0.01^{c}	0.3±0.00^{c}
C20:5n-3 (EPA)	7.3±0.16^{a}	2.0±0.04^{b}	1.8±0.08^{bc}	1.5±0.03^{c}	1.8±0.05^{bc}	1.7±0.02^{bc}	1.5±0.07^{c}	1.6±0.01^{c}
C22:6n-3 (DHA)	11.8±0.23^{a}	5.4±0.09^{b}	5.1±0.07^{b}	5.0±0.11^{b}	5.0±0.06^{b}	4.9±0.11^{b}	5.0±0.06^{b}	5.0±0.01^{b}

Experimental diets nomenclature as in Table 7.1.

Values are means±SEM of triplicate pooled abdominal fat samples from five fish. Values in the same row with different letters are significantly different ($P<0.05$) according to ANOVA.

[a]Sum of isomers.

CHAPTER 8

GENERAL DISCUSSION

Fish oil (FO) replacement by vegetable oils (VO) has been long and widely studied, this research effort being a big step taken towards a more sustainable aquaculture. A few studies, however, have assessed the use of VO by-products as feed ingredients. Despite the considerable load of these commodities generated by the VO refining industry every year, studies including them as potential dietary lipid sources in terrestrial and aquatic animals are scarce. A previous European project (ref. FP6 FOOD-CT-2004-007020) on the quality and safety of fats from by- or co-products from the food chain has evaluated the effects of the dietary inclusion of recycled VO by-products on poultry and rabbit production. Results showed that this inclusion had a direct influence on meat fatty acid composition but no effects on animal performance and health (htttp://www.ub.edu/feedfat/).

Acid oils from the chemical refining of crude VO and have led to controversial results when used as dietary lipid sources in terrestrial monogastric animals. Acids oils can be re-esterified with glycerol, a by-product from the biofuels industry. The characteristics of the final product will depend on the conditions at which the reaction is held, therefore, variable proportions of mono- (MAG) and diacylglycerols (DAG) can be obtained. This process, furthermore, can imply the positioning of more saturated fatty acids (SFA) at the sn-2 position of the acylglycerol molecules when compared to its corresponding native VO. We hypothesized that re-esterification would give acid VO an added value, and that the inclusion of re-esterified VO in diets for fish would exert a beneficial effect on lipid digestibility and absorption.

Given all the above, the objective of the present work was to study the effects of the inclusion of acid and re-esterified VO in fish diets on growth, feed conversion, diet digestibility and quality of the final product. Results obtained indicate that acid and re-esterified oils from unsaturated VO (rapeseed oil) can be included in fish diets as the main lipid source.

Results have been discussed in the different chapters of the present PhD dissertation, therefore, this general discussion will bring them together to highlight the most interesting findings and gain insight into them. The limitations that have emerged along the way and the possible changes to overcome them in a future will be discussed as well, to introduce improvements in this area of research.

8.1. Characterization of the experimental oils and diets

Characterization of fats and oils was the first thing to be carried out because the experimental oils and diets represented an essential part of the present work. Most of the analytical techniques previously implemented for mammals and/or poultry diets had to be firstly validated to make them suitable for oils and diets containing fish products. This represented a considerable time-consuming workload that let to obtain interesting and, in some cases, unexpected results as well.

The content of MAG, DAG and TAG in re-esterified VO was determined by setting the stoichiometric glycerol-to-fatty acid ratio prior to start the reaction. It is important to point out that the proportion of each acylglycerol molecule is very difficult to predetermine. In addition, re-esterified oils contain a blend of MAG and DAG and, consequently, it is not possible to differentiate between their individual effects. Pure MAG or DAG oils can be obtained by distillation (Parini, personal communication).

Partial acylglycerols (MAG and DAG) were expected to be 2- and 1(2), respectively, but in our study we found mainly 1(3)- and 1,3-partial acylglycerols *(Chapters 3 and 4)*. In fact, commercial emulsifiers are mainly constituted of 1(3)-MAG and 1,3-DAG (Krog and Vang Sparsø, 1997; Taguchi et al., 2001; Martin et al., 2014), which seems to be related to the higher stability than sn-1(2) esters. These proportions, in addition, can vary depending on the temperature (Tº) at which the production process is carried out (Krog and Vang Sparsø, 1997). In the present work, therefore, Tº could have probably been a determinant factor in the final proportion of MAG and DAG and in the proportions of 1(3)- and 1(2)-partial acylglycerols. Our results suggest that migration from the central to the external positions of the glycerol molecule did probably occur during esterification.

As we discussed in *Chapter 3*, a proportion of one-third (33%) of each fatty acid was initially expected to be in each position of the TAG molecules of the re-esterified VO (Berry, 2009) because the re-arrangement of fatty acid during the re-esterification reaction takes place in a random manner. It is necessary to produce a TAG oil to obtain a 33% of each fatty acid in sn-2 and to observe an evident “sn-2 fatty acid effect”. Presence of partial acylglcyerols (MAG and DAG), that are mainly 1(3)- and 1,3-, limits the sn-2 positions in the glycerol molecule available for the attachment of fatty acid.

We did not find changes in the fatty acid composition among experimental oils in *trials 1*, *2* and *3*. The re-esterified oils showed, however, a lower percentage of total unsaturated fatty acids (UFA) than the rest of the oils, especially in those from *trial 2,* and this was accompanied by a higher proportion of SFA (%). Losses of UFA during the esterification reaction emerged, possibly caused by the high temperatures reached during the reaction.

Regarding the determination of the sn-2 fatty acid composition of the experimental oils (*Chapters 3, 4* and *7*), the first step was the hydrolysis of the oils by pancreatic lipase from porcine pancreas. Although this is the method of choice, it has to be taken into account that porcine lipase has 1,3-specificity, which has not been described for all fish species. As quite extensively discussed in the present work, even though a 1,3-specific pancreatic lipase has been reported to be the main enzyme in fish this is still controversial, as it seems that there are many exceptions within different species (Kurtovic et al., 2009). Hence, it is important to bear in mind that the described analytical determination of the sn-2 fatty acid composition could be limiting for certain fish species.

8.2. Digestibility

In *trials 1* and *2 (Chapters 3 and 4)*, re-esterified VO were included in the experimental diets as a sole lipid source, to make possible to relate the results obtained to the presence of these oils in the diets.

One of the objectives of the dietary inclusion of re-esterified VO was to evaluate the possible emulsifying effect of partial acylglycerols on fat digestion. This was assessed by comparing the re-esterified oil, the low-in-MAG/DAG oil, the high-in-MAG/DAG oil between them and with the native oil. The emulsifying action of MAG and DAG was not apparent because there were no differences in digestibility between fish fed the native oil diets and those fed the re-esterified oils diets. Similarly, we did not observe any difference between the low and the high in MAG/DAG oils on digestibility. The exception to this was

the superior total fatty acid digestibility of the palm re-esterified oil high in MAG and DAG (74.4%) compared to the native oil in sea bream (61.8%).

In *trial 1*, the high total fatty acid digestibility coefficient obtained for the rapeseed acid oil diet (96.4±0.1%) was unexpected because this oil is rich in FFA (53.4%). This finding made evident the stronger effect that the degree of unsaturation of a fatty acid has on lipid digestibility compared to the lipid class composition. Acid oils available in the market can be of different qualities, which depend mainly on their corresponding original oil and process of production (i.e. chemical vs. physical), their FFA content and the presence of variable amounts of non-desired compounds such as impurities and polymers. Acid VO used in the present work were of high quality because of their low content of moisture, impurities and unsaponifiable matter.

The potential use of acid VO in animal diets, along with the lack of data about their composition and quality standards, has set the starting point for the next step in this area of research. In fact, there is a need to characterize acid oils from different origins and to assess the impact of their use on the nutritive value, productive parameters and final product quality in monogastric animal nutrition.

An improvement in digestibility of the acid VO diet when re-esterified VO diet was only present in fish fed the re-esterified palm oil high in MAG and DAG in sea bream (*Chapter 4*). Given that palm is a saturated source this indicated that free SFA have a more compromised digestibility than free UFA. Differently, rapeseed diets (unsaturated source) had good results in digestibility regardless of the dietary type of oil (crude, acid and re-esterified), suggesting that re-esterification would be advantageous only in the case of saturated sources.

We have observed and reported in the present discussion, that the degree of unsaturation is the main factor to determine fatty acid digestibility in fish. Differences in fatty acid digestibility between species however, as mentioned in *Chapter 4*, could have been caused by particularities in their digestive physiology. Possible differences in digestive lipase hydrolytic specificity, for instance, could be responsible for the differences in fatty acid digestibility between species.

Another possible factor affecting fatty acid digestibility is the melting point of the oils. Composition and distribution of the TAG type influences the crystallization behaviour of the different solid phases of a fat (deMan, 1982). In our case, the higher diversity of TAG molecules present in the re-esterified than in the native oil might have affected its melting point. Re-esterified oils (Vilarrasa et al., 2014), like interesterified fats (Karabulut et al., 2004), have a more expanded melting range than their corresponding native oils. Re-esterified oils might have a different SFC than native VO at a given temperature (Tº) because the melting point of a fat determines its solid fat content (SFC).

It is difficult to see, from our results, an effect on digestibility either between re-esterified VO (low in MAG/DAG vs. high in MAG/DAG) or compared to native oils. To carry out a trial with fish kept at different Tº would probably maximise differences on lipid digestibility between animals fed the native VO or the re-esterified VO, because re-esterified oils have a more expanded melting range than their corresponding native oils. To use oils from palm would be the source of choice at the time of producing the experimental oils because differences in digestibility among palm oils diets were the highest.

Re-esterified VO have a different positional distribution of fatty acids in the acylglycerols molecules, in addition to the variable proportions of MAG and DAG, compared to native oils. Although this implies the incorporation of a high proportion of SFA at the sn-2 position of the acylglycerols (especially in saturated sources such as palm), we did not obtain differences in fatty acid digestibility between re-esterified and native VO diets. As discussed in *Chapter 3*, the re-esterified VO studied in the present work had a lower proportion of SFA in sn-2 (12.5-29.8%) than the expected (33%) because of their richness in MAG and DAG. It is important to realize that it was not possible to differentiate between the effects of the potential emulsifying effect of MAG/DAG on digestion and that of the higher proportion of SFA in sn-2 in position of re-esterified VO compared to native VO. Results can only be regarded, therefore, as a product of the combination of two factors: presence of MAG and DAG and proportion of SFA in sn-2, albeit they have been discussed separately for an easier and clearer understanding.

As reported, in *trials 1* and *2*, rapeseed acid and re-esterified VO had obtained very good results in digestibility and this was the main reason for choosing rapeseed as the main source of the experimental oils in *trial 3*.

In *trial 3* (*Chapters 5, 6* and *7)*, the use of acid and re-esterified rapeseed oils, as a sole lipid source or in blends, was assessed. All the experimental diets were equally digested (*Chapter 5*). Differently as in *trials 1* and *2*, diets tested in *trial 3* contained 5% of FO in addition to the experimental oils. FO was necessary to ensure the minimum dietary input of essential fatty acids, as in this trial not only fat and fatty acid digestibility but also fish growth performance was evaluated. Having this proportion of FO produced a decrease of the content of partial acylglycerols and the high content of SFA in sn-2 than the native VO and, therefore, of their potential effects on digestibility. Its presence implied differences in the lipid class composition (increase of TAG and decrease of MAG/DAG) and the sn-2 fatty acid composition between oils and diets.

8.3. Growth performance

In *trial 3* (*Chapters 5, 6* and *7)*, the effects of the dietary inclusion of acid and re-esterified rapeseed oils, as a sole lipid source or in blends, in a longer term trial including assessment of their effects on fish growth, plasmatic parameters, fillet final quality and composition of tissues represented the next step in the study.

Fish fed the different experimental diets did not present differences in final weight, feed utilization and biometrical parameters among them. In spite of this, fish fed the acid oil diet resulted in a lower final weight (375.9 g) than fish fed the rest of diets (380.7 - 394.6), while they had obtained high fat and fatty acid digestibility values.

A possible cause for the lower final weight of fish fed the acid oil diet could be related to the fact that the proportion of FFA in the acid oil could cause a “saturation effect” at the time of their incorporation into the mixed micelles during digestion, because their amount would surpass that of MAG and DAG, responsible of expanding the micelle in order to allow the solubilisation of other products. The impairment in digestion, therefore, would compromise the growth performance.

A longer experimental period (>72 days; i.e. minimum 90 days) will possibly reveal significant differences in the growth and in the specific growth rate (SGR) and feed conversion ratio (FCR) between fish fed the acid oils and the rest.

8.4. Quality characteristics of fillets

In *trial 3* (*Chapter 6*), the dietary incorporation of rapeseed acid and re-esterified oils did not result in relevant changes in the fillets physico-chemical properties. Colour was the only parameter analysed on fresh and thawed fillets while the rest of determinations were performed only on thawed fillets. Rainbow trout fillets are found in fresh and thawed form in the market. In the present study, however, we were not able to perform the determination of the physico-chemical parameters analysed on fresh fillets due to the trial location conditions and logistic difficulties. To carry out these determinations on fresh fillet would have been of choice, because freezing can influence certain parameters such as texture, liquid holding capacity and/or oxidation. To analyse thawed fillets should not suppose, however, an issue in terms of observing differences among fish fed the different experimental diets.

The experimental diets used in the present work were not supplemented with astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), which is a lipid-soluble carotenoid from dietary origin that gives salmonids flesh the typical red to pink muscle colour (Bjerkeng et al., 1999). Its absorption and deposition can be determined by the amount and the fatty acid profile of dietary lipids and its concentration influences the colour of the fillet (Choubert et al., 2006). Although some authors did not find differences in astaxanthin concentration in fish fed rapeseed oil (Bell et al., 2001) or palm oil (Bell et al., 2002) compared to fish fed FO, it would have been interesting to evaluate the possible effects of the dietary experimental oils on astaxanthin deposition. Because acid oils contain pigments from the chemical refining, as mentioned in *Chapter 6*, an interaction between them and astaxanthin might be observed.

Results of the concentration of α- and β+γ-tocopherols were confusing, as values from diets tended to be in contradiction with those of fillets, especially for α-tocopherol.

For instance, fillets of fish fed the re-esterified oil diet presented the lowest concentration of α-tocopherol, which seemed to be in accordance with the loss of tocopherol that had been described in oils result of interesterification reactions (Park et al., 1983). This was not in correspondence, however, with concentrations of α-tocopherol in the diet. , to determine the concentrations of tocopherol in the experimental oils could provide helpful information because the reason for the results obtained are not clear.

8.5. Tissue fatty acid composition

The fact that all the experimental oils from *trial 2* were from rapeseed did reduce the possible effect of the higher positioning of SFA in sn-2 in the re-esterified oil on the tissues FA composition, because rapeseed is poor in SFA. Fatty acid positional distribution and the partial acylglycerols do not seem to exert an important effect on fat and total fatty acid digestibility because the degree of unsaturation is the main factor determining digestibility, To choose a more saturated source such as palm would be more indicated to observe an effect of acid and re-esterified VO on tissues fatty acid composition.

Lack of relevant changes in the sn-2 fatty acid composition of diets was translated to tissues. To evaluate the possible conservation of the fatty acid located at the sn-2 position of the acylglycerols through the digestion, absorption and metabolism processes was not easy because differences among tissues of fish fed the different were small. We estimated tissue-to-feed ratios to have an idea of the changes in the fatty acid located at the sn-2 position in tissues, and the possible conservation of the fatty acid located at this position, (**Figure 8.1.**). For a fatty acid, a ratio equal to 1 means total conservation of that fatty acid in sn-2 from the diet to the tissue. Values above and below 1 indicate gain and loss, respectively, of that fatty acid in sn-2 from the diet to the tissue. The most remarkable finding was the higher presence of SFA and C18:1n-9 in sn-2 of the fillets of fish fed diets that contained the highest presence of the acid oil than fillets of fish fed the rest of experimental diets. The higher amount of dietary FFA the higher the relocation of SFA and MUFA to sn-2 from diet to tissue. Tissue-to-feed ratios were close to 1 for SFA, especially in the fillet, indicating a high conservation of these fatty acids during the absorption and metabolism processes.

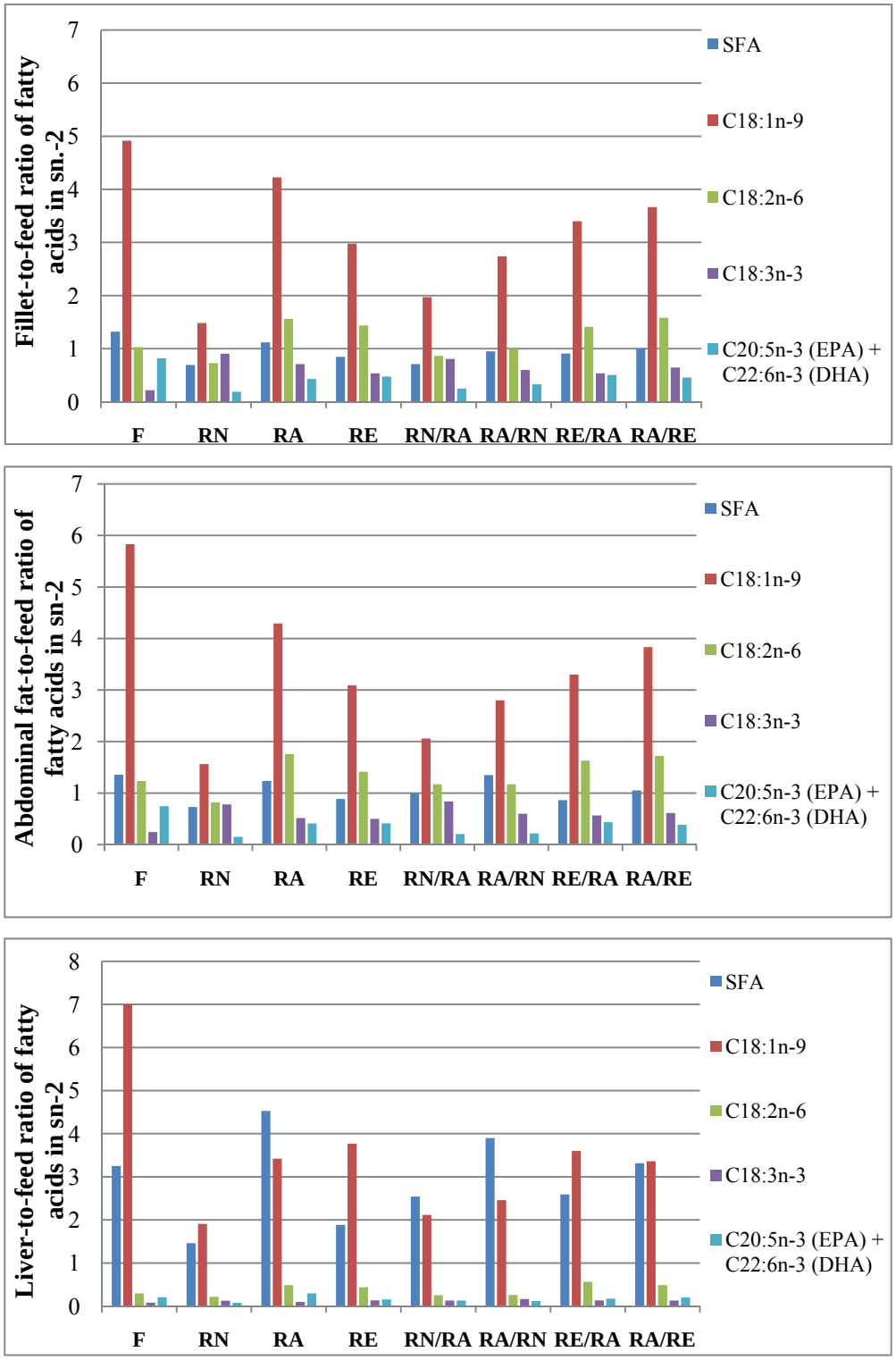

Figure 8.1. Fillet-to-feed ratio (A), abdominal fat -to-feed ratio (B) and liver-to-feed ratio (C) for the content of the two main saturated fatty acids (SFA, C16:0 + C18:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-6), linolenic acid (C18:3n-3) and eicosapentaenoic acid (EPA, C20:5n-3) + docosahexaenoic acid (DHA, C22:6n-3) located at the sn-2 position of acylglycerols by diet. Ratios were calculated with the fatty acid composition of the experimental oils breaking down only sn-2 fatty acids (indicated in brackets in Tables 7.4. and 7.1. from *Chapter 7*).

8.6. Economic considerations

Re-esterified and acid VO evaluated in the present work are adequate lipid sources to replace native VO in aqua feeds, especially in the case of rapeseed oil. To take the decision to use them will largely depend on their price. The acid VO is the most economical of the three (native, re-esterified and acid), although official re-esterified and acid VO current prices are not available.

According to the Malaysian Palm Oil Council (MPOC), current price of crude palm oil (native) is 459 €/t (MPOC, 2015). Regarding esterification, implies a cost of about 100 €/t, added to the price of the acid oil (Parini, personal communication). What decides whether the esterification can be advantageous, therefore, is the price differential between the native and the acid oils. Regarding acid oils we have been able to find only data by Cheah et al. (2010), who reported that before October 2009, the discount exceeded $200/ton between palm oil and palm acid oil. If the price differential is high, therefore, the extra cost of the esterification process can be compensated by the low cost of the acid oil.

CHAPTER 9

CONCLUSIONS

The following conclusions can be drawn from the results presented in this dissertation:

1. Re-esterified palm and rapeseed oils produced by means of a random chemical esterification reaction between an acid oil and glycerol:
    a) Have a higher proportion of partial acylglycerols (mono- and diacylglycerols) than their corresponding native oil. These partial acylglycerols are mainly 1(3)-MAG and 1,3-DAG.
    b) Incorporate a higher proportion of saturated fatty acids in the sn-2 position of their acylglycerol molecules than their corresponding native oil.

2. The random chemical esterification process between an acid vegetable oil and glycerol improves total fatty acid apparent digestibility coefficients of the acid vegetable oil, especially in palm oils.

3. Fatty acid digestibility of the experimental oils is more affected by their degree of unsaturation than by their positional distribution or lipid class composition.

4. In palm:
    a) Diets containing the acid oil have a lower apparent digestibility coefficient of total fatty acid than the native oil diets in rainbow trout and gilthead sea bream.
    b) Diets containing re-esterified oils have higher total fatty acid apparent digestibility coefficients than their corresponding acid oils in rainbow trout and gilthead sea bream.
    c) In rainbow trout, re-esterified oils diets do not present differences in total fatty acid apparent digestibility coefficients compared to the native oil diet.
    d) In gilthead sea bream, the re-esterified oil high in MAG and DAG diet has a higher total fatty acid apparent digestibility coefficient than the native oil diet, while those of the of re-esterified oil low in MAG and DAG and the native oil do not differ.

5. In rapeseed:
    a) Diets containing the acid oil have high total fatty acid apparent digestibility coefficients, especially in rainbow trout (96.4%). However, they have a lower total fatty acid apparent digestibility coefficient of total fatty acid than native oil diets.
    b) Diets containing re-esterified oils have higher total fatty acid apparent digestibility coefficients than their corresponding acid oils in rainbow trout and gilthead sea bream.
    c) In rainbow trout, total fatty acid apparent digestibility coefficients of the experimental diets are all high (96.4-98.1%). However, the re-esterified low in MAG diet has a lower total fatty acid apparent digestibility coefficient than the native oil diet, while no differences are observed between the re-esterified high in MAG and the native oils diets. Given the high digestibility values obtained, the statistically significant difference found is not considered to be of significance to the animals.
    d) In gilthead sea bream, re-esterified oil diets do not present differences in total fatty acid apparent digestibility coefficients compared to the native oil diet.

6. In rainbow trout, the dietary inclusion of rapeseed acid and re-esterified oils, both sole or in blends, and combined with 5% of fish oil:
    a) Does not produce differences in fat and fatty acid apparent digestibility coefficients compared to the native oil diet.
    b) Does not produce differences in performance parameters compared to their corresponding native oil, even though final weights of fish fed rapeseed diets were lower than that of fish fed the fish oil diet. A certain detrimental effect on final weights of fish fed diets with presence of the acid oil was observed.
    c) Does not produce relevant changes in plasma parameters or in the morphology of liver and intestine compared to their corresponding native oil. All the experimental rapeseed diets resulted in lower low

density lipoproteins (LDL)-cholesterol, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) plasma levels than the fish oil diet.

d) Does not produce relevant changes in the fillet quality (colour, texture, total fat, protein, moisture, pH, liquid holding capacity, TBARS and tocopherol concentration) compared to the native oil diet.

e) Does not produce differences in total and sn-2 fatty acid composition of liver, fillet and abdominal fat compared to the native oil diet.

7. Fatty acids located in the sn-2 position of acylglycerols of acid and re-esterified rapeseed oils seems to be conserved from diets to tissues.

CHAPTER 10

REFERENCES

“Quan llegeixes no aprens coses: et converteixes en.
El lector, en acabar una lectura, sap si aquell text l’ha transformat.
Probablement no es pot fer més preguntes: ho sap i prou.”

Jaume Cabré, *La matèria de l’esperit*

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CHAPTER 11

CURRICULUM VITAE OF THE AUTHOR

Surname / Name: Trullàs Huguet, Clara
Address: C/ Nadal Meroles, 17 4B 25008 Lleida (Spain)
Telephone: +34 676294367 (personal) / +34 935811897 (work)
E-mail address: clara.trullas@uab.cat / claratruu@gmail.com
Nationality: Spanish
Date of birth: 12.08.1985
Linkedin: https://es.linkedin.com/pub/clara-trullàs-huguet/b3/172/25a
ResearchGate: https://www.researchgate.net/profile/Clara_Trullas2

CURRENT SITUATION

Sept. 2011 – Present

- **PhD Student in Animal Production –** Universitat Autònoma de Barcelona (UAB). Funded by a Formación de Personal Investigador (FPI) Grant from Ministerio de Economía y Competitividad del Gobierno de España (MINECO)
  - **Project:** Use of re-esterified vegetable oils in fish nutrition

ACADEMIC FORMATION

Sept. 2003 – June 2009

- **Degree in Veterinary Science –** Universitat Autònoma de Barcelona (UAB)

OTHER ACADEMIC FORMATION

March 2013

- **Teaching, Master in Aquaculture: Practical classes –** Universitat de Barcelona (UB).

Febr. 2013

- **Teaching, Aquaculture: Practical classes –** 3rd grade of Veterinary Medicine degree. Universitat Autònoma de Barcelona (UAB)

Sept. 2013

- **Animal Ethics Course: Research or Teaching involving animals,** Deakin University, VIC, Australia

Nov. 2012

- **Fish Nutrition: sustainability and product quality** course - Instituto Agronómico Mediterráneo de Zaragoza **–** Centre International de Hautes Etudes Agronomiques Méditerranéennes (IAMZ -CIHEAM)

Oct. 2012

- **Formation for Laboratory Animals Users and other Scientific Purposes**. *European Qualification* **–** Universitat Autònoma de Barcelona (UAB)

Sept. 2009 – Sept. 2010

- **Master of Science (Aquaculture) –** Universitat de Barcelona (UB), Universitat Autònoma de Barcelona (UAB) and Universitat Politècnica de Catalunya (UPC)

ACADEMIC GRANTS

Sept. 2011 – Present

- **Formación de Personal Investigador (FPI)**, **Predoctoral Grant –** Ministerio de Economía y Competitividad del Gobierno de España (Ref. BES-2011-046806)

Apr. 2013 • **Ayuda para la realización de Estancias Breves del subprograma FPI –** Ministerio de Economía y Competitividad del Gobierno de España. (Ref. EEBB-I-13-07236)

June 2003 – June 2004 • **Honour High School Degree –** Departament d'Ensenyament de la Generalitat de Catalunya

EMPLOYMENT

Nov. 2010 – June 2011 • **Regulatory Affairs and Market Quality Assistant**, Nestlé Purina Petcare España S.A. **–** Castellbisbal, Barcelona, España

CONGRESSES, CONFERENCES and OTHER SCIENTIFIC EVENTS

- **As an assistant**

21-24 Nov. 2011 • **XIIIth Congreso Nacional de Acuicultura (CNA) –** Castelldefels, Barcelona, Spain

- **As an author**

20-24 Oct. 2015 • **Aquaculture Europe (AE)** – Rotterdam, The Netherlands
  • Native, acid and re-esterified rapeseed oils on diets for rainbow trout: fillet quality and tissue composition". *Oral presentation*

13-16 Oct. 2015 • **XVth Congreso Nacional de Acuicultura (CNA) –** Huelva, Spain
  • Aceites ácido y re-esterificado de colza en dietas para trucha arcoíris: efectos sobre la salud y la composición de los tejidos. *Poster*

27-30 Sept. 2015 • **Euro Fed Lipid Congress –** Firenze, Italy
  • Use of rapeseed re-esterified oils in fish nutrition. *Oral presentation*

14-17 Oct. 2014 • **Aquaculture Europe (AE) –** San Sebastián, País Vasco, Spain
  • Potential use of rapeseed re-esterified acid oil and rapeseed acid oil in diets for rainbow trout. *Poster*

25-30 May 2014 • **XVIth International Symposium on Fish Nutrition and Feeding (ISFNF) –** Cairns, QLD, Australia
  • Dietary origin and metabolic fate of omega 3 long-chain polyunsaturated fatty acids (EPA and DHA) in Atlantic salmon. *Poster*

10 Dec. 2013 • **The 3rd Deakin Mini-conference on Omega 3 Fatty acids –** Geelong, VIC, Australia
  • Effects of the use of random esterified acid oils in fish nutrition. *Oral presentation*

14 Oct. 2013 • **SRC Day – Centre for Chemistry and Biotechnology –** Fyansford, Geelong, VIC, Australia
  • Effects of the use of random esterified acid oils in fish nutrition. *Oral presentation*

23-25 Sept. 2013 • **XIVth Congreso Nacional de Acuicultura (CNA) –** Gijón, Asturias, Spain
  • Digestibilidad de aceites ácidos esterificados de origen vegetal con diferente proporción de mono- y diglicéridos en dietas de trucha arcoíris (*Oncorhynchus*

mykiss) y dorada (*Sparus aurata*). *Poster*

4-7 June 2012

- **XVth International Symposium on Fish Nutrition and Feeding (ISFNF) –** Mølde, Norway
  - Effects of the use of esterified acid oils with different saturation degree and different monoglyceride content in rainbow trout (*Oncorhynchus mykiss*). *Poster*

SCIENTIFIC PUBLICATIONS

- **Abstracts and Proceedings**

20-24 Oct. 2015

- **Aquaculture Europe (AE)** – Rotterdam, The Netherlands
  - Trullàs, C., Fontanillas, R., Tres, A., Sala, R. Native, acid and re-esterified rapeseed oils on diets for rainbow trout: fillet quality and tissue composition. *Oral presentation*

13-16 Oct. 2015

- **XVth Congreso Nacional de Acuicultura (CNA) –** Huelva, Spain
  - Trullàs, C., Fontanillas, R., Tres, A., Sala, R. Aceites ácido y re-esterificado de colza en dietas para trucha arcoíris: efectos sobre la salud y la composición de los tejidos. *Poster*

27-30 Sept. 2015

- **Euro Fed Lipid Congress –** Firenze, Italy
  - Trullàs, C., Fontanillas, R., Codony, R., Gómez, A., Sala, R. Use of rapeseed re-esterified oils in fish nutrition. *Oral presentation*

14-17 Oct. 2014

- **Aquaculture Europe (AE) –** San Sebastián, País Vasco, España
  - Trullàs, C., Fontanillas, R., Tres, A., Sala, R. Potential use of rapeseed re-esterified acid oil and rapeseed acid oil in diets for rainbow trout. *Poster*

7-11 June 2014

- **World Aquaculture (WA) –** Adelaide, SA, Australia
  - Norambuena, F., Morais, S., Emery, J.A., Trullàs, C., Strachan, J.F., Turchini, G.M. Dietary ARA/EPA ratio and water temperature in juvenile Atlantic salmon. *Oral presentation*

25-30 May 2014

- **XVIth International Symposium on Fish Nutrition and Feeding (ISFNF) –** Cairns, QLD, Australia
  - Emery, J.A., Norambuena, F., Trullàs, C., Trushenski, J., Turchini, G.M. EPA and DHA supplementation in Atlantic salmon: redifining essential fatty acid requirements. *Oral presentation*

25-30 May 2014

- **XVIth International Symposium on Fish Nutrition and Feeding (ISFNF) –** Cairns, QLD, Australia
  - Trullàs, C., Emery, J., Hermon, K., Norambuena, F., Trushenski, J.,Turchini, G.M. Dietary origin and metabolic fate of omega 3 long-chain polyunsaturated fatty acids (EPA and DHA) in Atlantic salmon. *Poster*

10 Dec. 13

- **The 3rd Deakin Mini-conference on Omega 3 Fatty acids –** Geelong, VIC, Australia
  - Trullàs, C., Fontanillas, R., Sala, R. Effects of the use of random esterified acid oils in fish nutrition. *Oral presentation*

14 Oct. 2013
- **SRC Day – Centre for Chemistry and Biotechnology –** Fyansford, Geelong, VIC, Australia
  • Trullàs, C., Fontanillas, R., Sala, R. Effects of the use of random esterified acid oils in fish nutrition. *Oral presentation*

23-25 Sept. 2013
- **XVIth Congreso Nacional de Acuicultura (CNA) –** Gijón, Asturias, España.
  • Trullàs, C., Fontanillas, R., Sala, R. Digestibilidad de aceites ácidos esterificados de origen vegetal con diferente proporción de mono- y diglicéridos en dietas de trucha arcoíris (*Oncorhynchus mykiss*) y dorada (*Sparus aurata*). *Poster*

19-21 Sept. 2013
- **European Society of Veterinary and Comparative Nutrition (ESVCN) –** Ghent, Bélgica
  • Vilarrasa, E., Trullàs, C., Fragua, V., Barroeta, A.C. Use of palm esterified acid oils in monogastric animal nutrition. *Poster*

4-7 June 2012
- **XVth International Symposium on Fish Nutrition and Feeding (ISNF) –** Mølde, Noruega
  • Trullàs, C., Fontanillas, R., Koppe, W., Sala, R. Effects of the use of esterified acid oils with different saturation degree and different monoglyceride content in rainbow trout (*Oncorhynchus mykiss*). *Poster*

- **Journal papers**

Nov. 2015
- **Fatty acid digestibility in gilthead sea bream fed diets containing native, re-esterified or acid vegetable oils**
  • Trullàs, C., Fontanillas, R., Tres, Sala, R., 2015. Aquaculture Nutrition, *Accepted*.

Sept. 2015
- **Acid and re-esterified rapeseed oils as alternative vegetable oils for rainbow trout diets: Effects on lipid digestibility and growth.**
  • Trullàs, C., Fontanillas, R., Tres, A., Barroeta, A. C., Sala, R., 2016. Aquaculture, 451: 186-194. doi: 10.1016/j.aquaculture.2015.09.021

March 2015
- **Vegetable re-esterified oils in diets for rainbow trout: Effects on fatty acid digestibility.**
  • Trullàs, C., Fontanillas, R., Tres, A., Sala, R., 2015. Aquaculture, 444: 28-35. doi: 10.1016/j.aquaculture.2015.09.021

Jan. 2015
- **Dietary n-6/n-3 LCPUFA ratio, temperature and time interactions on nutrients and fatty acid digestibility in Atlantic salmon.**
  • Trullàs, C., Norambuena, F., Emery, J. A., Hermon, K., Turchini, G., 2015. Aquaculture, 436: 160-166 http://dx.doi.org/10.1016/j.aquaculture.2014.11.011

COLLABORATIONS

Aug. 2013 – March 2014
- **Internship, Visitor PhD student** – Fish Nutrition Group, Deakin University, Warrnambool, VIC, Australia

Sept. 2011–Present	• **Monogastric animal nutrition projects** – Animal Nutrition and Welfare Service (SNiBA), Universitat Autònoma de Barcelona (UAB)

LANGUAGES

Spanish	• Native
Catalan	• Native
English	• Fluent / Basic Professional / Official title - Escola Oficial d'Idiomes (EOI), Lleida, Spain
French	• Basic

OTHER STAYS and TRAININGS

May – June 2009	• **L'Oceanogràfic** (Parques Reunidos) **–** Valencia, Spain
Feb. – Apr. 2009	• **L'Aquàrium de Barcelona** (Aspro) **–** Barcelona, Spain
July – Aug. 2008	• **Servei Central de Suport a la Investigació Experimental (SCSIE) –** Universitat de València, Valencia, Spain

AWARDS

June 2003	• **Regional Award for High School Research Projects –** Delegació Territorial de Lleida, Departament d'Ensenyament, Lleida, Spain
May 2000	• **Special mention, Joan Santamaria Youth Brief Literature Award –** Penya Joan Santamaria and La Busca Edicions, Barcelona, Spain

Barcelona, 21st December 2015

“Arriba un moment que, veient que el periple és infinit, sense estar-ne del tot segur, penso que he de donar per finalitzat el trajecte i que he de considerar que aquest revolt del camí on sóc és el final del viatge”

Jaume Cabré, *La matèria de l’esperit*