Enzymatic degreasing of whale bones *(Balaenoptera physalus)*

Dégraissage par voie enzymatique d'os de baleine (Balaenoptera physalus)

Laurent POISSON[1*], Françoise ERGAN[1], Gwenaël LEMOINE[2], Elodie GUIMINOT[2]

[*] corresponding author
[1] Unité Mer, Molécules, Santé, EA 2160, IUT de Laval, 52 rue des Drs Calmette et Guérin, BP 2045, 53020 Laval Cedex 9, France
laurent.poisson@univ-lemans.fr
[2] Laboratoire Arc'Antique, 26 rue de la Haute Forêt, 44300 Nantes, France

Abstract: Whale skeletons insufficiently degreased show a reduction of their long term conservation due to lipid degradation. The natural history museum of Nantes initiated a research program to investigate new and efficient methods for whale bone degreasing. Therefore an enzymatic approach has been proposed.
First, a biochemical analysis has been carried out showing that the lipids present on and in the bones were almost exclusively composed of free fatty acids, mainly oleic and palmitic acids. Then, a screening has been implemented to determine among 11 commercial lipase preparations the most efficient to catalyze esterification of free fatty acids with ethanol. The lipase preparation DF "Amano" 15 gave the best results and was chosen for further trials of enzymatic degreasing of whale bone samples. Comparing with solvent degreasing in acetone, the enzymatic treatment appeared to be not very efficient. This could be explain by the enzyme inability to cross the periosteum thus to facilitate the migration of the grease from the inner of the bone to the surface.

Résumé : Les lipides présents sur les os de baleines insuffisamment dégraissés peuvent, en se dégradant, altérer la bonne conservation des squelettes exposés en muséum. C'est pourquoi le Muséum d'histoire naturelle de Nantes a initié un programme de recherche visant à établir de nouvelles méthodes de dégraissage des squelettes gras de baleines. Dans ce cadre, une approche enzymatique a été proposée.
Une étude biochimique a tout d'abord été réalisée, permettant de montrer que la graisse présente à la surface et à l'intérieur de l'os était presque exclusivement constituée d'acides gras libres et principalement des acides oléique et palmitique. Ensuite, un criblage d'enzymes a permis de sélectionner, parmi 11 préparations commerciales, la lipase DF « Amano » 15 car elle donnait les meilleurs résultats dans le cadre de l'estérification d'acides gras et avec de l'éthanol. Cette préparation enzymatique a été retenue pour des essais de dégraissage sur des échantillons d'os de baleine. En comparant avec un dégraissage à l'acétone, le traitement enzymatique ne semble pas vraiment concluant, vraisemblablement à cause de l'incapacité de l'enzyme à traverser le périoste et donc à avoir un effet sur la migration de la graisse de l'intérieur de l'os vers la surface.

1. Introduction

Like many other museums, the natural history museum of Nantes encounters difficulties in the exhibition and preservation of skeletons insufficiently degreased. This is especially the case of a common rorqual (*Balaenoptera physalus*) skeleton exposed since 1995.
Indeed, despite a preparation consisting in 18 months of maceration and 6 months of degreasing in organic solvent baths, large brown spots of grease coming by seepage from the bone core, can be observed on the skeleton. Apart a loss of esthetic quality, the biochemical degradation of lipids (hydrolysis, oxidation...) strengthened by an microorganism development, may lead to the spoilage of the collagen fibers and to a reduction of the long-term conservation of the skeleton.
In this area, investigations on alternative degreasing methods have recently been initiated with a

view to restore the rorqual skeleton of the natural history museum of Nantes and more generally all the greasy cetacean skeletons exposed in museums [1].
In this article, a biochemical analysis of the lipids found on/in the bones of the rorqual skeleton is first implemented. Then, in a second part, an enzymatic approach for degreasing of whale bones is presented.

2. Materials and methods

2.1. Materials

Surface lipid samples were collected by softly scratching greasy areas of the bones with a scalpel. Inner lipids were collected by core sampling using a trocar. Core-samples were approximately 3 mm in diameter and 130 mm long. Enzymatic trials were conducted using pieces of bones of an approximate mass of 15g coming from a whale vertebra (Research Centre on Marine Mammalians, La Rochelle) sliced with a bandsaw. All solvents, chemicals and fatty acid standards were obtained commercially and were of analytical grade. Enzymatic preparations used for this work are presented in table 1.

	Brand name	**Physical form**	**Source**	**Company**
1	Lipase AY 30	Free, Powder	**Candida rugosa**	Amano Enzyme, UK
2	Lipase F-AP 15	Free, Powder	*Rhizopus oryzae*	Amano Enzyme, UK
3	Lipase M 10	Free, Powder	**Muco javanicus**	Amano Enzyme, UK
4	Lipase DF 15	Free, Powder	**Rhizopus oryzae**	Amano Enzyme, UK
5	Lipase A 12	Free, Powder	**Aspergillus niger**	Amano Enzyme, UK
6	Lipozyme TL IM	Immobilised, Resin granulate	**Thermomyces lanuginosus**	Novozymes, DK
7	Lipase Acrylic Resin	Immobilised, Resin granulate	*Candida antarctica*	Novozymes, DK
8	Novozyme 435	***Immobilised, Resin granulate***	**Candida antarctica**	Novozymes, DK
9	Lipozyme RM IM	Immobilised, Resin granulate	*Rhizomucor miehei*	Novozymes, DK
10	Lecitase 10L	***Free, Liquid***	***Porcine pancreas***	Novozymes, DK
11	Lipozyme TL 100L	***Free, Liquid***	**Thermomyces lanuginosus**	Novozymes, DK

Table 1: Features of enzyme preparations tested in the screening

2.2. Total lipid analysis

Lipid analysis was carried out through thin layer chromatography (TLC) on 20 x 20 cm silica plates (Silica Gel 60, Merck, Fontenay-sous-Bois, France). Separation of components was performed by a double development in one dimension [8]. The first development was performed in methyl acetate/propan-2-ol/chloroform/methanol/0.25 % aqueous KCl (25:25:25:10:9; v/v/v/v/v) to half final distance. The plate was dried at room temperature for about 10 minutes and the second

development was performed in hexane / diethyl ether / glacial acetic acid (80:20:2; v/v/v) on the full plate length. After drying at room temperature, components were visualised by spraying an α-naphtol solution (0.25 g α-naphtol/50 mL ethanol / 50 mL sulphuric acid 20 %) and incubating 10 minutes at 100 °C. Stains were identified by comparison of their R_f values with those of standards analysed under the same conditions.

2.3. Fatty acid analysis

Fatty acid composition of lipids was analysed through saponification followed by methylation of the fatty acids and gas chromatography (GC) analysis of the esters [9].
The lipid extract was first dried under a stream of nitrogen and the residue was collected in 1 mL of methanolic sodium hydroxide (0.5 M). Saponification of fatty acids was conducted at 80 °C for 15 minutes.
The sample was then cooled and 2 mL of 12 % methanolic boron trifluoride (BF_3) were added. Fatty acids were methylated at 80 °C for 20 minutes. Fatty acid methyl esters (FAME) were then extracted twice with 2 mL of isooctane. The upper isooctane layer (containing FAME) was recovered and rinsed with water to wash out the remaining sodium hydroxide and BF_3. Rinsing was performed until pH of water reached 7. The lipid mixture was then dried on sodium sulphate before GC analysis.
FAME analyses were carried out by GC using a GC Focus (Thermo Electron Corporation, Les Ulis, France) equipped with a split injector (1:15) at 250 °C and a Flame Ionization Detector at 280 °C. Separation was performed with a CP-SIL 88 column (25 m x 0.25 mm, 0.2 µm film thickness, Varian, Les Ulis, France). Nitrogen was used as carrier gas (1 $mL.min^{-1}$). Oven temperature was maintained at 120 °C for 4 minutes, then raised to 220 °C at 6 $C.min^{-1}$. Peaks were identified by comparison of their retention times to those of standards analysed under the same conditions.

2.4 Enzymatic trials

For enzymatic degreasing on bones, bones samples (see 2.1.), of similar shape (4.5 x 3 x 1 cm) and mass (15 g) with approximately the same proportion of spongiosa and periosteum, have been submerged in closed vessels in different solutions:

- Dry ethanol (dried under molecular sieves) : negative blank
- Acetone : positive blank
- Enzyme preparation in dry ethanol 10 % (m/vol): assay

Each vessel was then incubated at 30 °C and shaken at 170 rpm.

3. Results and discussion

3.1. Lipid analyses

In whales, lipids are considered to play important physiological functions like thermal insulation, energy storage or buoyancy.
The lipids could mainly be found in the blubber, an underskin fat layer and in the spermaceti, a waxy substance located in a cavity of the head of the animal. Lower lipid content could also be found in milk, liver and muscles. Lipid composition of these fat deposits has been described in several papers [2-7] but few data are available in literature concerning lipids in whale bones.
Double development thin layer chromatography allowed the resolution of neutral and polar lipids on a single plate. The first development allowed the separation of polar lipids on the bottom of the plate whereas the second development allowed the separation of neutral lipids on the top of the plate.

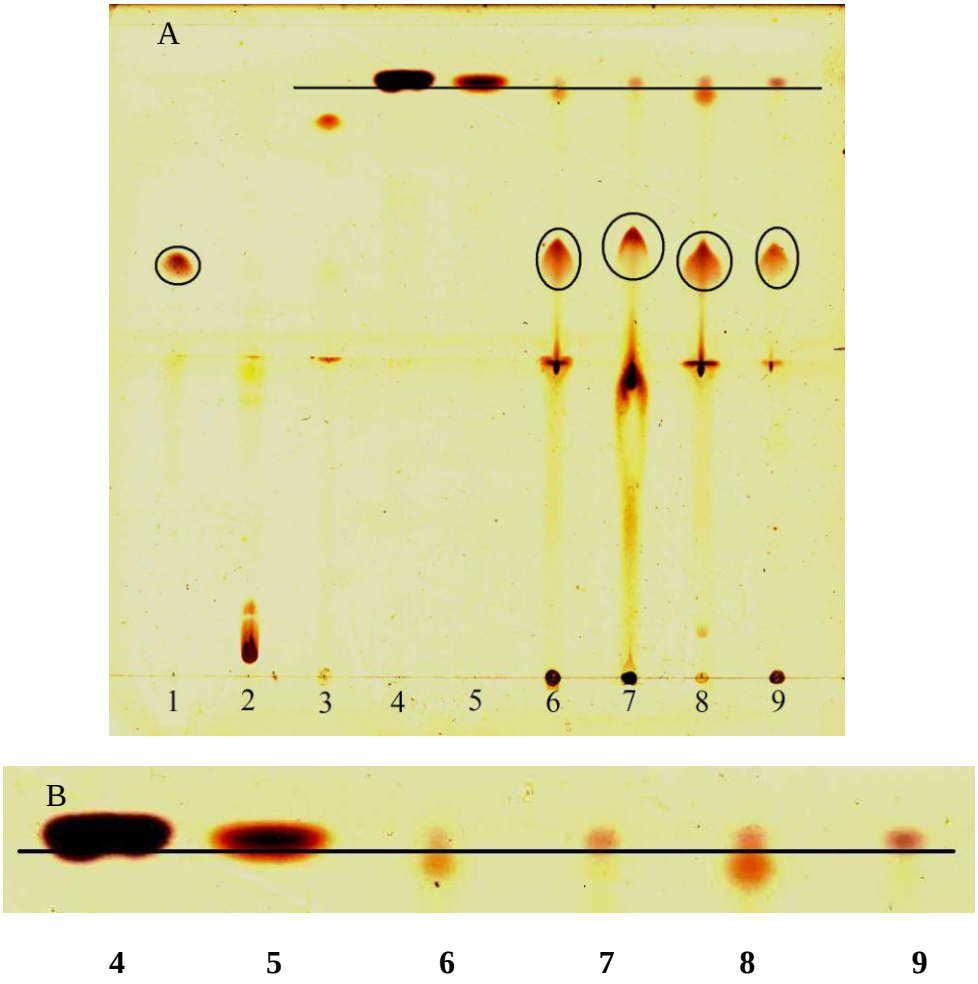

Figure 1: Two-dimensional Thin Layer Chromatography of lipid collected on/in cetacean bones.
A. whole chomatographic plate. 1. free fatty acid - 22:6, 2. phospholipids - phosphatidyl choline, 3. triglycerides - triolein, 4. cholesterol ester - 5. wax - oleyl oleate, 6. surface lipids of cetacean vertebra, 7. surface lipids collected on a shoulder blade of the roqual exposed in Nantes, 8. inner lipids of cetacean vertebra, 9. inner lipids collected on a spare bone (fin) of the roqual exposed in Nantes. B. Top spot focus.

The figure 1A shows on lanes 6 to 9, the chromatographic separation obtained with surface and core -samples collected on an unknown cetacean vertebra and on the skeleton of the roqual exhibited in Nantes natural history museum. No significant difference is observed between the chromatographic profiles of the four samples. As compared to the standards spotted on line 1 to 5, no phospholipids seem to be present in the rorqual or cetacean lipids whatever the type of sample. Besides, no other polar lipid can be noticed on the bottom of the plate. At the solvent front level corresponding to the first development, a streak of an undefined component is observed mainly on lane 7. It might be associated with a non lipidic component or a product of lipid degradation. In Henderson and Tocher (1992) [8], equivalent *Rf* are given for pigments. Fig. 1A also highlights the fact that triglycerides are not present whereas important spots of free fatty acids can be observed. Ambiguous spots near the solvent front have been focused on fig 1B. which reveals that waxes were likely present in each of the four samples. In the lipids collected on/in the vertebra of the unknown cetacean, a component corresponding to none of the spotted standards can also be noticed.
Fatty acid analyses (Table 2) shows that 2 fatty acids are mainly present in all samples: oleic acid (18:1) and palmitic acid (16:0). Four other fatty acids exhibit contents of minor importance: palmitoleic acid (16:1), gadoleic acid (20:1), myristic acid (14:0) and cetoleic acid (22:1). This profile is rather consistent with data published in literature [2, 5].

Table 2: Fatty acid composition (%, w/w) of bones from cetacean skeletons (n = at least 3 replicates)

Fatty acid	Cetacean 1		Cetacean 2		Rorqual 1	Rorqual 2	Rorqual exposed in Nantes			
	Vertebra Surface	Vertebra Inner	Rib Surface	Rib Inner	Metacarpus Surface	Skull Surface	Oil	Shoulder blade surface	Vertebra surface	Rib surface
12:0	1,2 ± 0,06	0,93 ± 0,1	0,16 ± 0,01		0,16 ± 0,03		0,21 ± 0,01	0,37 ± 0,01	0,2 ± 0,01	0,44 ± 0,1
14:0	10,08 ± 0,35	8,3 ± 0,23	4,85 ± 0,06	5,93 ± 0,08	3,71 ± 0,05	2,3 ± 0,02	5,7 ± 0,12	7,24 ± 0,09	6,2 ± 0,09	5,47 ± 0,14
							1,14 ± 0,02			
16:0	31,69 ± 0,5	25,97 ± 0,29	22,43 ± 0,46	32,63 ± 0,18	7,33 ± 0,08	4,74 ± 0,13	10,41 ± 0,07	11,3 ± 0,12	8,82 ± 0,12	5,89 ± 0,11
									1,23 ± 0,04	
16:1	3,8 ± 0,1	3,87 ± 0,05	4,38 ± 0,19	4,2 ± 0,02	11,41 ± 0,13	4,69 ± 0,03	7,85 ± 0,05	13,14 ± 0,13	3,39 ± 0,17	3,56 ± 0,05
17:0	1,55 ± 0,01	1,52 ± 0,01	0,96 ± 0,1	1,53 ± 0,02	0,69 ± 0,01	1,14 ± 0,01	0,88 ± 0,01	0,55 ± 0,38	0,31 ± 0,21	0,44 ± 0,06
							1,24 ± 0,01		0,96 ± 0,02	
									0,75 ± 0,02	
18:0	6,94 ± 0,09	5,89 ± 0,06	4,72 ± 0,12	7,02 ± 0,15	1,47 ± 0,01	0,83 ± 0,01	2,2 ± 0,02	2,06 ± 0,02	11,3 ± 0,17	1,46 ± 0,03
18:1	12,53 ± 0,12	13,38 ± 0,5	20,97 ± 0,28	21,2 ± 0,36	22,31 ± 0,06	33,97 ± 0,54	39,73 ± 0,24	28,14 ± 0,56	25,58 ± 0,31	20,56 ± 0,54
						1,21 ± 0,01				
18:2	0,81 ± 0,05	0,77 ± 0,07	1,36 ± 0,02	1,23 ± 0,02	0,44 ± 0,04	1,87 ± 0,03	2,09 ± 0,01	1,05 ± 0,36	0,81 ± 0,03	1,1 ± 0,02
18:3	0,03 ± 0		0,27 ± 0,01	0,27 ± 0,01			0,78 ± 0,01	0,18 ± 0,02	0,11 ± 0	0,15 ± 0,01
20:1	9,41 ± 0,33	9,55 ± 0,19	7,79 ± 0,02	7,31 ± 0,15	20,32 ± 0,26	15,6 ± 0,09	5,89 ± 0,04	6,27 ± 0,17	5,43 ± 0,34	4,73 ± 0,09
									1,64 ± 0,07	2,82 ± 0,06
			1,41 ± 0,04			1,05 ± 0,03			6,83 ± 0,24	5,44 ± 0,21
										2,41 ± 0,16
22:1	6,74 ± 0,3	7,08 ± 0,13	7,85 ± 0,15	7,85 ± 0,14	18,48 ± 0,07	17,73 ± 0,06	2,24 ± 0,06	2,57 ± 0,11	2,34 ± 0,03	2,04 ± 0,12
										2,75 ± 0,23
20:5	0,47 ± 0,01	0,53 ± 0,07	0,75 ± 0,27	0,87 ± 0,02	0,49 ± 0	0,49 ± 0,01	5,51 ± 0,03	0,29 ± 0,02	0,16 ± 0,08	0,27 ± 0,11
						1,37 ± 0,07				2,45 ± 0,11
			1,18 ± 0,15		1,14 ± 0,02					2,88 ± 0,18
							2,28 ± 0,02			
22:6	0,03 ± 0		0, 7 ± 0,03	0,53 ± 0,01	0,07 ± 0,01	0,43 ±0,14	0,47 ± 0,02	0,56 ± 0,05	0,13 ± 0,01	0,55 ± 0,02
										2,27 ± 0,08
										1,63 ± 0,43
	4,48 ± 0,37	10,88 ± 0,36	2,95 ± 0,07	1,39 ± 0,03	0,12 ± 0,01	0,81 ± 0,01		5,31 ± 0,1	4,98 ± 0,26	6,69 ± 0,73
Total [a]	93,11 ± 2,4	88,67 ± 2,06	82,65 ± 1,98	91,96 ± 1,19	88,14 ± 0,78	88,23 ± 1,19	88,62 ± 0,74	79,03 ± 2,14	81,17 ± 2,22	76,00 ± 3,59

[a] Total does not account for 100% since minor peaks were not assigned

3.2. Lipase catalysed degreasing

Lipid hydrolysis could be considered as the "natural" reaction of lipases. This reaction mainly consists in the degradation of triglycerides in aqueous medium to release fatty acids and glycerol. Lipases could also catalyzed the reversed reaction of hydrolysis: the esterification. Nevertheless to allow this reaction certain conditions, like a low water content of the medium, need to be carefully considered.Since lipids found on the skeleton are very degraded and only composed of free fatty acids, the implementation of a hydrolysis reaction appeared to be inappropriate. Nevertheless the solubility of a fatty acid is much lower than the solubility of its corresponding ethyl ester (Fig. 2). For this reason a lipase-catalyzed esterification of fatty acids with ethanol was chosen as a strategy to try to dissolve the grease contained in the whale bones.

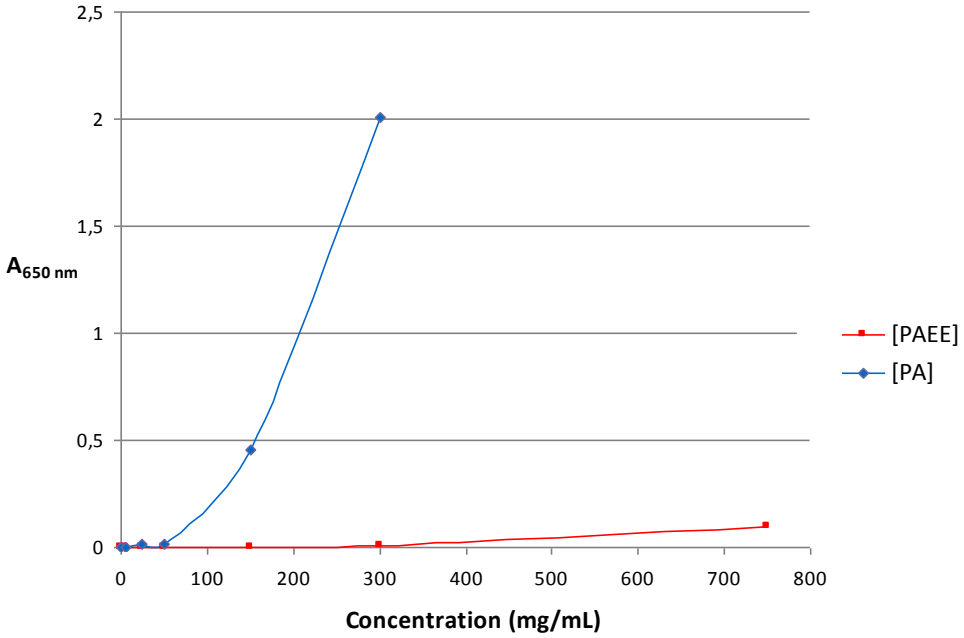

Figure 2: Solubility of Palmitic Acid (PA) and Palmitic Acid Ethyl Ester (PAEE) in ethanol evaluated by solution turbidity at 650nm

The figure 3 shows the results of the screening of 11 lipase preparations. Among the lipase preparations tested, Lipase DF Amano 15 gave the best results that is to say the most important ester production from fatty acids and ethanol. This enzymatic preparation was then chosen for an enzymatic degreasing on whale bone.

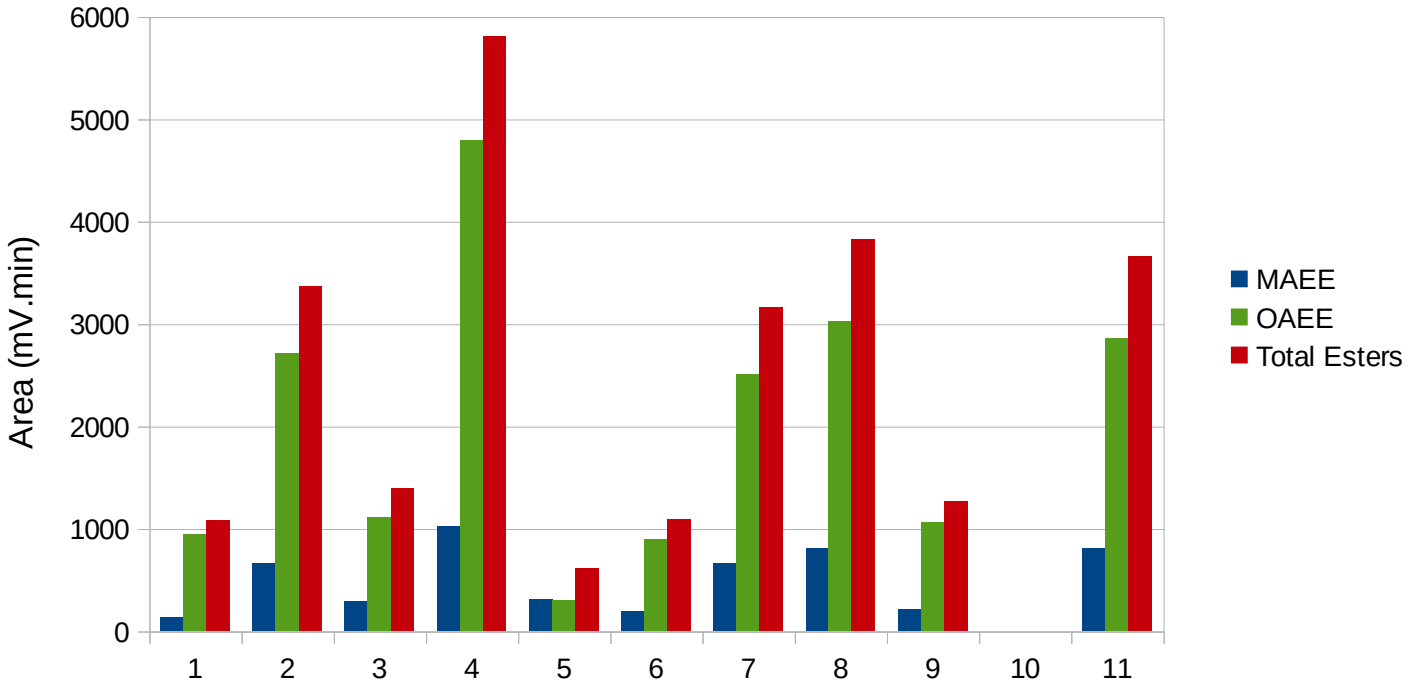

Figure 3: Lipase screening. Ability of different enzyme preparations (see table 1) to synthesise ethyl esters from fatty acids and ethanol. MAEE: Myristic Acid Ethyl Ester. OAEE: Oleic Acid Ethyl Ester.

After 72h of incubation, bone pieces were recovered and their changes in aspect and mass were examined. The results (Fig.4.) show that the efficiency of the enzymatic degreasing is very weak and close to acetone effect for the visual aspect as well as for the mass loss that is to say for the loss of grease. In fact it seems that the enzymatic treatment is slightly more efficient than acetone on the

bone surface but less efficient in the inner (data not shown) and this is especially emphasized when the proportion of periosteum is important. In fact, the enzyme seemed to be efficient only on the bone surface and seemed unable to reach the inner core of the bone. As the migration of the grease from the inner of the bone to the surface seemed to be a more important factor for the efficiency of the degreasing than the solubilization of the grease on surface, an enzymatic treatment appears to be an inadapted solution.

	T0	**T=72h**	**Weight loss (%)**
Dry ethanol			10.7
Acetone			13.4
Lipase DF 15 in dry ethanol (10% m/vol)			14.6

Figure 4: Aspect of the bone samples and weight loss after 72 h of immersion in different solutions under agitation at 30°C.

4. Conclusion

This work allows for a definition of the nature of lipids present on the whale bones. Moreover, after this study, we could assess that an enzymatic treatment does not appear to be convenient compared with solvent engineering for a proper degreasing due to the enzyme inability to penetrate the bone structure and especially the periosteum.

However the use of lipases could be of interest for future work in the field of the preparation of skeleton before its exhibition. Indeed, in that case the “natural” reaction of lipases, lipid hydrolysis, could be implemented. This may lead to a better grease dispersion and then to a more efficient subsequent solvent treatment of the skeleton.

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