Optimization of hydrolysis of sardine (*Sardina pilchardus*) heads with Protamex: enhancement of lipid and phospholipid extraction

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Abstract:

BACKGROUND: Fish by-products are not considered as valuable raw materials even if they usually contain valuable components such as lipids. Fish lipids are well known for their nutritional potential and health effects but their extraction remains problematic due to the use of organic solvents. Enzymatic hydrolysis such as the proteolysis of tissues can lead to lipid extraction.

RESULTS: Hydrolysis of sardine heads by Protamex was studied (temperature, hydrolysis time and enzyme-substrate ratio) using response surface methodology in order to obtain the highest release of lipids and particularly phospholipids. No relation between the degree of hydrolysis and lipid recovery were depicted; however, optimum conditions for both the release of lipids and phospholipids were found to be similar (29 min, 31 °C with 2.6 g kg-1 enzyme). Under these hydrolysis conditions, rich lipid and phospholipid fractions (oily and aqueous fractions) can be recovered when time, temperature and enzyme consumption are minimized. Analytical data have revealed that they contain high-quality lipids, especially 3 fatty acid.

CONCLUSION: This study demonstrated that proteolysis can be used for high lipid recovery from little-exploited biomass like fish heads without requiring solvent or thermal treatment. Resulting phospholipids, fatty acids and peptides could be utilized for nutritional or feed purposes.

Keywords: phospholipids • fatty acids • by-product • sardine • proteolysis • response surface methodology

1. Introduction

Worldwide, total fisheries production was about 140 million tons.[1] In France, sardine (*Sardina pilchardus*) represents the first landed fish: about 22 000 tons in 2004 [2] and it is mainly consumed canned. Such processing leads to high volume of by-products (about 30 % of the whole fish),[3] corresponding to an annual French production of more than 6 000 tons. Fish processing by-products are commonly recognized as low value resources with negligible market value. Nowadays, they are mainly used for meal, oil or pet food mince production. Hence, there is a great opportunity here to increase the added value of these by-products.

Biochemical compositions of fish have been widely described and have shown great nutritional values and their by-products possess quite similar composition.[4,5] Among the different nutritional compounds lipids, and particularly ω3 and phospholipids, from fatty fish are of interest. Indeed, in addition to their nutritional value, marine phospholipids have also exhibited antitumoral activities [6] while synthetic phospholipids were realised for antiviral uses.[7,8] Fatty acids also offer potential values. A large number of articles have described benefits of ω3 fatty acids: on blood circulation, thrombose, hypertriglycemia,[9] on blood pressure regulation,[10] against schizophrenia,[11] stress and depression,[12] on foetal development[13] and the most widely discussed is the benefit against cardiovascular disease.[14-17] Marine organisms seem to be a wide source of such fatty acids[18] and marine by-products as well.[19-21] Thus, the improvement on lipid recovery from fishery discards offers a large opportunity to reduce waste and increase profit from a highly valuable sub-product.

Hydrolysis processes have been developed to convert under-utilized fish and fish by-products into a marketable and acceptable form, which can be widely used in food rather than as animal feed or fertilizer. Enzymatic tissue disruption may be an efficient alternative technique for releasing lipids. Previous studies on fish and fish by-products have shown that, when compared to classical organic extraction, lipid extraction is enhanced by a pre-hydrolysis step using wide-spectrum proteases [22] and a part of the oil could be obtained after hydrolysis and centrifugation.[23] A recent study conducted on sardine viscera has shown that hydrolysis with proteases allows and improves lipid and phospholipid release as compared with common method [24] avoiding the use of drastic conditions (chemicals and heat).

Response surface methodology (RSM) is a useful technique for the investigation of complex processes such as enzymatic hydrolysis. Indeed, some papers have reported the optimisation of processing conditions, like pH, temperature, enzyme concentration but they are essentially based on protein recovery [25] or peptide biological activities.[26]

The purpose of this study is to develop efficient conditions for hydrolysis regarding the hydrolysis of sardine heads with Protamex. Hydrolysis time, temperature and enzyme concentration have been studied according to the experimental design procedure in order to remove the highest quantities of lipid and phospholipid from the by-product.

2. Experimental

All analyses have been performed in triplicates.

Materials

Sardines (*Sardina pilchardus*) were provided from Pêcheries Océanes (Nantes, France) and were caught in the Atlantic North-East in April and June 2004. Fish were stored under ice less than 24 h and then filleted. Heads were collected and stored under vacuum at – 80°C. Due to high variation in chemical composition of fish according to season, sex and length,[27,28] samples coming from April and June were mixed together and homogenized with a blender (Waring Commercial, USA) for 1 min. Samples were then stored at – 80°C.

Enzyme used for hydrolysis was kindly provided by Novozymes AS (Denmark). Protamex is a *Bacillus* protease complex (EC numbers: 3.4.21.62 and 3.4.24.28). Protamex is developed for the hydrolysis of food proteins and fulfils the purity demands for food-grade enzymes, set by the joint FAO/WHO Expert Committee on Food Additives (JEFTA) and the Food Chemicals Codex (FCC).[29] Optimal conditions for Protamex were determined in a previous study.[3] Its deactivation temperature is reported by manufacturer to be 85°C for 10 min.

Hydrolysis

The hydrolysis experiments were carried out using the pHstat method (TIM 854, Radiometer analytical, SAS, France) in a 250 mL glass reactor under controlled conditions (pH, temperature and stirring speed). Mixed by-products were homogenized with water (ratio 1/1, w/v). Enzyme was added to the mixture and the system was continuously stirred at 700 rpm during hydrolysis. pH was maintained at the desired value (pH 8) by addition of NaOH 2M. Therefore, the degree of hydrolysis (DH) could be obtained following the formula:

$$DH = \frac{V.N.100}{\alpha.mp.h_{tot}}$$

Where V: volume of sodium hydroxide consumed during hydrolysis in mL
 N: normality of sodium hydroxide
 α: dissociation factor for α-NH$_2$ groups = 0.88 at 50°C [30]
 mp: mass of protein in g in the fresh matter introduced in the system (protein content was determined according to Crooke *et al.* [31]
h_{tot}: total number of peptide bounds in the protein = 8,6 eq.g.kg^{-1} protein in fish samples
After hydrolysis, the temperature was increased to 85°C for 10 min to inactivate the enzyme. The hydrolysate was then centrifuged at 20,000 g for 30 min at 4°C. After centrifugation, the tubes were put upright in a freezer (-20°C) and all fractions: sludges (or insoluble phases), oily phase and aqueous (or soluble) phase were separated by cutting the frozen content of the tubes. Each fraction was then freeze-dried and stored at – 20°C until analysis.

Dry matter content

Dry matter content was estimated gravimetrically after freeze-drying.

Ash content

Ash content was determined by heating samples at 600°C overnight.

Lipid extraction

Lipid extraction was carried out according to the method described by Folch *et al.* [32] About 1 g (exactly measured) of dry sample was homogenized in 4 mL distilled water. Methanol (Technical grade, Carlo Erba, France) was added to the mixture (ratio mixture/methanol: 3/20, w/v). Samples were then stirred for 30 min before adding chloroform (Technical grade, Carlo Erba, France) (ratio mixture/chloroform: 3/40, w/v). The system was stirred for 30 min before filtration and addition of 0.2 volume of water with 0.9% NaCl. After 15 hours decantation at + 4°C in separating funnel and in darkness, lipids were recovered in the organic phase. Lipid extracts were dried under vacuum in a rotary evaporator (temperature < 35°C). Lipids were weighted and results expressed as g of lipid per g of dry matter. Lipids extracts were kept in 10 mL chloroform and stored at – 80°C until analysis.

Phospholipid content of total lipid extracts

Phospholipid content was evaluated by the colorimetric method of Stewart [33] based on the formation of a complex between phospholipids and ammonium ferrothiocyanate. Ammonium ferrothiocyanate consisted of ferric chloride (27 g.L^{-1}) and ammonium thiocyanate (30 g.L^{-1}) in distilled water. One milliliter of this reagent was added to 2 mL of lipid extract in chloroform (0.25 mg/mL). The mixture was homogenized and centrifuged for 10 min at 750 *g*. The absorbance of the lower phase was read at 488 nm against the blank. A standard curve was plotted with standard phosphatidylcholine in chloroform (5 to 50 µg/mL) and results were expressed as g phosphatidylcholine equivalent / g of dry matter.

Lipid class distribution

Lipid class was analysed twice, by TLC-FID to obtain a precise but destructive distribution and by solid phase extraction to recover neutral lipids and phospholipids.
The TLC-FID technique was realized according to an adaptation of the methods described by Parrish (1987) and Ackman *et al.* (1990) with a Iatroscan MK-6 (Shell, USA). [34,35]
The solid phase extraction and separation of lipid classes was performed using open glass column (internal diameter: 8-10 mm, length: 20 cm) containing glass wool and silica gel (ratio silica gel/ oily sample: 30/1, w/w). Chloroform (1 mL/mg of oil sample), acetone/methanol (9/1, v/v; 1.5 mL/mg of oil sample) then methanol (1 mL/mg of oil sample) were used to recover respectively neutral lipids, glycolipids and phospholipids. Each fraction was then stored at -80°C until analysis.

Fatty acid analysis

Aliquot of lipid was evaporated under nitrogen and transmethylated by contact with methanol/sulphuric acid (98:2, V/V) in excess at 50°C overnight. After cooling, 2 mL of hexane and 1 mL of water were added and vortexed. The upper organic phase containing fatty acid methyl esters (FAMEs) was collected and assayed by GC using a Perkin Elmer Autosystem equipped with a FID detector. Separation was done using helium as carrier gas on a fused silica column (BPX-70, 60 m long, 0.25 mm i.d, 0.25 µm film thickness, SGE) programmed from 55°C (for 2 min) to 150°C at 20°C/min then to 230°C at 1.5°C/min. Sample was injected with a programmable split/splitless inlet and large volume injection system (PSS) using the following temperature program: 55°C (for 2 min) to 350°C at 200°C/min. FAMEs were identified by comparison of their equivalent

chain length with those of authentic standards. Quantification was done using margaric acid (17:0) as internal standard.

Protein content

Crude protein content (N x 6.25) was estimated in the heads and determined colorimetrically after Kjeldahl digestion using a method described by Crooke et al. [31]

Experimental design and evaluation

Response surface methodology (RSM) was used to investigate the influence of the hydrolysis variables: temperature, hydrolysis time and enzyme-substrate (E/S) ratio (g.kg^{-1} of raw heads) on the digestion of sardine heads and to optimize the influence of these three variables. In a 2^3 Central Composite Design (CDD), the starting temperature, hydrolysis time and E/S ratio were varied systematically at high and low levels (Tables

Table 1). Two parallel center groups with medium levels of all the three factors studied were used to check the linear relationship between high and low levels of the variables tested. The measured variable responses were degree of hydrolysis (DH), lipid and phospholipid release. A total of 16 experiments were performed. The order of the experiments has been fully randomized. Analytical determinations were performed in triplicate. The mathematical analysis of data was conducted by the software Statgraphics plus v.5 experimental design. The accuracy of the model was further tested by conducting hydrolysis experiments.

3. Results

The proximate chemical composition of sardine heads was: 749 ± 1 g.kg^{-1} moisture; 64 ± 0 g.kg^{-1} ash; 58 ± 2 g.kg^{-1} lipids and 137 ± 5 g.kg^{-1} proteins.

Experimental design

Responses (DH, lipids and phospholipids) obtained for each experiment are reported in Table 2. Experiment 1 gave highest DH, 28%, corresponding to a long hydrolysis (22h), a low temperature (36°C) and a high enzyme concentration (2.3 g.kg^{-1}). At the other extreme, the lowest DH (7%) was obtained conducting the experiment 11 with a short hydrolysis time (6h), a low temperature (36°C) and low enzyme concentration (0.7 g.kg^{-1}).

Regarding lipids and phospholipids, two responses have been examined: the total lipids for the evaluation of the total lipid quantity in the matrix, and the lipids released, corresponding to the part recovered into the liquid fractions (oily and soluble fractions). The 9th experiment resulted in the highest lipid quantity with a short hydrolysis time (6h), a low temperature (36°C) and a high enzyme concentration (2.3 g.kg^{-1}). Under such conditions, 0.29 g of lipids per g of dry matter was extracted and 0.20 g in the liquid fractions. The 4th experiment provided the lowest lipids recoveries (0.19 g of total lipids per g of dry matter and only 0.09 g in the liquid fractions) even with a long hydrolysis, and moderate temperature and enzyme concentration (27h30, 43°C, 2.3 g.kg^{-1}).

Concerning phospholipids, similar results were observed for total phospholipids extraction and their recovery into liquid fractions. The 11th experiment appears to be the

best one. Under such conditions, the total phospholipids has increased by 50% compared to chemical extraction and 33% of these phospholipids were recovered in the liquid fractions. At the opposite, conditions used in the first experiment conducted to the worst results (0.006 g of total phospholipids per g of dry heads and 0.003 g in the liquid fractions).

Predicted responses obtained

According to predicted responses, none of the conditions were found suitable for an optimisation of both DH, lipids and phospholipids recoveries. Thus the optimisation procedures have been carried out considering only on lipid responses. Conditions resulting from the canonical analysis of the data were reported in Table 3. The first part of this table indicates the conditions required to obtain the highest value for all responses studied and the regression factor (R^2 adjusted) of these predictions. The other part of Table 3 indicates the theoretical values for each studied response according to the conditions mentioned above. As example, to obtain the highest DH, the hydrolysis should be conducted during 27h (1605 min), at 33°C and with an enzyme concentration of 2.0 g.kg^{-1}. According to the prediction, the DH should be around 30%, the total lipids quantity about 0.155 g.g^{-1} of dry matter and the phospholipids about 0.004 g.g^{-1} of dry matter. The software also indicated around 93.9% hardiness, which indicates the reliability. The regression given for the lipid response was a little lower ($R^2 = 75.2\%$) and very low for the phospholipids ($R^2 = 48.7\%$) but it is important to notice that the conditions indicated for this response were closed to those needed for the lipid response. Canonical analysis revealed that the experimental conditions for the highest release of lipids and phospholipids were: hydrolysis time = 29 min, temperature = 31°C and E/S ratio = 2.6 g.kg^{-1} at pH 8 and with one volume of water added, following the pHstat method. The best explanatory model equation for the lipid release became:
Lipid releasing = 0,2260669 + 0,000186345 time + 1,82655 E/S - 0,0135053 T° - 0,00048255 time*E/S – 0,00000333432 time*T° - 0,0334821 E/S*T° + 0,000241924 T°^{2}
Three dimensional graphs, showing the influence of each variable on the lipid releasing are presented on Figure 1 (A: enzyme concentration and temperature; B: hydrolysis time and temperature; C: enzyme concentration and hydrolysis time). Profiles obtained here confirm values predicted in Table 3, where lipids will be released in higher quantity with a short-time hydrolysis, a high enzyme concentration and at a moderate temperature. Thus, these graphs emphasize the strong influence of temperature and enzyme concentration. The accuracy of the model was further tested by conducting hydrolysis experiment.
Validation of the predicted value: hydrolysis conduced at optimised conditions for lipids release

DH and release of lipids and phospholipids

Results obtained after the conduction of the hydrolysis are reported in Table 4. Under such conditions, for a 29 min hydrolysis, the DH reached 3.5%. Only 27% of the total matter was located into the liquid fractions after proteolysis. However, 80% of extracted lipids were located into liquid fractions and 60% in the oily one. Regarding phospholipids, they are mainly located in liquid fractions (56%) as well, and especially into the soluble fraction.

Total fatty acid analysis

Fatty acids of lipids recovered in each fraction were analysed by gas chromatography (Table 5). In all fractions, palmitic acid is the main fatty acid (more than 20%). In the insoluble fraction, main fatty acid families were the ω3 (~24%) and the ω9 (~21%). Concerning the liquid fractions, the principal difference observed with the insoluble part was the PUFA proportion, more prominent in liquid fractions (~28%) corresponding to a decrease of SFA (~40%). Thus, the ω3 fatty acid family was the most important in the soluble fraction (27.02 ± 0.46%) followed by the oily fraction (26.80 ± 0.04%). The ω9 fatty acid family represented around 20% of total fatty acids in both liquid fractions.

Lipid class distribution

Results from TLC-FID analysis of sardine heads hydrolysis fractions were depicted in Figure 2. No free fatty acids, ketones, hydrocarbons or waxes were found on our samples and sterol esters were estimated as trace levels. The insoluble fraction was the most diversified. The tri-acylglycerols (TAGs) represented 36% of total lipids, followed by phospholipids (29%). Other polar lipids (glycolipids (GLs), pigments, mono-acylglycerols (MAGs)) were found predominantly in the insoluble fraction (19%). The sterol fraction was also mainly found in this fraction, representing 15% of total lipids. The oily fraction is almost exclusively composed of acylglycerols, especially TAG (68% of total lipids). Less than 10% of the lipid composition of this fraction was represented by the phospholipids and the sterols. In the soluble fraction, 46% of the total lipids were TAG and 25% were represented by the GLs, MAGs, and pigments group. Phospholipids in this fraction represented around 20% of the total lipids.

Fatty acid analysis of lipid classes from liquid fractions

Fatty acid distribution of the neutral lipids and phospholipids resulted from the three fractions of the hydrolysis were then studied (Table 6, Table 7 and Table 8).
Insoluble fraction: neutral lipid fatty acids (Table 6) were mainly represented by SFAs (39%) especially with palmitic acid (26%). PUFAs were highly represented (36%) and principally by the ω3 and ω6 families. Phospholipids possessed mainly SFAs (56%). Phospholipid PUFAs were almost exclusively represented by EPA and DHA.
Oily fraction: neutral lipids were the only lipid class found significantly. Fatty acids (Table 7) were mainly represented by PUFAs (42% of total fatty acids), essentially belonged to the ω3 family (75% of PUFAs).
Soluble fraction: fatty acids of neutral lipids and phospholipids were reported in Table 8. Neutral lipids were essentially represented by PUFAs (41%) with EPA and DHA as major components (around 23% of total fatty acids). SFAs represented 36% of total fatty acids, with 23% were palmitic acid. Phospholipids of the same fraction were depicted mainly by SFA (47%), essentially with palmitic (31%) and stearic (14%) acids. Oleic acid is the major MUFA, where ω9 fatty acids family represented more than 25% of the total fatty acid. While PUFAs were the minor fatty acid class, they were constituted exclusively by EPA and DHA.

4. Discussion

Results obtained in the first part of the study have shown that hydrolysis could increase the extraction of lipids by 27% compared to classical extraction. Phospholipid quantity is also enhanced (up to 50%), inducing the benefit of hydrolysis for phospholipid release.

Natively, phospholipids are combined with proteins to form the membrane. Hydrolyses realized with proteases induce the membrane breakdown and consequently gave a more efficient phospholipid extraction, as shown before on other by-product matrixes.[22] These results have also demonstrated that the lipids recovered into liquid fractions could reach 70% of the total quantity for lipids and 33% for phospholipids, avoiding solvent utilisation. Thanks to results obtained, the enzyme concentration was the most influencial factor. Hydrolysis time is the second influent factor, but it leads to different effects according to the response. It is not beneficial to perform a long hydrolysis to obtain the highest lipid release. Indeed, the tissue disruption obtained at the beginning of the proteolysis appears sufficient to release the lipids. Moreover, the response surface profiles indicated that the lipid recovery on liquid fractions decreased with long hydrolysis. This phenomenon is due to the small peptides generation during long time hydrolysis, suggesting that these peptides produced had more accessible hydrophobic regions to interact with the lipids. Such complexes were then recovered in the sludge. Temperature variation does not affect the responses. While the contrary has been demonstrated in previous studies,[25,26,36] it is surely due to the fact that our chosen temperature area is not wide enough and centered around the high enzyme activity temperature area (Protamex has an optimal temperature range from 31 to 55°C). Other studies have also shown, with experimental design procedure, that temperature did not possess a significant effect on hydrolysis (DH) when the temperature corresponded to the enzyme temperature range.[37,38]

All these tendencies have been confirmed with the canonical analysis. Data treatment concerning the lipid responses indicates preferential operating conditions. The prediction was not as robust as the one obtained for DH. Anyway, this analysis gave an indication of the conditions to use. Concerning phospholipid recovery, results obtained do not permit to obtain an accurate prediction, but the values of the parameters indicated for the phospholipids were the same as those predicted for the lipid response.

Thus, this statistical treatment demonstrated that lipid extraction was not linked to proteolysis (DH). Indeed, the hypothesis formulated at the beginning (the higher the DH is, the higher the breakdown of the matrix is, the smaller the peptides is and the higher the lipid release is) was contradicted by the present results because a low hydrolysis time was sufficient to obtain the highest lipid recovery. It was also demonstrated that DH and lipids were not only linked but were antinomic: experiments which have led to high DH values have led to low lipid recovery and *vice versa*. Recently, Šližyte *et al.* have shown that it was difficult to obtain, with the same conditions, the maximum lipid and protein recoveries.[39] Nowadays, few works have been published focusing on lipid extraction by enzymatic way, most of the hydrolyses being devoted to protein fractions to produce hydrolysates with high nutritional value [37,38,40,41] or to find any biological activity, such as antioxidant one [42,43,44], anti-anaemia [45] or secretagogue [46] for example.

Hydrolysis conducted here has led to low solubility of the matter, which can be explained by the short hydrolysis. Quaglia and Orban, in 1987, have shown that hydrolysate solubility increases with DH. [47] However, hydrolyses have enabled to extract a high amount of lipids. After centrifugation, the main part was released in the liquid fractions, which was our principal objective. Additional mild steps (likewise ultrafiltration) could be used for purifying those liquid fractions. Phospholipids were extracted in higher quantity after hydrolysis. Despite the fact that a lot of phospholipids are still located in the insoluble fraction, an amount as high as the one obtained without hydrolysis is located in the liquid fractions, especially in the soluble fraction. Similar results were obtained after conducting a proteolysis (with Protamex and Flavourzyme) on marine by-products (salmon backbone and cod viscera) which is in accordance with our results. [48,49] Thus, both liquid fractions present an interest in the lipid area.

The fatty acid study showed that PUFAs were concentrated in liquid fractions. ω3 Fatty acids represented 27% of the total fatty acids in liquid fractions. Phospholipid results were located in soluble and insoluble fractions. Moreover, lipid class results obtained with both analytic methods gave the same results. Those analyses showed that insoluble fraction possess a higher lipid diversity (TAGs, sterols, MAGs, phospholipids) than the others.

Fatty acids contained in the neutral lipid of insoluble fraction were mainly SFAs and PUFAs. The recovery of those compounds could be studied in a further study, with additional hydrolysis, for example. Indeed, previous works realized in the Laboratory on other marine by-products have highlighted that a second hydrolysis has an interest. Hence, after conducting a hydrolysis on cod milt with Flavourzyme, the resulting sludge was more solubilized with a secondary hydrolysis with Protamex and more lipids were released. Other processes are using several hydrolyses, in order to increase functional properties of hydrolysate or to reduce their bitterness for example. [50,51] This process could also be used, for the insoluble fraction, to release lipids, proteins, carbohydrates…

Liquid fractions were less diversified because the oily fraction was only composed of neutral lipids and the soluble fraction is composed of MAGs, TAGs and phospholipids. Fatty acid composition of oily fraction neutral lipids was mainly PUFAs and especially ω3 fatty acids. Similar fatty acid composition was obtained concerning neutral lipid soluble fraction. Phospholipid fatty acids of soluble fraction were mainly SFA. However, PUFA were only EPA and DHA.

Sardine heads hydrolysis has shown a large interest for lipid recovery. Extracted lipid quantities were not more significant than those obtained with classical chemical extraction, but the hydrolysis conducted with Protamex permitted to release the lipids in the liquid fractions. Soluble and oily fractions had moreover a qualitative interest. Indeed, oily fraction is mainly composed of ω3 fatty acid rich neutral lipids and soluble fraction possesses three main cards: ω3 fatty acid rich neutral lipids, a high phospholipid quantity, and its protein content. While oily fraction can be recovered including a centrifugation step and then a micro-filtration, the soluble fraction should be finely purified using ultrafiltration techniques, in order to separate lipid and peptides parts. [52]

Acknowledgements

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Tables

Table 1
Experimental design levels of the independent variables used in the hydrolysis of sardine heads with Protamex

Levels of independant variables used	Hydrolysis time (min)	Temperature (°C)	E/S ratio $(g.kg^{-1})$
-1.68	29	31	0.2
-1	358	36	0.7
0	840	43	1.5
+1	1322	50	2.3
+1.68	1651	55	2.8

Table 2
Proteolysis conditions and responses for Degree of Hydrolysis, Lipid and Phospholipids (sum of all fractions and sum of oily and aqueous fractions *i.e.* liquid fractions) obtained for the Central Composite Design

| Experiment number | Proteolysis conditions | | | Responses obtained | | | | |
| | Hydrolysis time | Temperature | E/S ratio | DH | Lipids (g/g of dry heads) | | Phospholipids (g/g of dry heads) | |
	(min)	(°C)	(g.kg-1)		All fractions	liquid fractions	All fractions	liquid fractions
1	1322	36	2.3	28%	0.22	0.14	0.006	0.003
2	840	43	2.8	24%	0.19	0.11	0.010	0.004
3	840	43	1.5	24%	0.21	0.13	0.012	0.006
4	1651	43	1.5	23%	0.19	0.09	0.010	0.002
5	29	43	1.5	8%	0.22	0.13	0.018	0.004
6	840	43	1.5.2	13%	0.20	0.13	0.019	0.004
7	358	50	1.5.7	9%	0.23	0.17	0.016	0.005
8	840	43	1.5	22%	0.21	0.13	0.012	0.004
9	358	36	2.3	9%	0.29	0.20	0.015	0.007
10	840	31	1.5	21%	0.22	0.15	0.016	0.009
11	358	36	1.5.7	7%	0.22	0.11	0.021	0.007
12	840	55	1.5	11%	0.23	0.15	0.020	0.006
13	1322	36	1.5.7	23%	0.23	0.15	0.010	0.003
14	1322	50	2.3	17%	0.21	0.08	0.012	0.004
15	358	50	2.3	12%	0.22	0.16	0.020	0.008
16	1322	50	1.5.7	12%	0.22	0.14	0.018	0.005

Table 3
Optimal conditions calculated for each response and values predicted statistically according to those optimal conditions

	Response	DH	Lipid released	PL released
Conditions	Time (min)	1605	29	29
	Enzyme (g.kg^{-1} of raw material)	2.0	2.6	2.8
	Temperature (°C)	33	31	32
	R² adjusted	93.9	75.2	48.7
Predicted values	DH (%)	30	Out of range	Out of range
	Lipids released (g.g^{-1} of dry heads)	0.155	0.239	0.255
	PL* released (g.g^{-1} of dry heads)	0.004	0.009	0.009

* PL: Phospholipids

Table 4
Degree of hydrolysis, dry matter distribution, lipids and phospholipid recoveries obtained after processing the optimal hydrolysis

	Aqueous	Oily	Insoluble	Total
DH	-	-	-	3.5%
Dry matter distribution	27%	20%	53%	100%
Lipids (g.g^{-1} of dry heads)	0.03	0.11	0.04	0.18
Phospholipids (g.g^{-1} dry heads)	0.012	0.002	0.011	0.025

Table 5
Fatty acid distribution (%) among fractions obtained after optimized hydrolysis. SFA: saturated fatty acids, MUFA: mono-unsaturated fatty acids, PUFA: poly-unsaturated fatty acids

	Insoluble	Oily	Aqueous
C14:0	8.5 ± 0.1	11.7 ± 0.3	8.5 ± 0.2
C16:0	28.2 ± 0.5	23.7 ± 0.1	25.5 ± 0.3
C18:0	6.2 ± 0.1	4.0 ± 0.0	5.6 ± 0.0
C20:0	0.6 ± 0.0	0.4 ± 0.3	0.6 ± 0.0
Σ SFA	**43.5 ± 0.7**	**39.8 ± 0.1**	**40.2 ± 0.5**
C16:1 c ω7	6.1 ± 0.1	7.4 ± 0.0	6.4 ± 0.1
C18:1 c ω9	10.4 ± 0.1	8.0 ± 0.0	10.1 ± 0.1
C18:1 c ω7	3.0 ± 0.0	2.6 ± 0.0	2.7 ± 0.0
C20:1 c ω9	4.6 ± 0.0	5.5 ± 0.0	4.6 ± 0.0
C22:1 c ω9	4.4 ± 0.1	5.7 ± 0.0	4.5 ± 0.0
C24:1 c ω9	1.6 ± 0.1	0.7 ± 0.0	1.4 ± 0.1
Σ MUFA	**29.8 ± 0.1**	**29.9 ± 0.0**	**30.0 ± 0.3**
C16:2 c ω4	0.6 ± 0.0	0.9 ± 0.0	0.7 ± 0.0
C18:2 c ω6	1.5 ± 0.0	1.6 ± 0.0	1.5 ± 0.0
C18:3 c ω3	0.9 ± 0.0	1.3 ± 0.0	1.0 ± 0.0
C20:3 c ω3	0.8 ± 0.0	0.5 ± 0.0	0.8 ± 0.0
C18:4 c ω3	2.7 ± 0.1	4.4 ± 0.0	3.4 ± 0.0
C20:4 c ω5	0.7 ± 0.0	0.9 ± 0.0	0.8 ± 0.0
C20:5 c ω3	6.7 ± 0.2	9.3 ± 0.0	8.3 ± 0.1
C22:5 c ω3	0.8 ± 0.0	1.0 ± 0.0	0.9 ± 0.0
C22:6 c ω3	12.0 ± 0.5	10.3 ± 0.0	12.6 ± 0.4
Σ PUFA	**24.5 ± 0.9**	**27.7 ± 0.1**	**27.9 ± 0.5**

Table 6
Neutral lipid and phospholipid fatty acid composition (%) in the insoluble fraction generated during the optimized hydrolysis of sardine heads by Protamex. SFA: saturated fatty acids, MUFA: mono-unsaturated fatty acids, PUFA: poly-unsaturated fatty acids

	Neutral lipids	Phospholipids
C14:0	7.3 ± 0.2	2.0 ± 0.2
C16:0	25.8 ± 1.0	42.0 ± 1.2
C18:0	5.9 ± 0.1	12.2 ± 0.3
Σ **SFA**	**39.0 ± 1.0**	**56.2 ± 1.2**
C16:1 c ω7	7.5 ± 0.1	3.8 ± 0.2
C18:1 c ω9	12.5 ± 0.2	20.8 ± 0.2
C18:1 c ω7	3.8 ± 0.1	4.6 ± 0.0
C22:1 c ω9	1.0 ± 0.0	- -
Σ **MUFA**	**24.8 ± 0.4**	**29.1 ± 0.3**
C18:2 c ω6	1.4 ± 0.0	- -
C22:2 c ω6	6.3 ± 0.1	- -
C20:3 c ω6	1.0 ± 0.0	- -
C18:4 c ω3	2.2 ± 0.1	- -
C20:4 c ω3	5.3 ± 0.1	- -
C20:5 c ω3	7.2 ± 0.4	4.0 ± 0.3
C22:5 c ω3	0.9 ± 0.1	- -
C22:6 c ω3	12.1 ± 0.6	10.7 ± 1.2
Σ **PUFA**	**36.3 ± 1.1**	**14.7 ± 1.5**

Table 7
Neutral lipid fatty acid composition (%) in the oily fraction generated during the optimized hydrolysis of sardine heads by Protamex

	Mean		Standard deviation
C14:0	8.3	±	0.2
C16:0	21.7	±	0.3
C18:0	4.5	±	0.1
Σ SFA	**34.4**	**±**	**0.3**
C16:1 c ω7	8.6	±	0.1
C18:1 c ω9	11.0	±	0.1
C18:1 c ω7	3.6	±	0.0
C22:1 c ω9	0.9	±	0.0
Σ MUFA	**24.0**	**±**	**0.2**
C18:2 c ω6	1.5	±	0.0
C22:2 c ω6	7.8	±	0.1
C20:3 c ω6	1.8	±	0.0
C18:4 c ω3	3.0	±	0.0
C20:4 c ω3	6.0	±	0.1
C20:5 c ω3	9.9	±	0.2
C22:5 c ω3	1.1	±	0.0
C22:6 c ω3	11.2	±	0.3
Σ PUFA	**41.7**	**±**	**0.4**

Table 8
Neutral lipid and phospholipid fatty acid composition (%) in the soluble fraction generated during the optimized hydrolysis of sardine heads by Protamex

	Neutral lipids	Phospholipids
C14:0	7.6 ± 0.4	1.6 ± 0.2
C16:0	23.3 ± 1.1	31.1 ± 0.8
C18:0	5.1 ± 0.0	14.1 ± 0.3
Σ SFA	**35.9 ± 1.4**	**46.8 ± 0.9**
C16:1 c ω7	8.0 ± 0.3	3.3 ± 0.2
C18:1 c ω9	11.8 ± 0.2	23.1 ± 0.5
C18:1 c ω7	3.6 ± 0.1	3.8 ± 0.1
C24:1 c ω9	- -	2.7 ± 0.1
Σ MUFA	**23.4 ± 0.5**	**32.9 ± 0.3**
C18:2 c ω6	1.5 ± 0.0	- -
C22:2 c ω6	6.2 ± 0.5	- -
C20:3 c ω6	0.8 ± 0.7	- -
C18:4 c ω3	2.9 ± 0.1	- -
C20:4 c ω3	5.6 ± 0.2	- -
C20:5 c ω3	9.7 ± 0.2	5.0 ± 0.1
C22:5 c ω3	0.7 ± 0.6	- -
C22:6 c ω3	13.4 ± 0.5	15.3 ± 0.4
Σ PUFA	**40.8 ± 1.7**	**20.3 ± 0.5**

Figures

Estimated Response Surface

Time = 29 min

Estimated Response Surface

Enzyme = 2.6 g/kg

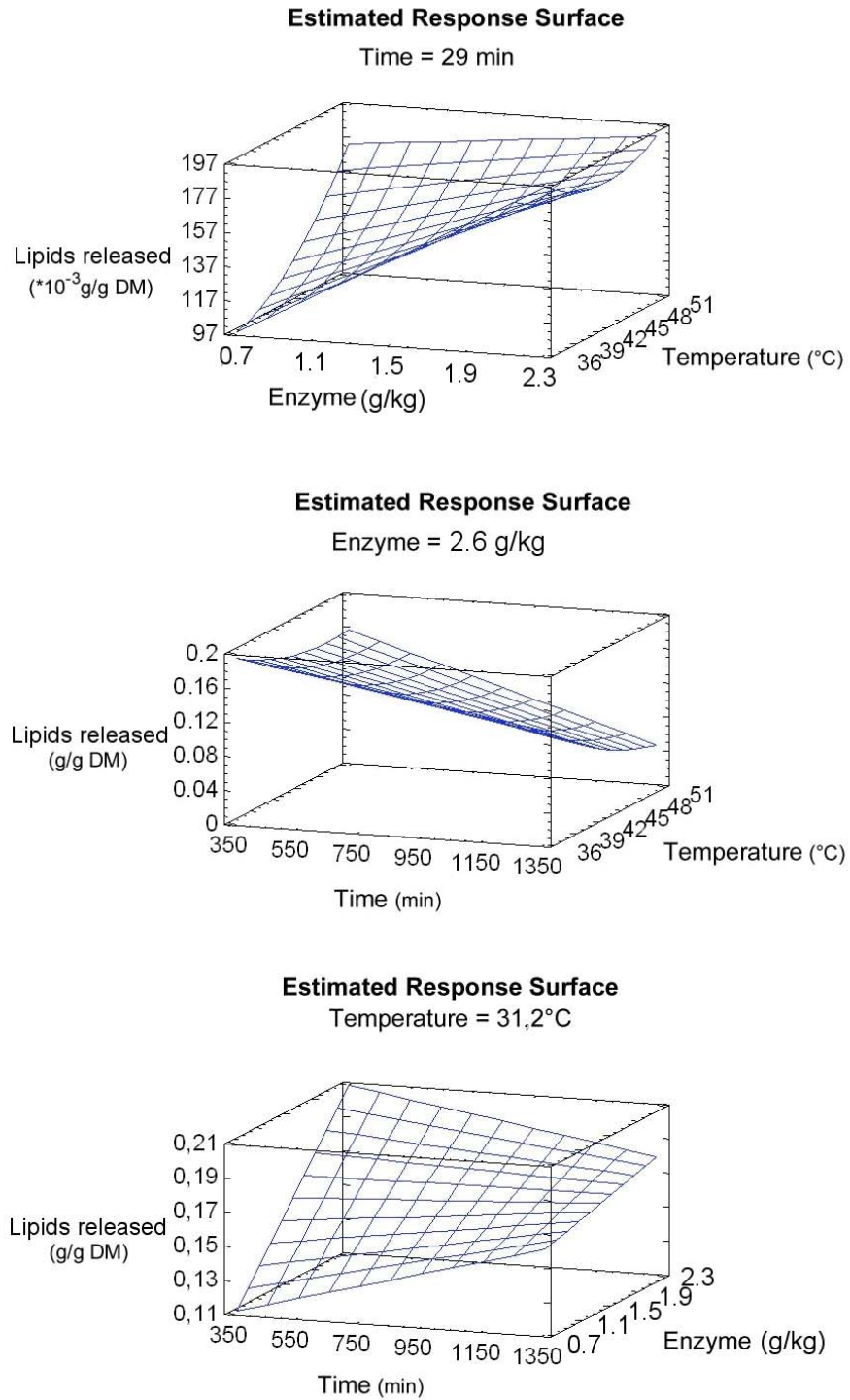**Estimated Response Surface**

Temperature = 31,2°C

Figure 1

Estimated Response Surfaces according to the time, enzyme and temperature parameters
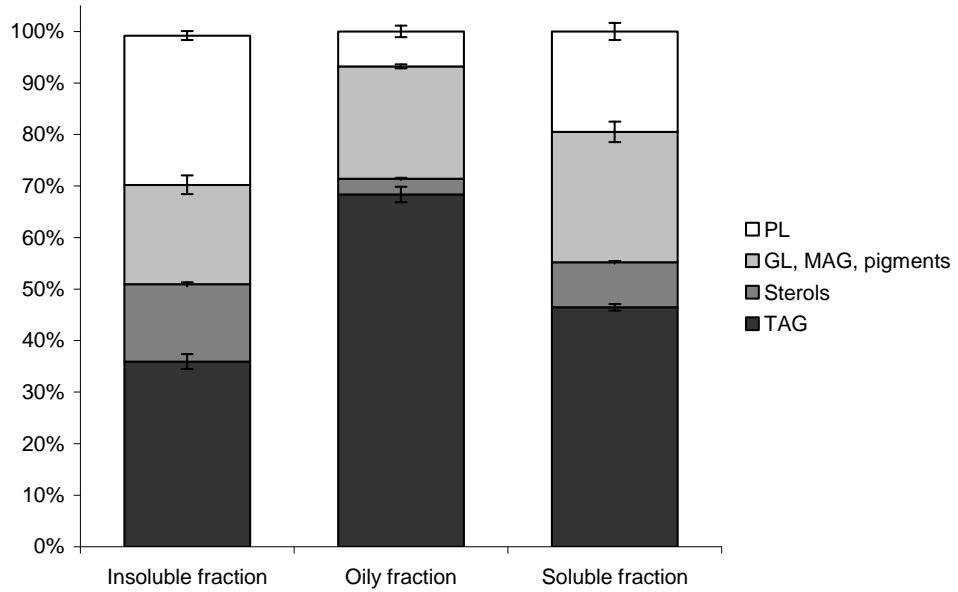
Figure 2
Lipid class distribution obtained by TLC-FID of fractions resulted from hydrolyzed sardine heads. PL: phospholipids, GL: glycolipids, MAG: monoacylglycerols, TAG: triacylglycerols