Food Chemistry 211 (2016) 17–26

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

A study by ^{1}H NMR on the influence of some factors affecting lipid *in vitro* digestion

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ARTICLE INFO

Article history:
Received 11 December 2015
Received in revised form 11 April 2016
Accepted 4 May 2016
Available online 4 May 2016

Chemical compounds studied in this article:
Ammonium chloride (PubChem CID: 25517)
Glucosamine hydrochloride (PubChem CID: 2830832)
Glucose (PubChem CID: 5793)
Glucuronic acid (PubChem CID: 94715)
Glycerol (PubChem CID: 753)
Monosodium phosphate (PubChem CID: 23672064)
Sodium bicarbonate (PubChem CID: 516892)
Sodium sulfate (Pubchem CID: 24436)
Urea (PubChem CID: 1176)
Uric acid (PubChem CID: 1175)

Keywords:
^{1}H NMR
Lipolysis
In vitro digestion
Fish lipids
Bile salts

ABSTRACT

This article focuses on the impact of several experimental factors, including gastric acidification, intestinal transit time, presence of gastric lipase, sample/digestive fluids ratio, concentration and nature of the enzymes in intestinal juice, and bile concentration, on the extent of *in vitro* lipolysis when using a static model that simulates human digestion processes in mouth, stomach and small intestine. The study was carried out by Proton Nuclear Magnetic Resonance (^{1}H NMR). This technique provides a complete molecular picture of lipolysis, evidencing for the first time, whether preferential hydrolysis of certain glycerides over others occurs. A lipolysis degree similar to that reported *in vivo* was reached by varying certain variables within a physiological range; among them, bile concentration was found to be crucial. The holistic view of this ^{1}H NMR study provides information of paramount importance to design sound *in vitro* digestion models to determine the bioaccessibility and bioavailability of lipophilic compounds.
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1. Introduction

In recent years, *in vitro* digestion has attracted great interest in multiple fields, including food technology and nutrition, and has become a valuable research tool in studying the bioaccessibility and bioavailability of relevant nutrients and toxic compounds (Garrett, Failla, & Sarama, 1999; Goicoechea, Brandon, Blokland, & Guillén, 2011; Roman, Burri, & Singh, 2012; Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005). However, gastrointestinal digestion is a very complex and dynamic process where ingested food components are submitted to mechanical forces and to digestive juices until they are transformed into small bioavailable molecules, some of which can also be metabolized by the gut microbiota. Thus, an accurate reflection of the human physiological environment within the digestive tract in order to mimic naturally occurring events is very difficult and the performance of *in vitro* digestion can be influenced by several experimental factors.

The *in vitro* digestion models proposed in the literature greatly differ in their complexity level, varying from static to dynamic, and from one step procedures to models that simulate sequentially all of the digestive process, that is, those taking place in the mouth, stomach and gut, including colonic fermentation (Kong & Singh, 2010; Li, Hu, & McClements, 2011; Minekus, Marteau, Havenaar, & Huis in 't Veld, 1995; Molly, Woestyne, & Verstraete, 1993; Versantvoort, Van de Kamp, & Rompelberg, 2004). Depending on the research topic and objectives of the study, a wide variety of conditions has been assayed. Therefore, differences can be

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http://dx.doi.org/10.1016/j.foodchem.2016.05.021
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observed between the proportions of samples/digestive fluids, the composition of digestive juices, the transit times performed in each step, or the intensity of the mechanical forces applied (Hur, Lim, Decker, & McClements, 2011). Recently, an attempt to homogenize experimental conditions for *in vitro* digestion was made (Minekus et al., 2014). Nevertheless, the first requirement for all the *in vitro* methodologies should be to mimic *in vivo* macronutrient digestion extent (Golding & Wooster, 2010; Hur, Decker, & McClements, 2009), and for this purpose it is of paramount importance to analyze the influence of the factors affecting it.

Regarding lipid digestion, the human body shows a high efficiency for this process. After the enzymatic hydrolysis that takes place in the stomach and mainly in the small intestine, >95% of dietary triglycerides are absorbed as monoglycerides or fatty acids (Golding & Wooster, 2010). *In vitro* lipolysis levels reported in the literature are usually far lower than those occurring *in vivo*, especially with regard to fish lipids (Martin, Nieto-Fuentes, Señoráns, Reglero, & Soler-Rivas, 2010; Marze, Meynier, & Anton, 2013; Zhu, Ye, Verrier, & Singh, 2013). The high resistance of long chain polyunsaturated acyl groups to *in vitro* hydrolysis by pancreatic lipase could explain the low rates of lipolysis reported for fish oils (Bläckberg, Hernell, Bengtsson, & Olivecrona, 1979). Thus, the improvement of lipolysis under *in vitro* conditions is a challenge that deserves a deeper knowledge of the factors affecting lipase activity. Furthermore, this deeper knowledge of lipid digestion would help in the future to design food products with specific performance during digestion; that is to say, with special properties like targeted delivery.

In this context, the effect of different experimental factors on lipid *in vitro* digestion extent is studied in this paper, in order to find those conditions under which a lipolysis degree similar to that occurred *in vivo* is reached. The starting point method was that described by Versantvoort et al. (2004, 2005). Although initially designed for assessing bioavailability of food mycotoxins, it has since been employed for several purposes, mainly related to lipid research, such as the study of microstructural changes in emulsified lipids (Hur et al., 2009), the fate of toxic compounds resulting from lipid oxidation (Goicoechea et al., 2008, 2011), the influence of the cheese matrix on lipid digestion (Lamothe, Corbeil, Turgeon, & Britten, 2012), the effects of antioxidants on lipid oxidation during digestion (Tarvainen, Phuphusit, Suomela, Kuksis, & Kallio, 2012), the digestion of fish oil emulsions (Marze et al., 2013), and milk macronutrient decomposition (Kopf-Bolanz et al., 2012). Attention was paid to different experimental factors, including gastric acidification, intestinal transit time, presence of gastric lipase, sample/digestive fluids ratio, intestinal enzymes concentration and bile concentration. Their influence on the lipolysis advance was quantified by means of Proton Nuclear Magnetic Resonance (^{1}H NMR). This technique was selected because it was previously successfully employed to quantify triglycerides, diglycerides, monoglycerides, fatty acids and glycerol in lipid mixtures and to evaluate the advance of lipolysis during *in vitro* digestion (Nieva-Echevarría, Goicoechea, Manzanos, & Guillén, 2014, 2015).

2. Materials and methods

2.1. Samples, reagents and enzymes

Farmed European sea bass (*Dicentrarchus labrax*) specimens were purchased from a local supermarket. After cleaning, gutting, filleting and skinning, they were submitted to *in vitro* digestion. The average weight of a fillet was 252.9 ± 22.0 g and their average lipid content was 8.2 ± 1.0% (ww).

Reagents and enzymes for the preparation of digestive juices were acquired from Sigma-Aldrich (St. Louis, MO, USA): *Aspergillus oryzae* α-amylase (10065); pepsin from porcine gastric mucosa (P7125); lipases from *Aspergillus niger* (534781) and *Candida rugosa* (62316); pancreatin from porcine pancreas (P1750); lipase type II crude from porcine pancreas (L3126) and bovine bile extract (B3883).

2.2. In vitro digestion experiments

The starting point for this study was the *in vitro* digestion model developed by Versantvoort et al. (2004, 2005) for the fed state. The composition of digestive juices (saliva, gastric, duodenal and bile) is given in Table 1. Just before the *in vitro* digestion experiments, the juices were heated to 37 ± 2 °C. The fish sample was prepared by mincing in a grinder, to simulate mechanical disintegration that occurs in the mouth. The digestion experiment started with the addition of 6 ml of saliva to 9 g of minced sea bass sample. After 5 min of incubation, 12 ml of simulated gastric juice (GJ) were added and the mixture was rotated head-over-heels at 40 rpm for 2 h at 37 ± 2 °C. Thirty minutes after starting the gastric digestion, pH was set between 2 and 3 with HCl (37%), simulating the gradual acidification of the chyme that occurs *in vivo*. After 2 h of gastric digestion, 2 ml of sodium bicarbonate solution (1 M), 12 ml of duodenal juice (DJ) and 6 ml of bile juice (BJ) were added. Subsequently, pH was set between 6 and 7, and the mixture was rotated again at 40 rpm and incubated at 37 ± 2 °C for 2 h.

The influence of some experimental factors on the fish lipolysis was evaluated. These were: gastric pH, intestinal residence time, presence of lipase in the GJ, sample/digestive fluids ratio, enzymatic composition of the DJ and bile concentration in the BJ. Although each variable can be affected by the others, the influence of each experimental factor on the lipolysis extent was studied sequentially, keeping the rest of the experimental conditions constant but including the selected conditions for the factor previously tested. This selection was made considering the improvement of lipolysis, the reflection of physiological conditions, as well as practical and economical reasons. Each digestion experiment was carried out in triplicate, except for that using a lower amount of

Table 1
Composition of the juices employed in the model described by Versantvoort et al. (2004, 2005) used as a starting point in this study.

Components	Saliva	Gastric Juice (GJ)	Duodenal Juice (DJ)	Bile Juice (BJ)
KCl (mmol/l)	12.02	11.06	7.57	5.05
NaCl (mmol/l)	5.10	47.09	119.98	89.99
$NaHCO_3$ (mmol/l)	20.17	–	40.33	68.86
NaH_2PO_4 (mmol/l)	7.40	0.22	–	–
NH_4Cl (mmol/l)	–	5.72	–	–
KH_2PO_4 (mmol/l)	–	–	0.59	–
Na_2SO_4 (mmol/l)	4.79	–	–	–
KSCN (mmol/l)	2.06	–	–	–
$MgCl_2$ (mmol/l)	–	–	0.53	–
$CaCl_2 \cdot 2H_2O$ (mmol/l)	–	2.72	1.36	1.51
HCl (37%) (ml/l)	–	6.50	0.18	0.15
Urea (mmol/l)	3.33	1.42	1.67	4.16
Glucose (mmol/l)	–	3.61	–	–
Glucuronic acid (mmol/l)	–	0.10	–	–
Uric acid (mmol/l)	0.09	–	–	–
Glucosamine hydrochloride (mmol/l)	–	1.53	–	–
Bovine serum albumin (g/l)	–	1.00	1.00	1.80
Mucin (g/l)	0.025	3.00	–	–
α-amylase (g/l)	0.29	–	–	–
Pepsin (g/l)	–	2.50	–	–
Pancreatin (g/l)	–	–	9.00	–
Pancreatic lipase (g/l)	–	–	1.50	–
Bile (g/l)	–	–	–	30.00
pH	6.8 ± 0.2	1.3 ± 0.2	8.1 ± 0.2	8.2 ± 0.2

sample/digestive fluids ratio that was performed in duplicate (4.5 g of fish meat: 6 ml of saliva: 12 ml of GJ with lipase of *A. niger* added: 12 ml of DJ proposed by Versantvoort: 6 ml of BJ with bile at 30 g/l).

2.3. Lipid extraction and ¹H NMR spectra acquisition

Lipids contained in minced fish and in digested samples were extracted using dichloromethane as solvent (CH_2Cl_2, HPLC grade, Sigma-Aldrich, St. Louis, MO, USA), as in a previous study (Nieva-Echevarría et al., 2015). Afterwards, the ¹H NMR spectra of the lipid extracts were acquired using a Bruker Avance 400 spectrometer operating at 400 MHz. The sample preparation, the acquisition conditions and the study of the spectral data were the same as in previous studies (Guillén & Ruiz, 2004; Nieva-Echevarría et al., 2014, 2015). Each spectrum was recorded in duplicate. Table 2 gives the assignment of the different ¹H NMR signals to the corresponding protons, in agreement with the above-mentioned studies. The ¹H NMR spectra shown in the figures were plotted at a fixed value of absolute intensity to be valid for comparative purposes using the MestreNova program (Mestrelab Research, Santiago de Compostela, Spain).

2.4. Equations derived from ¹H NMR spectral data employed for the quantification of the several lipolytic products in the digestates and the extent of lipid digestion

Bearing in mind that the signal areas in the spectra are proportional to the number of protons that generate them and the proportionality constant is the same for all kinds of protons, the number of moles (**N**) of 2-monoglycerides (**2-MG**), 1-monoglycerides (**1-MG**), 1,2-diglycerides (**1,2-DG**), triglycerides (**TG**), fatty acids (**FA**) and glycerol (**Gol**) in each sample can be expressed as follows (Nieva-Echevarría et al., 2014):

$$N_{2\text{-}MG} = Pc^{*}A_{K}/4 \quad (1)$$

$$N_{1\text{-}MG} = Pc^{*}A_{L} \quad (2)$$

$$N_{1,2\text{-}DG} = \left(Pc^{*}A_{I+J} - 2N_{1\text{-}MG}\right)/2 \quad (3)$$

Table 2
Chemical shift assignments and multiplicities of the ¹H NMR signals in $CDCl_3$ of the main protons of glycerides and fatty acids present in fish lipid samples before and after *in vitro* digestion. The signal letters agree with those given in Fig. 1.

Signal	Chemical shift (ppm)	Multiplicity	Functional group	
			Type of protons	Compound
A	0.88	t	$-C\underline{\mathbf{H_3}}$	Saturated, monounsaturated **ω-9** and/or **ω-7 acyl groups** and **FA**
	0.89	t	$-C\underline{\mathbf{H_3}}$	Unsaturated **ω-6 acyl groups** and **FA**
B	0.97	t	$-C\underline{\mathbf{H_3}}$	Unsaturated **ω-3 acyl groups** and **FA**
C	1.19–1.42	m*	$-(C\underline{\mathbf{H_2}})_n-$	**Acyl groups** and **FA**
D1	1.61	m	$-OCO-CH_2-C\underline{\mathbf{H_2}}-$	**Acyl groups** in **TG**, except for DHA, EPA and ARA acyl groups
	1.62	m	$-OCO-CH_2-C\underline{\mathbf{H_2}}-$	**Acyl groups** in **1,2-DG**, except for DHA, EPA and ARA acyl groups
	1.63	m	$-OCO-CH_2-C\underline{\mathbf{H_2}}-$, $COOH-CH_2-C\underline{\mathbf{H_2}}-$	**Acyl groups** in **1-MG** and **FA**, except for DHA, EPA and ARA acyl groups
	1.64	m	$-OCO-CH_2-C\underline{\mathbf{H_2}}-$	**Acyl groups** in **2-MG**, except for DHA, EPA and ARA acyl groups
D2	1.69	m	$-OCO-CH_2-C\underline{\mathbf{H_2}}-$	EPA and ARA **acyl groups** in **TG**
	1.72	m	$COOH-CH_2-C\underline{\mathbf{H_2}}-$	EPA and ARA **acids**
E	1.92–2.15	m**	$-C\underline{\mathbf{H_2}}-CH{=}CH-$	**Acyl groups** and **FA**, except for $-CH_2-$ of DHA acyl group in β-position in relation to carbonyl group
F1	2.26–2.36	dt	$-OCO-C\underline{\mathbf{H_2}}-$	**Acyl groups** in **TG**, except for DHA acyl groups
	2.33	m	$-OCO-C\underline{\mathbf{H_2}}-$	**Acyl groups** in **1,2-DG**, except for DHA acyl groups
	2.35	t	$-OCO-C\underline{\mathbf{H_2}}-$ $COOH-C\underline{\mathbf{H_2}}-$	**Acyl groups** in **1-MG** and **FA**, except for DHA acyl groups
	2.38	t	$-OCO-C\underline{\mathbf{H_2}}-$	**Acyl groups** in **2-MG**, except for DHA acyl groups
F2	2.37–2.41	m	$-OCO-C\underline{\mathbf{H_2}}-C\underline{\mathbf{H_2}}-$	DHA **acyl groups** in **TG**
	2.39–2.44	m	$COOH-C\underline{\mathbf{H_2}}-C\underline{\mathbf{H_2}}-$	DHA **acid**
G	2.77	t	$=HC-C\underline{\mathbf{H_2}}-CH=$	**Diunsaturated ω-6 acyl groups** and **FA**
H	2.77–2.90	m	$=HC-C\underline{\mathbf{H_2}}-CH=$	**Polyunsaturated ω-6 and ω-3 acyl groups** and **FA**
I	3.65	ddd	$ROCH_2-CHOH-C\underline{\mathbf{H_2}}OH$	Glyceryl group in **1-MG**
J	3.73	m***	$ROCH_2-CH(OR')-C\underline{\mathbf{H_2}}OH$	Glyceryl group in **1,2-DG**
K	3.84	m***	$HOC\underline{\mathbf{H_2}}-CH(OR)-C\underline{\mathbf{H_2}}OH$	Glyceryl group in **2-MG**
L	3.94	m	$ROCH_2-C\underline{\mathbf{H}}OH-CH_2OH$	Glyceryl group in **1-MG**
N	4.18	ddd	$ROC\underline{\mathbf{H_2}}-CHOH-CH_2OH$	Glyceryl group in **1-MG**
O	4.22	dd,dd	$ROC\underline{\mathbf{H_2}}-CH(OR')-C\underline{\mathbf{H_2}}OR''$	Glyceryl group in **TG**
P	4.28	ddd	$ROC\underline{\mathbf{H_2}}-CH(OR')-CH_2OH$	Glyceryl group in **1,2-DG**
Q	4.93	m	$HOCH_2-C\underline{\mathbf{H}}(OR)-CH_2OH$	Glyceryl group in **2-MG**
R	5.08	m	$ROCH_2-C\underline{\mathbf{H}}(OR')-CH_2OH$	Glyceryl group in **1,2-DG**
S	5.27	m	$ROCH_2-C\underline{\mathbf{H}}(OR')-CH_2OR''$	Glyceryl group in **TG**
T	5.28–5.46	m	$-C\underline{\mathbf{H}}{=}C\underline{\mathbf{H}}-$	**Acyl groups** and **FA**

Abbreviations: t: triplet; m: multiplet; TG: triglycerides; DHA: docosahexaenoate; EPA: eicosapentaenoate; ARA: arachidonate; 1-MG: 1-monoglyceride; FA: fatty acid; 1,2-DG: 1,2-diglyceride; 2-MG: 2-monoglyceride; d: doublet.

* Overlapping of multiplets of methylenic protons in the different acyl groups or fatty acids either in β-position, or further, in relation to double bonds, or in γ-position, or further, in relation to the carbonyl group.

** Overlapping of multiplets of the α-methylenic protons in relation to a single double bond of the different unsaturated acyl groups or fatty acids.

*** This signal shows different multiplicity if the spectrum is acquired from the pure compound or taking part in the mixture.

$$N_{TG} = (Pc^{*}A_{N+O+P} - 2N_{1,2\text{-}DG} - 2N_{1\text{-}MG})/4 \quad (4)$$

$$N_{FA} = (Pc^{*}10A_{2.26-2.37} + Pc^{*}5A_{2.37-2.44} - 60N_{TG} - 40N_{1,2\text{-}DG} - 18N_{1\text{-}MG} - 13N_{2\text{-}MG})/20 \quad (5)$$

$$N_{Gol} = (N_{FA} - N_{1,2\text{-}DG} - 2N_{2\text{-}MG} - 2N_{1\text{-}MG})/3 \quad (6)$$

where **Pc** is the proportionality constant relating the number of protons that generate a signal, **A** is the area of the signal involved (see Table 2) and $\mathbf{A_{2.26-2.37}}$ and $\mathbf{A_{2.37-2.44}}$ are the areas of signals at 2.26–2.37 and 2.37–2.44 ppm respectively. These equations were used in order to quantify the several products generated during lipolysis and the extent of lipid digestion in the digestates, following the approaches outlined in the Sections 3.2.1–3.2.3 of the Results and Discussion section.

2.5. Statistical analysis

The significance of the differences on the molar percentages of the different kinds of lipolytic products present in the digestates, and on the hydrolysis level, triglyceride transformation and lipid bioaccessibility were determined by one-way variance analysis (ANOVA) followed by Tukey *b* test at $p < 0.05$, using SPSS v.19 (IBM, NY, USA).

3. Results and discussion

3.1. General

Due to the broad range of applications of *in vitro* digestion methodology, very different models have been proposed to mimic this complex physiological process. The selected model should not only be able to reflect as closely as possible the human physiology in order to generate consistent and realistic results, but should also be relatively simple, cost effective, easily applicable and reproducible. Likewise, the method employed to determine the extent of lipolysis must be sound, and the definition of the latter clear.

The model developed by Versantvoort et al. (2004, 2005) was used as the starting point for development of a new method. To evaluate the changes in the *in vitro* digestion lipolysis as a consequence of the variations in several experimental factors, the determination of the extent of lipolysis firstly in the starting method, and then in all of the subsequent *in vitro* digestion experiments, is required.

3.2. Determination of the extent of lipolysis in the digestates of sea bass meat obtained using Versantvoort conditions

Fig. 1 shows the ^{1}H NMR spectra of the fish lipids before *in vitro* digestion (BD) and after *in vitro* digestion using the Versantvoort conditions (DV). Some regions are sufficiently enlarged to show the most significant differences in the intensity of the signals, whose assignment is given in Table 2. The BD spectrum contains the typical signals (**A**, **B**, **C**, **D1**, **D2**, **E**, **F1**, **F2**, **G**, **H**, **T**) corresponding to the protons of the main acyl groups of fish lipids and to those specific to triglycerides (TG) (**O**, **S**).

Nevertheless, when comparing DV and BD spectra, noticeable differences are observed. Although certain signals (**A**, **B**, **C**, **E**, **G**, **H**, **T**) remain almost unchanged after digestion, those related to the glycerol backbone of TG (**O**, **S**) show lower intensities. Moreover, in the DV spectrum signals related to the protons in the glycerol backbone of newly formed glycerides appear; these are predominantly 1,2-diglycerides (1,2-DG) (**J**, **P**, **R**) and 2-monoglycerides (2-MG) (**K**, **Q**), which is in agreement with lipid hydrolysis that occurs within the human tract and the regiospecificity of the lipases added to the duodenal juice. Although in very

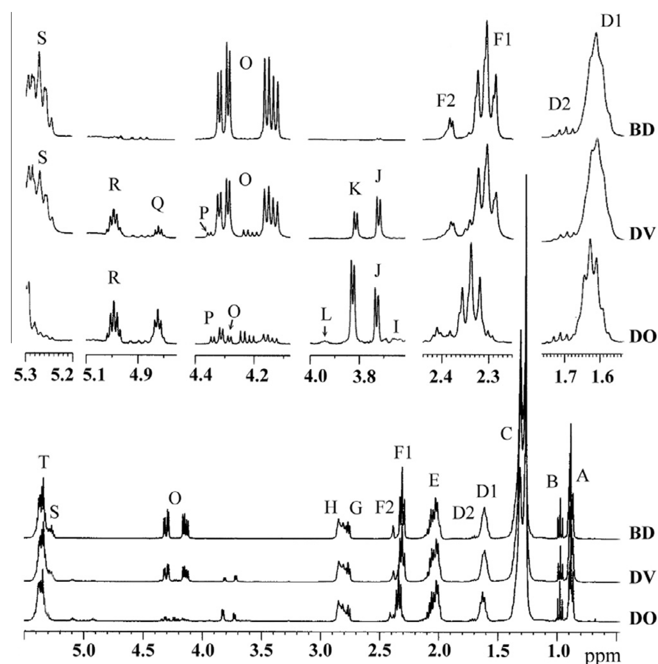

Fig. 1. ^{1}H NMR spectra of lipid extracts of sea bass before (BD) and after *in vitro* digestion following the conditions described by Versantvoort et al. (2004, 2005) (DV) and the ones proposed after studying the influence of certain factors (DO). Some spectral regions have been properly enlarged and the signal letters agree with those in Table 2.

low intensity, signals **I** and **L**, corresponding to 1-monoglycerides (1-MG), are also present in the DV spectrum. The occurrence of 1-MG in the digestates can be explained by the isomerization of 2-MG, as occurs *in vivo* (Mattson & Volpenhein, 1964).

In short, from the simple observation of the BD and DV spectra it can be deduced that the extent of lipolysis reached in the DV sample is rather limited, and that an important amount of TG remains unhydrolyzed.

All of the above, directly observable in the spectra, can also be quantified. Thus, two approaches can be used, either based on the glyceryl structures or on the fatty acids and acyl groups present.

3.2.1. Approach based on the glyceryl structures present in the digestates

Using the equations provided in the Materials and methods Section 2.4, the molar percentages of the different kinds of glycerides in relation to the total number of moles of glyceryl structures ($\mathbf{NT_{GS}}$) can be determined:

$$TG\% = 100N_{TG}/NT_{GS} \quad (7)$$

$$1,2\text{-}DG\% = 100N_{1,2\text{-}DG}/NT_{GS} \quad (8)$$

$$2\text{-}MG\% = 100N_{2\text{-}MG}/NT_{GS} \quad (9)$$

$$1\text{-}MG\% = 100N_{1\text{-}MG}/NT_{GS} \quad (10)$$

$$Gol\% = 100N_{Gol}/NT_{GS} \quad (11)$$

$$NT_{GS} = N_{TG} + N_{1,2\text{-}DG} + N_{2\text{-}MG} + N_{1\text{-}MG} + N_{Gol} \quad (12)$$

The results obtained are given in Table 3. Using Versantvoort conditions (DV) a considerable proportion of TG remained intact (69.2 ± 3.3%), far below the hydrolysis performance reported *in vivo* (95% of TG transformed into FA and MG) (Golding &

Table 3
Complete molecular picture of the lipolysis (CMP_L) undergone in the digestates of sea bass meat under different experimental conditions. Different letters within each column indicate a significant difference ($p < 0.05$).

Factors	Molar percentages of glycerides in relation to the total number of glyceryl structures present					Molar percentages of acyl groups (AG) and fatty acids (FA) in relation to the total number of AG + FA present				
	TG%	1,2-DG%	2-MG%	1-MG%	Gol%	AG_{TG}%	$AG_{1,2\text{-}DG}$%	$AG_{2\text{-}MG}$%	$AG_{1\text{-}MG}$%	FA%
Starting point (Sample DV) (Versantvoort conditions)	69.2 ± 3.3a	17.6 ± 1.0a	6.4 ± 2.0a	1.3 ± 0.4a	5.5 ± 0.2a	69.2 ± 3.3a	11.7 ± 0.7a	2.1 ± 0.7a	0.4 ± 0.1a	16.5 ± 1.9a
Delay of pH shifting in the gastric step (60 min)	69.0 ± 2.6a	18.1 ± 1.2a	6.0 ± 1.4a	1.2 ± 0.2a	5.7 ± 0.6a	69.0 ± 2.6a	12.1 ± 0.8a	2.0 ± 0.5a	0.4 ± 0.1a	16.5 ± 1.5a
Increase of intestinal residence time (4 h)	63.0 ± 3.1a	19.1 ± 1.6a	6.7 ± 1.3a	2.5 ± 0.6a	8.7 ± 1.6a	63.0 ± 3.1a	12.8 ± 1.1a	2.2 ± 0.4a	0.8 ± 0.2a	21.2 ± 2.2a
Addition of lipase in the GJ (100 U/ml of *A. niger* lipase)	48.9 ± 4.3b	23.5 ± 0.7a	12.0 ± 1.7b	2.2 ± 0.2a	13.4 ± 2.2b	48.9 ± 4.3b	15.7 ± 0.4a	4.0 ± 0.6b	0.7 ± 0.1a	30.7 ± 3.4b
Decrease of ratio food sample/digestive fluids volume (4.5 g food: 6 ml S: 12 ml GJ: 12 ml DJ: 6 ml BJ)	39.6 ± 5.3c	18.2 ± 2.9a	9.4 ± 1.5ab	2.0 ± 0.5a	30.8 ± 1.3c	39.6 ± 5.3c	12.1 ± 1.9a	3.1 ± 0.5ab	0.7 ± 0.2a	44.5 ± 3.0c
Increase of lipase concentration in the DJ (DJ2: 9 g/l of pancreatin and 9.6 g/l lipase)	39.9 ± 3.5c	18.7 ± 1.4a	8.7 ± 2.3ab	1.5 ± 0.6a	31.1 ± 0.9c	39.9 ± 3.5c	12.5 ± 1.0a	2.9 ± 0.8ab	0.5 ± 0.2a	44.2 ± 1.5c
Decrease of bile concentration in the BJ (Sample DO) (18.75 g/l)	4.6 ± 2.1d	23.0 ± 5.2a	23.2 ± 1.9c	5.4 ± 1.1b	43.8 ± 5.0d	4.6 ± 2.1d	15.3 ± 3.5a	7.7 ± 0.6c	1.8 ± 0.4b	70.5 ± 4.8d

Abbreviations: TG: triglycerides; 1,2-DG: 1,2-diglyceride; 2-MG: 2-monoglyceride; 1-MG: 1-monoglyceride; Gol: glycerol; DV: digested fish lipid extracts following Versantvoort conditions; S: saliva; GJ: gastric juice; DJ: duodenal juice; BJ: bile juice; DO: digested fish lipid extracts following the optimized conditions proposed in this study.

Wooster, 2010), thus providing evidence of limited lipolysis in the starting method. It is also noteworthy that 1,2-DG was the most abundant glyceride among the lipolytic products and that complete hydrolysis of TG into FA and Gol occurred to a slight extent. Moreover, valuable information concerning the preferential hydrolysis of the glyceryl structures during digestion is obtained from the comparative analysis of these molar percentages; greater hydrolysis of the ester bonds occurred in TG than it did in DG and in MG. Indeed, the positional specificity of pancreatic lipase would explain this decrease of the hydrolysis rate in partial glycerides (TG >1,2-DG >MG), because lipase finds two outer chains in TG, only one in 1,2-DG (and 1-MG) and none in 2-MG (Desnuelle & Savary, 1963).

3.2.2. Approach based on the fatty acids and acyl groups present in the digestates

Lipolysis can also be analyzed considering the acyl groups (AG) bound to the different kinds of glycerides and the FA present. The molar percentage of FA (**FA%**) and those of the AG bounded to TG (**AG_{TG}%**), 1,2-DG (**$AG_{1,2\text{-}DG}$%**), 2-MG (**$AG_{2\text{-}MG}$%**) and 1-MG (**$AG_{1\text{-}MG}$%**) in relation to the total number of moles of these AG plus FA (**NT_{AG+FA}**) can be determined:

$$AG_{TG}\% = 100(3N_{TG})/NT_{AG+FA} \tag{13}$$

$$AG_{1,2\text{-}DG}\% = 100(2N_{1,2\text{-}DG})/NT_{AG+FA} \tag{14}$$

$$AG_{2\text{-}MG}\% = 100N_{2\text{-}MG}/NT_{AG+FA} \tag{15}$$

$$AG_{1\text{-}MG}\% = 100N_{1\text{-}MG}/NT_{AG+FA} \tag{16}$$

$$FA\% = 100N_{FA}/NT_{AG+FA} \tag{17}$$

$$NT_{AG+FA} = 3N_{TG} + 2N_{1,2\text{-}DG} + N_{2\text{-}MG} + N_{1\text{-}MG} + N_{FA} \tag{18}$$

The results obtained using this second approach are also given in Table 3. The proportion of potentially non-absorbable structures (AG_{TG}% + $AG_{1,2\text{-}DG}$%) remained very high using Versantvoort conditions, this being approximately 4-fold higher than that of bioaccessible molecules (MG and FA). Moreover, these latter were mainly FA (see $AG_{2\text{-}MG}$%, $AG_{1\text{-}MG}$%, FA%). However, it must be noted that except for TG, different values are obtained due to the different significance of each quantification method. Indeed, a mass balance could be successfully applied with only the first approach. Despite this, all of the determinations obtained from ^{1}H NMR spectral data provide a ***complete molecular picture of lipolysis*** (***CMP_L***), which is a holistic view of the lipolysis at a molecular level, allowing a deeper study of lipid digestion.

3.2.3. Other parameters usually employed to describe the extent of lipolysis

The extent of fish lipid *in vitro* digestion can also be estimated using different parameters employed by other authors. One of them is the so-called **hydrolysis level** (**H_L%**) (Zhu et al., 2013), which can be calculated using this equation:

$$H_L\% = 100N_{FA}/NT_{AG+FA} \tag{19}$$

Another proposed parameter (Armand et al., 1999; Vinarov et al., 2012a, 2012b) is the **degree of transformation of TG** (**T_{TG}%**). This only considers the hydrolysis that occurred in TG and can be determined using the following equation:

$$T_{TG}\% = 100(N_{TGi} - N_{TG})/N_{TGi} \tag{20}$$

where N_{TGi} is the number of moles of TG initially present in the sample, which can be estimated in each sample as NT_{GS}, assuming that the lipids before digestion consist exclusively of TG

(99.2 ± 0.2%), that a total extraction of lipids is performed, and that the possible destruction of FA during digestion is negligible.

Finally, to estimate the extent of lipolysis in digestion from a physiological point of view, some authors proposed the parameter **lipid bioaccessibility (L_{BA}%)** (Kenmogne-Domguia, Meynier, Viau, Llamas, & Genot, 2012):

$$L_{BA}\% = 100(N_{1\text{-}MG} + N_{2\text{-}MG} + N_{FA})/NT_{AG+FA} \quad (21)$$

As can be observed in Table 4, the H_L% reached with the starting method is very low, releasing less than 20% of acyl groups. However, the proportion of triglyceride transformed into DG, MG or Gol (T_{TG}%) was approximately 30%. Finally, the L_{BA}% reached when using the starting method was close to 20%. The small difference between H_L% and L_{BA}% indicates that the molar percentage of AG supported on MG was much smaller than that of FA (see Table 3).

H_L% coincides exactly with the FA% given in Table 3 and that T_{TG}% is exactly (100-TG%) or (100-AG_{TG}%), also given in Table 3. These two parameters are included in the above-defined complete molecular picture of lipolysis (CMP_L), providing a detailed view of the molecular composition of the digestate.

3.3. Study of the influence of several factors on the extent of in vitro lipolysis

3.3.1. Effect of gastric pH acidification

Following the digestion model described above, the addition of 12 ml of gastric juice (GJ) to 9 g of minced sea bass led to an initial gastric pH of 4.7 ± 0.2 and 30 min later an acidification of the chyme to 2.5 ± 0.5, with HCl (37%), was carried out. However, *in vivo*, gastric pH was very low in basal conditions (1.8–1.9) and after 30 min of food ingestion it markedly increased up to 6.1, mainly due to the buffer capacity of dietary proteins. Then, after 3 h it progressively returned to basal values (Armand et al., 1999).

With this in mind, in the current study the acidification of the chyme to 2.5 ± 0.5 was performed after 60 min of the addition of GJ, instead of 30 min, with the aim of mimicking human conditions. This change did not cause any significant difference to the extent of lipolysis (Tables 3 and 4). Nevertheless, as it is closer to physiological conditions, this modification was maintained in the subsequent digestion experiments.

Similar results were obtained when rapeseed oil in water emulsion was *in vitro* digested and no significant difference relating to total lipolysis was observed when gastric pH was maintained either at 2.5 or 4.0 (Kenmogne-Domguia et al., 2012). The limited impact of gastric pH was attributed to the lack of gastric lipase.

3.3.2. Effect of intestinal transit time

The starting method proposes an intestinal residence time of 2 h; however, *in vivo* it is of the order of 3 h, ranging from 1 to 6 (Versantvoort et al., 2004). Moreover, when *in vitro* digestion models are used for marine foods, longer intestinal residence times are employed, usually up to 4 h (Hur et al., 2011). In line with this, an increase of intestinal residence time to 4 h was tested in order to ensure a greater degree of lipolysis.

As can be observed in Table 3, a slight increase ($p > 0.05$) in the extent of lipid *in vitro* digestion was obtained. As for molar percentages in relation to the total number glyceryl structures present, approximately 6% more TG were hydrolyzed mainly yielding MG and Gol, thereby releasing potentially absorbable lipid structures (MG, FA). It is also noticeable that the increase of intestinal residence time favours the isomerization of 2-MG to 1-MG. Indeed, 2-MG are very unstable in aqueous solution and at alkaline pH (Desnuelle & Savary, 1963), as they are at the intestinal step. Similar observations can be deduced by comparing molar percentages in relation to the total number of AG + FA. Although the percentage of non-absorbable AG (AG_{TG}% + $AG_{1,2\text{-}DG}$%) was slightly reduced, it still remained very high because approximately 75% of initial AG were not bioaccessible. Comparing the values of H_L%, T_{TG}% and L_{BA}% before and after increasing intestinal residence time (Table 4), a similar increase is observed, indicating that a longer residence time mainly led to the hydrolysis of TG into MG, Gol and FA. Moreover, the small difference between H_L% and L_{BA}% values shows that AG_{MG}% was much lower than FA%. These deductions can also be directly deduced from the CMP_L (Table 3). Considering all of the above, a 4 h-intestinal transit time was performed in the subsequent digestions experiments.

3.3.3. Effect of the addition of lipase to the gastric juice (GJ)

The model developed by Versantvoort et al. (2004, 2005) focuses on intestinal lipolysis and does not include any lipase in the gastric step. Nevertheless, several authors reported a notable contribution of gastric lipase to the extent of lipolysis in healthy adults. These authors indicate that although pancreatic lipase is mainly responsible for fat digestion, lipolysis can start in the stomach catalyzed by an acid-stable gastric lipase, which hydrolyzes 5–35% of TG (Armand et al., 1999; Carriere, Barrowman, Verger, & Laugier, 1993; Pafumi et al., 2002). This partial hydrolysis is believed to be of paramount importance for triggering the subsequent digestion in the small intestine, because gastric lipase promotes the pre-emulsification of lipids by altering the interfacial composition of lipid droplets through newly formed products, mainly DG and FA. Tarvainen et al. (2012), following the model proposed by Versantvoort et al. (2004, 2005), simulated this pre-emulsification effect by adding standard compounds, such as FA and DG, to the samples prior to digestion.

In order to evaluate the effect of gastric lipase, digestion experiments were carried out simultaneously using GJ without and with lipase. Taking into account that human gastric lipase (HGL) remains active in the duodenum, where it still shows a considerable hydrolytic activity (Carriere et al., 1993), the performance of gastric lipase on fish lipolysis was evaluated at the end of the whole digestion. Based on previous studies (Roman et al., 2012; van Aken, Bomhof, Zoet, Verbeek, & Oosterveld, 2011), lipase from *Aspergillus niger* was selected to simulate HGL, because this fungal

Table 4
Different parameters describing the lipolysis extent undergone in the *in vitro* digestion of sea bass meat under different experimental conditions. Different letters within each column indicate a significant difference (at $p < 0.05$).

Factors	H_L%	T_{TG}%	L_{BA}%
Starting point (Sample DV) (Versantvoort conditions)	16.5 ± 1.9a	30.8 ± 3.3a	19.0 ± 2.7a
Delay of pH shifting in the gastric step (60 min)	16.5 ± 1.5a	31.0 ± 2.6a	18.9 ± 1.9a
Increase of intestinal residence time (4 h)	21.2 ± 2.2a	37.0 ± 3.1a	24.3 ± 2.5a
Addition of lipase in the GJ (100 U/ml of *A. niger* lipase)	30.7 ± 3.4b	51.1 ± 4.3b	35.4 ± 3.9b
Decrease of ratio food sample/digestive fluids volume (4.5 g food: 6 ml S: 12 ml GJ: 12 ml DJ: 6 ml BJ)	44.5 ± 3.0c	60.4 ± 5.3bc	48.3 ± 3.3c
Increase of lipase concentration in the DJ (DJ2: 9 g/l of pancreatin and 9.6 g/l lipase)	44.2 ± 1.5c	60.1 ± 3.4c	47.6 ± 2.5c
Decrease of bile concentration in the BJ (Sample DO) (18.75 g/l)	70.5 ± 4.8d	95.4 ± 2.1d	80.1 ± 5.6d

Abbreviations: H_L: Hydrolysis level; T_{TG}: Triglyceride transformation; L_{BA}: Lipid bioaccessibility; DV: digested fish lipid extracts following Versantvoort conditions; S: saliva, GJ: gastric juice; DJ: duodenal juice; BJ: bile juice; DO: digested fish lipid extracts following the optimized conditions proposed in this study.

lipase shows a similar regiospecificity, a wide optimum pH range (2.5–5.5) and resistance against proteases.

As shown by the CMP_L (Table 3), the addition of 100 U/ml of *A. niger* lipase to the GJ provokes noticeably increased lipolysis, decreasing TG% significantly from 63.0 to 48.9%, in agreement with *in vivo* hydrolytic efficiency attributed to HGL (5–35% of TG). Consequently, 1,2-DG%, 2-MG% and Gol% increased in a similar proportion. It can also be observed in Table 3 that only one third of AG + FA present were potentially absorbable after this digestion experiment (see AG_{2-MG}%, AG_{1-MG}% and FA%). The parameters employed to describe lipolysis (Table 4) also show a significant increase in relation to the experiments carried out in the absence of gastric lipase. This increase is higher in T_{TG}% than in H_L% and L_{BA}%, suggesting again that the hydrolysis of the ester bond occurred to a greater extent in TG than in partial glycerides. Thus, like pancreatic lipase, this fungal lipase preferentially attacks the ester bonds of TG rather than those of partial glycerides. Moreover, it should be taken into account that TG are the main glyceryl structures present and thus might have the best chance of interacting with lipase.

Since the *in vitro* gastroduodenal lipolysis here performed by the GJ containing lipase, occurred to a lesser extent (FA% from 21.2 to 30.7, see Table 3) than that of van Aken et al. (2011), who reported 30% of FA% after *in vitro* gastric digestion of several food emulsions, a higher concentration of *A. niger* lipase (200 U/ml) was also assayed. However, no significant differences were observed (data not shown). This limited increase in the extent of lipolysis regardless of the amount of gastric lipase used could be explained by hindrance caused by protonated long chain FA accumulated at the surface of lipid droplets, which could inhibit further lipolysis (Pafumi et al., 2002).

Moreover, assays with unspecific *Candida rugosa* lipase were also performed, but with less successful results (data not shown). Thus, a gastric juice that included *A. niger* lipase at 100 U/ml was used for the following digestion experiments.

3.3.4. Effect of food/digestive fluids ratio

In the starting method, a proportion of 9 g (food): 6 ml (saliva): 12 ml (GJ): 12 ml (DJ): 6 ml (BJ) is proposed. Nevertheless, lower food/digestive fluids ratios have been employed in later studies for different food matrixes (Hur et al., 2009; Kopf-Bolanz et al., 2012; Lamothe et al., 2012; Marze et al., 2013).

Taking all the above considerations into account, *in vitro* digestion experiments were performed employing the conditions selected in the previous section, and using 9 g and 4.5 g of minced fish. Table 3 shows the CMP_L after digestion in both cases. The reduction of the sample amount provoked a significant decrease in TG% (≈10%). Likewise, 1,2-DG%, 2-MG% and 1-MG% were reduced due to complete hydrolysis of glycerides; in fact, it is worth noting the increase ($p < 0.05$) of Gol% and FA%; however, approximately half of the AG + FA present remained as non-absorbable (AG_{TG}% + $AG_{1,2-DG}$%). These observations are in agreement with the parameters of Table 4; H_L% and L_{BA}% underwent a higher increase than T_{TG}% did, which suggests that the use of 4.5 g of sample led to greater hydrolysis in DG and MG than when 9 g were used. Similar results were also observed by Li et al. (2011) using the pH-stat titration method. These authors submitted three different amounts of a corn oil emulsion to *in vitro* digestion and reported the lowest rate and extent of lipid hydrolysis when digesting the highest amount. Hence, the lowest food/digestive fluids ratio assayed was employed in the subsequent experiments.

3.3.5. Effect of the enzymatic composition of the duodenal juice (DJ)

Duodenal juice is a complex mixture in which at least three different types of pancreatic lipase enzymes are secreted, colipase-dependent lipase being the one mainly responsible for fat digestion. Carboxyl ester hydrolase (bile salt stimulated lipase) and phospholipase A2 are considered minor components of pancreatic juice (Reis, Holmberg, Watzke, Leser, & Miller, 2009). Moreover, wide compositional variations can be observed due to the amount and type of food ingested, the individual characteristics and the time of day (Clarysse et al., 2009; Kalantzi et al., 2006). Despite this variable composition, the concentration of pancreatic lipases *in vivo* is reported to always be in large excess over substrate, ensuring a complete lipid digestion (Reis et al., 2009).

For *in vitro* studies, different types of DJ have been proposed. These vary widely in ionic composition and type and amount of enzymes added (Kopf-Bolanz et al., 2012; Li et al., 2011; Versantvoort et al., 2004). Due to difficulties in obtaining human enzymes, in most studies purified commercial enzymes from mammal pancreas or fungi are used. The DJ proposed by Versantvoort et al. (2004, 2005) includes porcine pancreatin extract, together with additional porcine lipase to counteract the lower activity of lipase with respect to amylase in the commercial extract compared to that reported *in vivo*.

To investigate the effect of the nature and concentration of the enzymes of DJ on lipid digestion, four *in vitro* digestion experiments of 4.5 g of minced fish were carried out in parallel, employing either the DJ detailed in Table 1 (**DJ1**) or 3 different approaches (**DJ2-4**). These differ only in the content of pancreatin and porcine pancreatic lipase. Thus, **DJ2** contains 9 g/l of pancreatin and 9.6 g/l of lipase, based on Li et al. (2011), **DJ3** includes 18 g/l of pancreatin but no additional lipase, according to Kopf-Bolanz et al. (2012), and **DJ4**, 18 g/l and 9.6 g/l of pancreatin and lipase respectively.

Fig. 2a shows the molar percentages of the glyceryl structures present in the digestates after the use of DJ1-4. Quantitative data reported on Tables 3 and 4 correspond to the digestates obtained using DJ2. Thus, the increase in the concentration of duodenal enzymes did not lead to any significant increase of TG hydrolysis during *in vitro* digestion and the percentages of hydrolytic products obtained using DJ2-4 were very similar to those obtained using DJ1. This limited impact on extent of lipolysis could be evidence that the amount and type of enzymes present in DJ1 are already in excess over substrate. Similar results were obtained by Li et al. (2011) when examining the effect of lipase concentration (0–4.8 g/l) on the total amount of FA released during the *in vitro* digestion of a corn oil emulsion. The initial rate of lipolysis clearly increased as the amount of lipase increased, but in terms of the final amount of FA released, that is the extent of lipid digestion, similar results were obtained for lipase concentrations between 0.4 and 2.4 g/l. Since the enzymatic composition of DJ is not a key factor limiting the hydrolysis under the conditions of this study, no modification of duodenal juice composition was included in the later experiments and initial DJ1 was continued to be employed.

3.3.6. Effect of the bile concentration in the bile juice (BJ)

Bile salts are natural biosurfactants present in the gut lumen that play an important role in lipolysis by regulating the composition of lipid-water interface, where hydrolysis occurs (Golding & Wooster, 2010). Depending on bile concentration in relation to the critical micelle concentration, pancreatic lipase activity is enhanced or inhibited (Borgström & Erlanson, 1973). On the one hand, bile salts solubilize newly formed lipolytic products into bile-phospholipid mixed micelles, thus removing them from the interface together with other surfactants (proteins, phospholipids) that may also be present in the adsorption layer, displacing bound lipases. On the other hand, the highly surfactant nature of bile salts can restrict lipase adsorption at the interface, limiting fat digestion by sterical hindrance. This inhibitory effect can be relieved by the presence of colipase, a non-enzymatic protein cofactor that helps lipase to anchor at the interface and stabilizes lipase

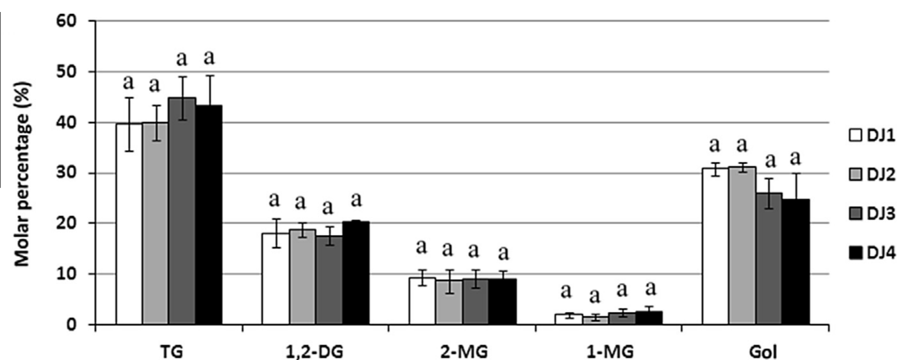

Fig. 2a. Molar percentages of the different glyceryl structures present in the lipid extracts of *in vitro* digested sea bass when varying the nature and concentration of enzymes in the duodenal juice (**DJ1**: 9 g/l of pancreatin and 1.5 g/l of lipase; **DJ2**: 9 g/l of pancreatin and 9.6 g/l lipase; **DJ3**: 18 g/l of pancreatin; **DJ4**: 18 g/l pancreatin and 9.6 g/l lipase). Different letters within each lipolytic product represent a significant difference ($p < 0.05$).

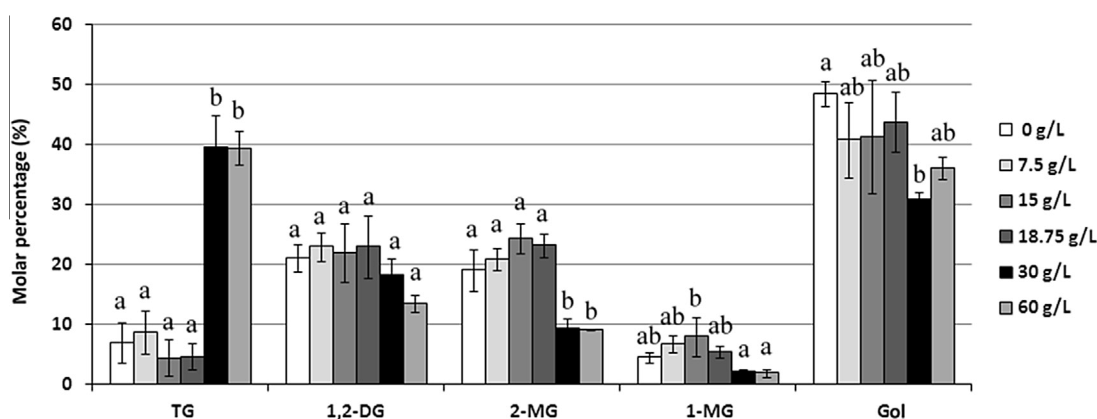

Fig. 2b. Molar percentages of the different glyceryl structures present in the lipid extracts of *in vitro* digested sea bass when varying bile concentration in the bile juice (0, 7.5, 15, 18.75, 30 and 60 g/l). Different letters within each lipolytic product represent a significant difference ($p < 0.05$).

conformational arrangement, allowing lipolysis to continue (Bläckberg et al., 1979; Reis et al., 2009).

A bile concentration of 30 g/l is employed in the starting model (Table 1); this corresponds to 8 mM in the chyme, which reproduces the biochemical environment of the small intestine during the fed state (Versantvoort et al., 2004). However, the amount of bile present in the small intestine fluctuates over digestion time, and this value is also influenced by food composition and by individual characteristics; in human postpandrial aspirates values ranging from 5 to 15 mM of bile have been observed (Clarysse et al., 2009; Kalantzi et al., 2006).

Bearing in mind the above mentioned, the impact of different bile concentrations (0, 7.5, 15.0, 18.75, 30.0 and 60.0 g/l in the BJ) on the extent of *in vitro* lipolysis was also investigated. Fig. 2b shows the molar percentages of the glyceryl structures present in the digestates obtained. Quantitative data reported in Table 3 and 4 correspond to the digestates obtained using a bile concentration of 18.75 g/l. As can be observed in the figure, the use of low bile concentrations (0–18.75 g/l) led to significantly lower TG% than the use of high ones (30, 60 g/l). This decrease of TG yielded mainly 2-MG and Gol, indicating that lower bile concentration greatly favoured the hydrolysis not only in TG, but also in partial glycerides, especially in DG. This can also be inferred from the CMP_L (Table 3) and the three parameters shown in Table 4.

The lesser extent of lipolysis observed at high bile concentrations is in agreement with previous studies (Borgström & Erlanson, 1973; Li et al., 2011). These results confirm the key role of bile salts in controlling lipase activity. It should be pointed out that this clear effect of bile concentration on lipolysis can be enhanced by the fact that as it is a static model, the digestion products generated are not removed from the media, in contrast to what occurs *in vivo*. When using bile at 30 and 60 g/l, critical micellar concentration could be reached that would limit the hydrolysis reaction dramatically because, although surfactants are removed from the interface, it is dominated by bile molecules. Vinarov et al., (2012a, 2012b) proposed a detailed inhibition mechanism of pancreatic lipase and revealed the roles played by emulsifiers and bile. Furthermore, the DJ used in this study lacks additional colipase (to that contained in pancreatin), which could explain in part the major impact of bile on the lipolysis (Bläckberg et al., 1979). At high bile concentrations, the absence of sufficient colipase meant that the electrostatic repulsion of lipases at bile salt covered interfaces could not be overcome, greatly influencing the hydrolysis reaction during the intestinal step.

Regarding the low bile concentrations tested, Fig. 2b shows that the use of bile at 0 and 7.5 g/l led to higher TG% and lower MG% than after using 15 and 18.75 g/l, although differences are not statistically significant. In fact, due to a worse dispersion of lipid droplets in the absence of bile, a smaller surface area would be available for lipase activity at the interface (Borgström & Erlanson, 1973). Moreover, without a bile dispersing effect, the presence of surface active molecules generated by lipases and proteases at the interface could hinder lipase-substrate direct contact (Mun, Decker, & McClements, 2007).

Taking into account the results obtained, high bile concentrations might explain the low *in vitro* lipolysis rates previously

reported (Kopf-Bolanz et al., 2012; Marze et al., 2013). By contrast, an *in vitro* digestion study on oil-in-water emulsions observed that by increasing bile concentration, the lipolysis rate is also increased (Mun et al., 2007). As for the improvement of the extent of fish lipolysis, a bile concentration of 18.75 g/l in the BJ (5 mM in the chyme according to Versantvoort et al. (2004)) would be suitable. Even if this concentration still remains lower than that used in *in vitro* protocols simulating fed state, it can be considered within the physiological range, since this concentration was reported in some *in vivo* duodenal aspirates (Clarysse et al., 2009; Lindahl, Ungell, Knutson, & Lennernäs, 1997).

3.4. Proposed conditions for a higher in vitro extent of lipolysis

Considering all the above studies, an optimized *in vitro* digestion method that allows one to obtain a TG hydrolysis level close to that occurring *in vivo,* can be proposed for fish lipid digestion. The modifications to carry out regarding the starting method include: delaying gastric pH acidification (from 30 to 60 min), increasing intestinal transit time (from 2 to 4 h), adding *A. niger* lipase to the gastric juice (100 U/ml), decreasing food/digestive fluids ratio (using 4.5 instead of 9 g of food), and decreasing bile concentration (from 30 to 18.75 g/l).

The improvements made can be observed in the lower part of Fig. 1, by comparing the spectra of digested fish lipid extracts following the starting protocol (DV) and the optimized one proposed (DO). Marked differences can be observed, especially in the intensity of specific signals of TG (**O**, **S**), almost imperceptible in DO, which indicates that they have undergone extensive hydrolysis. Likewise, the higher intensities of signals related to 1-MG (**L**, **I**), 2-MG (**K**, **Q**) and 1,2-DG (**J**, **P**, **R**) are further evidence for advanced lipolysis in DO. Differences in the chemical shift and multiplicity of signals **D1**, **D2**, **F1** and **F2** are also noticeable; in the DO spectrum higher chemical shifts (1.63, 1.71, 2.34 and 2.41 ppm) can be observed than in the DV spectrum (1.61, 1.69, 2.30 and 2.38 ppm). In fact, as the hydrolysis advances, signals **D1**, **D2**, **F1** and **F2** corresponding to TG gradually decrease whereas those corresponding to the same kinds of protons supported on 1,2-DG, 2-MG, 1-MG and FA appear centered at higher chemical shifts (Table 2).

This qualitative information agrees with the quantitative data reported. As shown by the CMP_L (Table 3), TG% in sample DO is very low (4.6 ± 2.1%), indicating that approximately 95% of TG initially present were hydrolyzed, close to *in vivo* performance. No significant modification of 1,2-DG% was noticed in relation to the starting method, whereas the occurrence of potentially absorbable glycerides (2-MG, 1-MG), increased significantly. Regarding this latter, it should be noted that 1-MG% remained almost constant in the previous experiments; therefore, its increase when using the new conditions might be mainly explained by the higher occurrence of 2-MG (23.2 ± 1.9%). Moreover, approximately 44% of the initial TG were completely hydrolyzed in sample DO (see Gol%), in agreement with levels reported *in vivo* (Borgström, Tryding, & Westöö, 1957). Hence, the present study highlights that the TG hydrolysis into Gol and 3 FA is not only restricted to *in vivo* conditions and cannot be dismissed in *in vitro* studies, as it has been previously (Lamothe et al., 2012; Li et al., 2011). Regarding molar percentages expressed in relation to the total number of FA + AG, more than two thirds of the total AG initially present were released as FA (70.5 ± 4.8%), which were the main form of absorbable lipids, considering the values obtained for $AG_{2\text{-}MG}$% and $AG_{1\text{-}MG}$%.

The parameters shown in Table 4 also reflect the above-commented in Table 3. Thus, H_L%, T_{TC}% and L_{BA}% in sample DO are three- or fourfold higher than that reached with the starting model (DV), highlighting the significant improvement of fish lipid digestion caused by varying, within a physiological range, the above-mentioned experimental factors. The differences observed between T_{TC}% and L_{BA}% (≈15%), and L_{BA}% and H_L% (≈10%), suggest that sample DO contains similar proportions of DG and MG, as shown by CMP_L (see 1,2-DG%, 2-MG% and 1-MG% in Table 3).

4. Conclusions

The present study provides further knowledge on the impact of experimental factors on lipid *in vit*ro digestion, which is of paramount importance in gaining a better insight into the limitations of the protocols currently employed in *in vitro* studies, and thus understanding the results obtained. The addition of gastric lipase, the decrease of food/digestive fluids ratio and the decrease of bile concentration significantly improved lipolysis. However, of all the experimental variables studied, the bile concentration was found to be the key factor for controlling *in vitro* lipolysis in the static model employed. With the modifications proposed, approximately 95% of triglycerides underwent hydrolysis and 80% of fish lipids acyl groups were bioaccessible. Since an accurate match of naturally occurring events is necessary for consistent statements and predictions, the optimization of *in vitro* digestion experimental conditions should be carried out for each kind of sample when required, especially in bioaccessibility and bioavailability studies. ^{1}H NMR was evidenced to be a sound technique in the lipid digestion field, providing a holistic molecular view of lipolysis, giving a great deal of information in a fast single-run and without any chemical modification of the sample. To the best of the authors' knowledge, this is the first time that the methodology proposed for the evaluation of the influence of certain factors on the lipolysis advance is able to provide information on the specific ester bond affected by each factor in each case.

Acknowledgements

This work has been supported by the Spanish Ministry of Economy and Competitiveness (MINECO, AGL2012-36466, AGL2015-65450-R), by the Basque Government (EJ-GV, GIC10/85-IT-463-10) and by the Unit for Education and Research "Food Quality and Safety" (UPV/EHU-UFI-11/21). All authors participate in the COST Action FA1005-INFOGEST. B.N-E thanks UPV/EHU for a predoctoral contract.

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