Production of High-Quality Fish Oil from Herring Byproducts

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When the Lord created the world and people to live in it- an enterprise which, according to modern science, took a very long time – I could well imagine that He reasoned with Himself as follows: “If I make everything predictable, these humans beings whom I have endowed with pretty good brains, will undoubtedly learn to predict everything, and they will thereupon have no motive to do anything at all, because they will recognize that the future is totally determined and cannot be influenced by any human action. On the other hand, if I make everything unpredictable, they will gradually discover that there is no rational basis for any decision whatsoever and, as in the first case, they will thereupon have no motive to do anything at all. Neither scheme would make sense. I must therefore create a mixture of the two. Let some things be predictable and let others be unpredictable. They will then, amongst many other things, have the very important task of finding out which is which.”

E. F. Schumacker, Small Is Beautiful (1973).
Published by Hartley and Marks, Vancouver, Canada.

Aos meus pais

À memória do meu avô, José dos Aidos

ABBREVIATIONS USED

a,	Number of analyses of each sample preparation n;
AAS,	Atomic absorption spectrophotometry;
AS,	Advanced screening stage;
AV,	Anisidine Value;
CD,	Conjugated dienes, absorbance measured at 235 nm;
CT,	Conjugated trienes, absorbance measured at 270 nm;
D,	Speed of the three-phase decanter;
DHA,	4, 7, 10, 13, 16, 19-Docosahexaenoic acid;
df,	Degrees of freedom;
EPA,	5, 8, 11, 14, 17-Eicosapentaenoic acid;
F,	F-test;
FC,	Lipid soluble fluorescent oxidation products;
FIA,	Flow injection analysis;
FFA,	Free Fatty Acids;
Fresh oil,	Oil extracted from fresh unsalted herring byproducts;
Frozen oil,	Oil extracted from unsalted byproducts originated from frozen herring.
GLM,	General Linear Models;
HPLC,	High-performance liquid chromatography;
L&H,	Long and Hammer's medium;
Maatjes oil,	Oil extracted from salted byproducts originated from frozen herring;
MP,	Speed of the mono-pump;
MS,	Mean squares;
MUFAs,	Monounsaturated fatty acids;
n,	Number of samples analyzed;
NL,	Neutral lipids;
P, p,	Probability level;
PC,	Principal component;
PCA,	Principal component analysis;
PL,	Phospholipids;
PLS,	Partial least square regression;
PO,	Process optimization stage;
PUFAs,	Polyunsaturated fatty acids;
PUFA,	PUFAs excluding EPA and DHA;
PV,	Peroxide Value;
Sat,	Saturated fatty acids;
SD,	Standard deviation,
SH,	Oil extracted from headless herring byproducts;
SM,	Oil extracted from mixed herring byproducts;
T,	Temperature of the heat exchanger.
TIC,	Total ion current;
Tocop.	α-Tocopherol;
TVB,	Total volatile bases.

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Isabel Aidos
Haarlem, August 2002.

Chapter 1

Introduction

The world annual catch of fish and marine invertebrates is approximately 100 million metric tons (*1*), from which some 20% are processed for food use. Approximately 30% of the latter amount is actually consumed; the rest is generally discarded as waste (*2, 3*). More complete utilization of the fish processed for human consumption can be achieved by conversion of heads, frames, fins and visceras into fish meal and fish oil. Fish meal is a ground solid product that is obtained by removing most of the water and some or all oil from fish or fish byproducts and is mainly used for the production of animal feeds (*4*). Fish oil is the lipid fraction that can be extracted from the fish or fish byproducts.

There is a good world market demand for high quality fish meal and oil, and production can be quite profitable if suitable raw material is available. Clupeids (e.g. herring and sprat) provide the largest single source of raw material for production of fish meal and oil. They may be classified as fatty although the fat content may vary from 2 to 30% depending on species and seasons (*5*). Industrial fisheries have large impact on the herring stocks and in a time that ocean depletion is all too real, fishing for exclusive production of fish meal and fish oil should not be encouraged. On the other hand, during fish processing, operations such as gutting and filleting, generate offal that is very often being considered waste and is therefore discarded. In order to maximize the utilization of the fish that is caught, the waste that is generated in the processing of fish, should be used as good as possible. Skåra and Cripps (*6*) found that byproducts from aquacultured salmon could be used to produce fish oil of a quality that is suited for human consumption. Sun and co-authors found that it is possible to concentrate ω-3 fatty acids in the oil extracted from salmon's viscera (*7*). Besides salmon byproducts, also byproducts from other fish species with high fat content such as herring and mackerel could be used as a raw material source for oil recovery.

In The Netherlands the main fatty fish species that is processed for human consumption is herring. The fat is not concentrated in the liver as in lean fish, but is distributed throughout the body (*5*). Presently, the Dutch fish industry sells their byproducts to fish meal plants in Germany and Denmark. The byproducts are transported to these countries and then converted into fish meal and fish oil of only moderate quality due to the long transportation times. An important factor in the

production of high quality fish oil is the condition of the starting raw material. If possible the fish should, after the catch, be stored under chilled conditions to minimize the effect of microbial, chemical and enzymatic attack on the fish tissue (*3*). This spoilage is responsible for, for example, increased free fatty acids content and increased oxidative breakdown by e.g., peroxidases (*8, 9*).

1.2 Fish oil and its usage

The difference between fish oil and other oils is mainly the unique variety of fatty acids it contains (*10-13*), including a high level of unsaturated fatty acids (*14*). Further, fish oil contains a high level of fatty acids with the first double bond between the third and fourth carbon atom, counted from the terminal methyl group. This family of fatty acids is called the ω-3, also sometimes marked as n-3, fatty acids and is found primary in oil of marine origin. Vegetable oils contain mainly unsaturated fatty acids from the ω-6 family and animal fat from the ω-9 family. The amount and variety of the fatty acids in fish oil varies from one fish species to another, and also with the biological stage, fish diet, fishing location, ocean temperature, nutritional and spawning state, etc. (*15-19*). In fish oil, the major ω-3 fatty acids present are the eicosapentaenoic acid (C20:5ω-3, commonly called EPA) and the docosahexaenoic acid (C22:6ω-3, commonly called DHA).

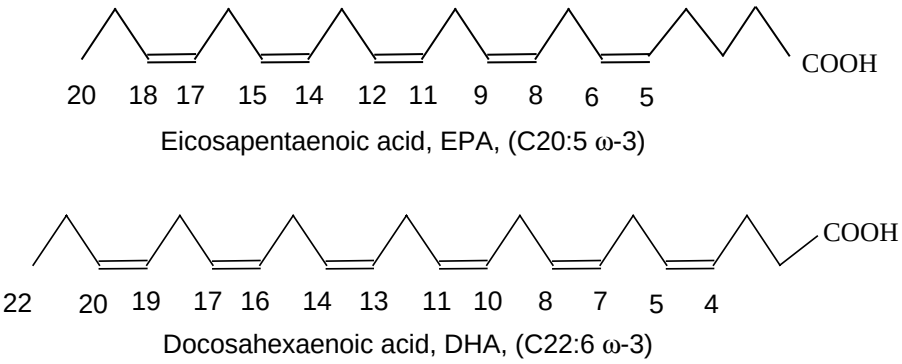

Figure 1- Schematic representation of EPA and DHA.

Human metabolism differentiates between the position of the first double bond, i.e., ω-3, ω-6, or ω-9. Discernable health implications from the consumption of these fatty acids have been reported (*20, 21*).

Marine oils are an important source of the already synthesized EPA and DHA. There are indications that EPA and DHA exert a positive influence on human health, related to proper neural development, the ability of seeing and learning and by modulation of eicosanoids synthesis decreasing the risk of cardiovascular diseases (atherosclerosis, thrombosis, stroke), certain cancers, diabetes, depression, immune disorders and others

(*22-28*). The indicated benefits of the intake of ω-3 caused an interest in high-quality fish oil with high levels of ω-3 fatty acids (*29*).

The market for liquid fish oil for human consumption can be divided into three areas: as a pharmaceutical component, as a health food component and as a commodity for the food industry. Further, fish oil can be an important constituent of aquaculture feeds, contributing with essential fatty acids needed by fish for normal growth, health and reproduction. In principle, fish oil may be used in any food item that contains fat. However, the use of fish oil may result in sensory problems: the products can obtain a "fishy" taste. Additionally, due to the susceptibility of fish oil to oxidation, the shelf life of the product may be seriously reduced.

Although the emphasis has been on the marketability of the ω-3 fatty acids related health benefits of fish oil, it is known that fish oil and fish liver oil contain other interesting compounds such as vitamin A and D. With improved separation techniques and more gentle processing methods, these oils might play an even more important role in the pharmaceutical and health food industry in the near future.

1.3 Processing of the herring and collection of the byproducts

In The Netherlands, maatjes and marinating herring have been applied as a conservation method for herring for centuries. Over the year, different herring products are processed according to the fat content. Figure 2 shows the flow chart of the two major processes.

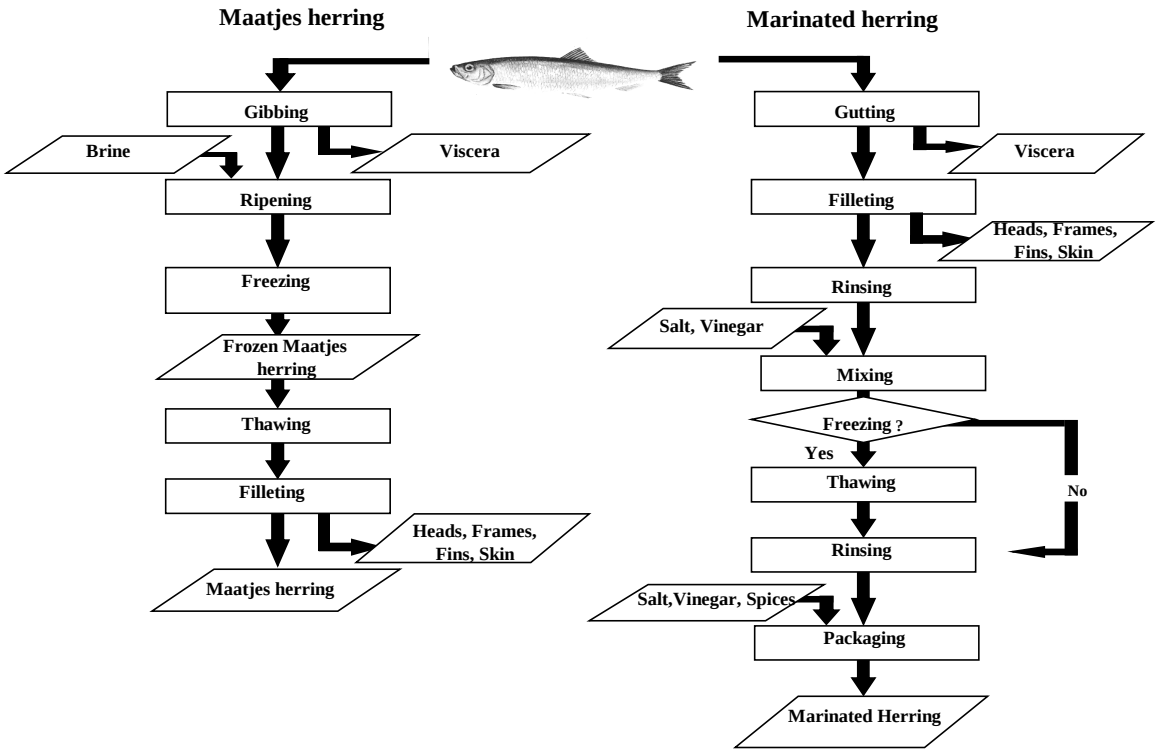

Figure 2- Flow chart of the maatjes and marinated herring production process.

Marinated herring

For this type of production, usually fresh herring is used as a raw material but frozen herring can be used as well. The first step in the process is gutting followed by filleting. After these steps, byproducts consisting of heads, fins, frames and organs are generated, which can be used for the oil recovery process. The byproducts derived from this process are free of any additives. After the filleting operation, the fillets are rinsed with water. The fillets are then put in a tumbler with a solution of salt, vinegar and water to make sure that the solution and the fillets are well mixed. The residence time of the fillets in the salt/vinegar solution depends on the concentration of salt used. Typically salt solutions are in the range of 3-4%. The salt concentration used is important for the effect that it has on the survival of nematodes in the fillets. The nematode *Anisakis marina* is killed by freezing for more than 24 hours at –20 °C or below. For this reason it depends on the salt concentration used in the solution whether the fillets have to be frozen prior to packaging. Before the herring is put into the jars it is rinsed with water. Then a mixture of water, vinegar, salt, pickles and spices is added and the process is complete.

Maatjes herring

The majority of the maatjes herring processed in the Netherlands requires a minimum fat content of 15% and is made from fresh herring landed and gibbed in Denmark and subsequently salted. The remainder is made from herring frozen in blocks immediately after the catch at sea. Gibbing is the manual removal of gills, liver, heart, intestines, stomach but not the pyloric appendix. In this production process, different salt concentrations can be used according to the demand of the buyers. In heavily salted herring, the pyloric appendix is left in order to achieve a high proteolytic enzyme activity during the ripening period, which induces a softening of the texture and also influences taste and odor. In lightly salted herring the ripening by the enzymes from the pyloric appendix affects mainly the texture of maatjes herring and not the taste and odor of the product (*30*).

The main production of maatjes herring uses lightly salted herring as a raw material. The herring is transported from Denmark to the Netherlands in barrels. After arrival it is sorted and packed in smaller buckets. Brine is added and the herring is frozen in order to kill the nematodes and to preserve the herring. Then the buckets are sold to fishmongers or ambulant traders. The byproducts from the maatjes herring that is sold in buckets are lost. Large processors also process maatjes herring fillets that are sold to the institutional market and to retailers. The byproducts of these processors consist of heads, viscera, and frames and may be available for further processing. The total amount of maatjes herring byproducts that is obtainable from the major Dutch

companies is 2100 tons/year. The byproducts from maatjes herring have been treated with salt, which may affect the quality of the oil, due to the presence of pro-oxidants such as copper and iron ions. These ions are often present as contaminants in the salt.

Lipid oxidation may be initiated by several organic and inorganic substances like e.g. Cu and Fe, often present in salt (*31*). The production of maatjes herring, a characteristic and popular product in The Netherlands and surrounding countries, is based on using salt for the conservation of the product (whole herring). In contrast, during the marinated process salt is only added after the filleting operation. Since the salt used in the industry is of a technical quality, a certain amount of pro-oxidants is expected to be present. These pro-oxidants (Cu and Fe) will be present in the herring fillets and probably also in the maatjes byproducts used for the fish oil recovery.

1.4 Fish oil extraction process

Presently, fish oil producers have several options for oil extraction, such as fractionation by high-speed centrifugation (*32*), low temperature solvent extraction (*33*), supercritical fluid extraction (*34*), as well as wet and steam rendering methods (*35-39*). In this research, the extraction of herring oil was performed using an adaptation of the Conkix Process from Alfa Laval®. This process has yielded good results with the extraction of fish oil from salmon byproducts (*6*). Figure 3 illustrates this process. The raw material is minced in order to ensure sufficiently small particle size. Then, the minced material is pumped to a heat exchanger in which the fish oil is released and the proteins denaturated. Immediately following this stage, a three-phase decanter is used to separate the stream into an oil phase, a diluted, aqueous phase, and a semi-solid, concentrated protein phase. Finally, the collected fish oil is cooled and stored. The oil produced from this process is defined as crude fish oil.

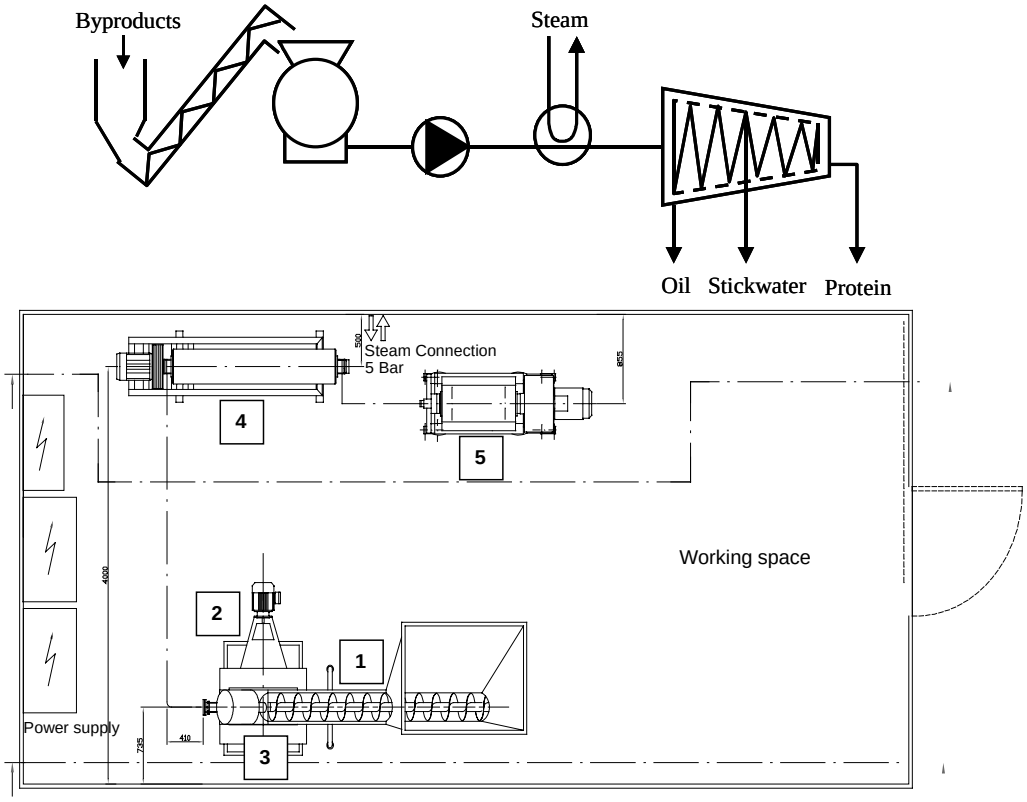

Figure 3- Layout of the pilot plant used for fish oil extraction process. 1: storage bin with feedscrew, 2: mincer, 3: mono-pump, 4: heat exchanger and 5: Decanter.

1.5 Preliminary costs estimation

The oil and fish meal industry requires a regular supply of raw material. It is therefore necessary to know the type and amount of fish species available, the length of the fishing season and the location of the fish and the variation in composition over the year. An inventory of the different herring products (composition and availability of the related byproducts) as well as a preliminary feasibility study for the byproducts in The Netherlands was carried out. In 2000 the total herring byproducts sold from the processing companies was approximately 27,300 tons, while in 2001 the figure dropped to 23,300 tons (*40*). The decrease is related to the decrease of herring processing due to the reduction of fishing quota. The market price of herring byproducts was between € 0.027 to € 0.036 per kg, resulting in a total turnover in byproducts of k€ 860 and 741, for 2000 and 2001, respectively. From an industrial point of view, of course it is important to obtain the best price for everything that leaves the plant. Hence, with herring byproducts containing at least ~ 13% of fat (*41*) it is of interest to process these byproducts into fish oil. Nowadays, with a high demand of fish oil, its price has increased from € 0.33 to € 0.39 per kg for the year 2000 and 2001, respectively (*42*). Considering the amount of available byproducts, the

oil alone would represent a revenue of k€ 1091 and k€ 1088 for the considered years. This is higher than the total price currently obtained for the total byproducts. Moreover, ~ 13% of the byproducts is protein (*41*). This protein phase could be processed further e.g. in fish meal. This could increase the revenue even more, since fish meal can be sold for more than € 0.56 per kg (*42*). Encouraged with this idea, a feasibility analysis for a complete byproducts fractionation plant, as shown in Figure 4, was performed. Given the mutual proximities of the fish processing companies, it is assume to transport and perform the upgrading of herring byproducts in one single unit rather than install several smaller plants. Therefore, in our estimation, a single plant with a capacity of 100 tons/day is studied.

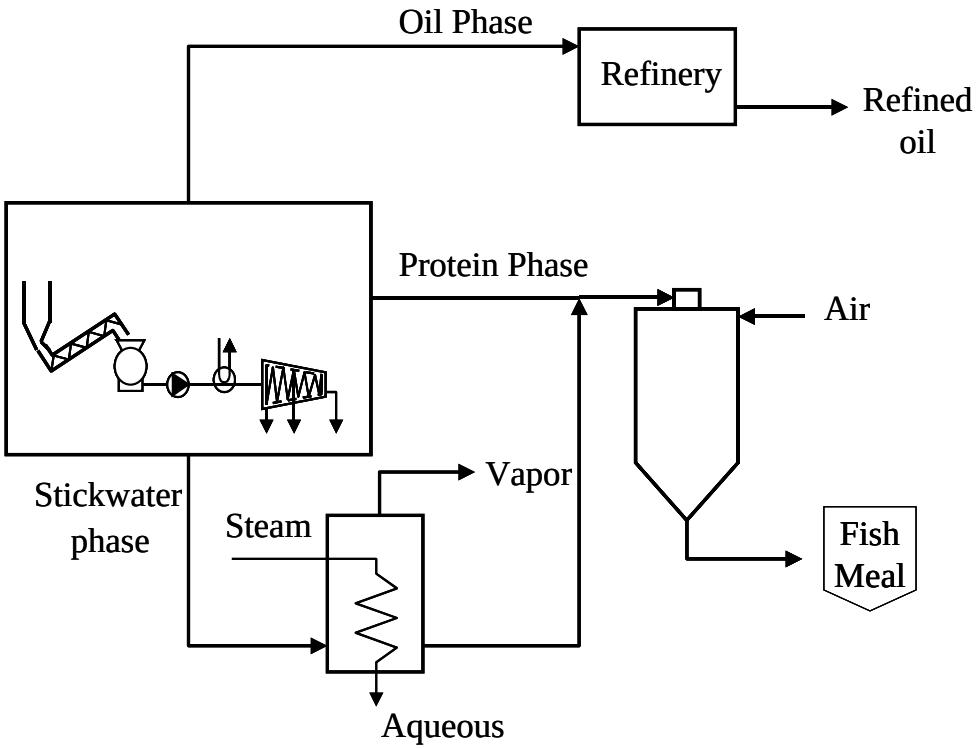

Figure 4- Flow diagram of a complete fish meal plant.

Table 1 shows results of a preliminary feasibility analysis. Generally, it can be concluded that from a financial point of view, the process appears to be feasible.

Table 1- Preliminary feasibility analysis for a complete fish meal plant with 100 tons/day capacity, calculations made by Alfa Laval Oil and Protein Technology (*43*).

Investment		k€
Plant type		
Conkix		705
Raw material		67
Evaporator		269
Dryer		403
Milling & Bagging		67
Other (steam boilers, hot water equipment, silos, etc.)		1,007
Erection & Commissioning		503
Fright, Insurance, Engineering, etc		13
Equipment investment		3,034
Building		268
Total investment		**3,302**
Raw material cost per year		336
Utilities and maintenance cost per year		1,553
Product sold per year		
Fish meal		2,454
Fish oil		1,510
Total		**3,964**
Operational Contribution First Year	62.84%	**2,075**
Pay back period on contribution		**1.6 years**
Interest 1st year	8%	264
Estimated return on Investment after interest 1st year	54.84%	1,811
Estimated pay back period after international interest rate of	8%	**2 years**

1.6 Oxidative changes

The high content of polyunsaturated fatty acids in fish oil, especially the ω-3 EPA and DHA, influences the stability and quality of the oil. Oxidative deterioration of the oil primary involves autoxidation accompanied by various other reactions (*44*). Primary autoxidation products are lipid hydroperoxides, generally referred to as peroxides, which decompose rapidly to a range of secondary oxidation products, such as ketones and alcohols, that may cause strong undesirable flavors in oils (*45*).

Lipids become rancid as a result of oxidation. High levels of oxidation products in the crude oil ensue in poor flavor stability in the final product. Special treatment may be needed to reduce them to acceptable levels. The lipid oxidation process is schematically illustrated in Figure 5. This process is a chain reaction that takes place through several intermediate stages: initiation, propagation and termination. The susceptibility and speed of oxidation of the fatty acids increase proportionally to their

degree of unsaturation (*44*). Fish oils, rich in polyunsaturated fatty acids, are therefore very sensitive to oxidation. In order to maintain the quality of the polyunsaturated fish oils during processing of the fish byproducts, the measurement of lipid oxidation products play an important role. In this study, the primary products were measured and followed with the peroxide value, conjugated dienes and trienes; secondary products with anisidine value, volatiles and sensory assessment, and the tertiary products were followed by measuring the concentration of fluorescence compounds.

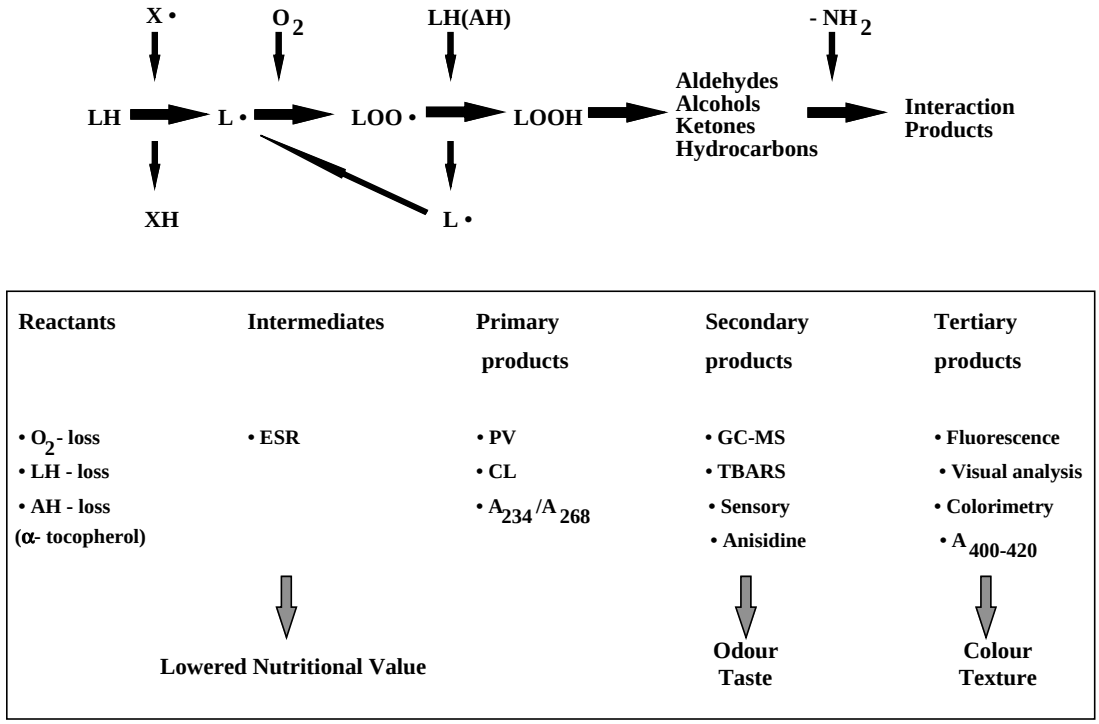

Figure 5– Schematic illustration of the lipid autoxidation process including different techniques that can be used to follow its progress and quality attributes that are affected. LH = fatty acid, X• = initiator, L• = alkyl radical, LOO•= peroxy radical, LOOH = hydroperoxide, ESR = electron spin resonance, PV = peroxide value, CL = chemiluminescence, $A_{234,268,\ 400\text{-}420}$ = absorbance at 234, 268 or 400-420 nm, GC-MS = gas chromatography with mass spectroscopy detection, TBARS = thiobarbituric acid reactive substance test, adapted from references (*46, 47*).

The measurement of the α-tocopherol content can be used as indicator of the total antioxidant content, which will be reduced during oxidation.

The fatty acid compositions of the oils vary widely with the fish species and, to some extent, with the composition of the plankton feed and the time of the year. These influence the suitability of the oils for edible and technical applications. The phospholipids content in fish oil is important because of the effects of these compounds as emulsifying agents in refining and as catalyst poisons during the hydrogenation step. Free fatty acids usually are controlled due to contractual reasons and also because it is still one of the most reliable parameters for oil quality, as an indication of the lipolysis extension, and yield assessment (*5*).

Hydroperoxides, which are the primary products of lipid oxidation, are very unstable and decompose into monomeric nonperoxidic products, polymers and volatile products (*48*). The decomposition products of hydroperoxides will be oxidized further. High temperatures, long cooking times and transition metal contaminants favor fast decomposition of the oil. The presence of antioxidants and related compounds may reduce the rate of decomposition of the oil.

The catalytic effect of trace transition metals on fat oxidation is well known. It is therefore important to know their concentration in all stages of the oil production process, before, during and after processing. Therefore, contents of copper and iron in the byproducts and in the oil have also been determined.

A flow chart describing the characterization of the experiments used during this project can be seen in Figure 6. In order to determine the oxidative state and quality of the oil, several stability tests including accelerated storage tests are frequently employed. Chemical, instrumental and sensory techniques are used to measure the oxidation of lipids. These methods are also used in this study.

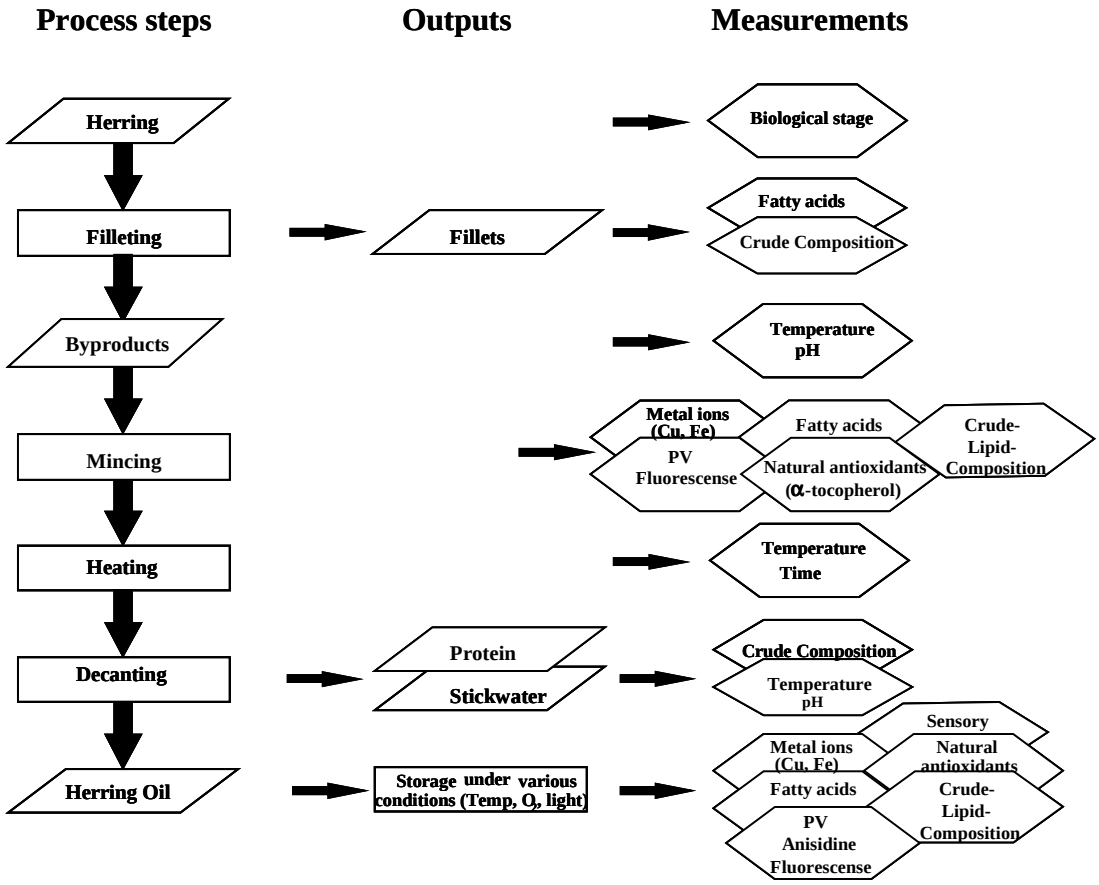

Figure 6– Flow chart for the characterization of the different experiments.

A number of analytical and sensory methods have been developed and implemented at our laboratory to determine the composition and the extent of deterioration as well as to predict the expected shelf life of the oil. In this study the herring oil was analyzed using different methods in order to perform an assessment of quality. The data collected from the different parameters allows characterization of the herring oil obtained from herring byproducts. In order to optimize the storage conditions that the oil should be kept, experiments related with the influence of light, oxygen and different temperatures were scheduled.

1.7 Outline of this Thesis

In this thesis, the upgrading of herring byproducts into high-quality crude oil is discussed and evaluated. The influence of the variability of the raw material quality is discussed and explained in **chapter 2**. Subsequently, the seasonal variation (chapter 2.1), the use of salted raw material (chapter 2.2) and a comparison between different extracted oils evaluated chemically and sensorial (chapter 2.3) are discussed. The processing of raw material into high-quality crude fish oil is described in **chapter 3**. In chapter 3.1 the effect of storage of herring byproducts at two different temperatures up to 72 hours is evaluated. The sorting of byproducts is presented in 3.2 and the optimization of the fish oil pilot plant is assessed and discussed in chapter 3.3. The oil product quality is evaluated in **chapter 4**. The first question addressed is until which extent storage temperature of the oil affects oil's quality. Therefore, the oil extracted from fresh herring byproducts was stored at three different temperatures in chapter 4.1. Volatiles that developed in the oil during storage were followed and evaluated in chapter 4.2. Final considerations, including a general discussion, are given in **chapter 5**. Attention is also given to the content of dioxins and PCB's present in the oil. Finally, a comparison is made between the extracted crude and refined herring oil and the commercially available menhaden oil.

Chapter 2

Influence of raw material quality

2.1 Seasonal Changes in Crude and Lipid Composition of Herring Fillets, Byproducts, and Respective Produced Oils

ABSTRACT

Crude and fatty acid composition analyses were performed on fillets, byproducts, and oil originating from herring (*Clupea harengus*) caught off the North Sea from June 1999 to January 2001. Monthly statistical differences were found in the fat content, the range of variation being larger in fillets than in byproducts. The most consistent change observed in fillets was an increase of unsaturation from May to September reflected in a reduced percentage of monounsaturated fatty acids, whereas for byproducts and oil this trend was not so well defined. The results indicated that the lowest values of the total amount of polyunsaturated fatty acids (PUFAs) in the oil were found from January to March (~ 14%), coinciding with the post-spawning and starvation period. In contrast, the highest values were found from June to August (~ 23%). Thus, the herring byproducts are all year an adequate raw material for fish oil production; however, during the summer they are richer in PUFAs.

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INTRODUCTION

The composition of several fish species varies from season to season due to its natural cycle, maturity stage, geographic location, etc. (*17, 19, 49-54*). Herring, being a typical pelagic fatty fish, goes through a natural cycle showing considerable variations in lipid content and composition as well (*11, 18, 55-58*). The industry takes advantage of this fact by producing respective typical products such as maatjes, marinated, frozen, cured herring, and kippers, which are characteristic for different times of the year and encouraged to be consumed by health authorities as a source of the beneficial polyunsaturated fatty acids (PUFAs). During the processing of herring a considerable amount of byproducts is originated, which has to be processed further or disposed of. Following successful experiences upgrading herring byproducts into stable crude fish oil (*59, 60*), showing that sorting is not required (*61*) and that good-quality fish oil could be produced from stored byproducts (*62*), knowledge is necessary to elucidate the oil composition changes over the year. An important question is whether it is possible to keep the composition of oils produced from herring byproducts constant when the lipid content and composition of herring fluctuates seasonally. Literature on the topic does not exist, and the availability of such data might bring practical implications to the fish-processing industry.

The present work was undertaken to examine possible seasonal changes over the year in crude composition and lipid content of herring with particular regard to the distribution and change of fatty acids composition among fillets, byproducts, and oil produced from byproducts.

MATERIALS AND METHODS

Sampling of herring (*Clupea harengus*) was carried out from June 1999 to January 2001 at the North Sea (Figure 1).

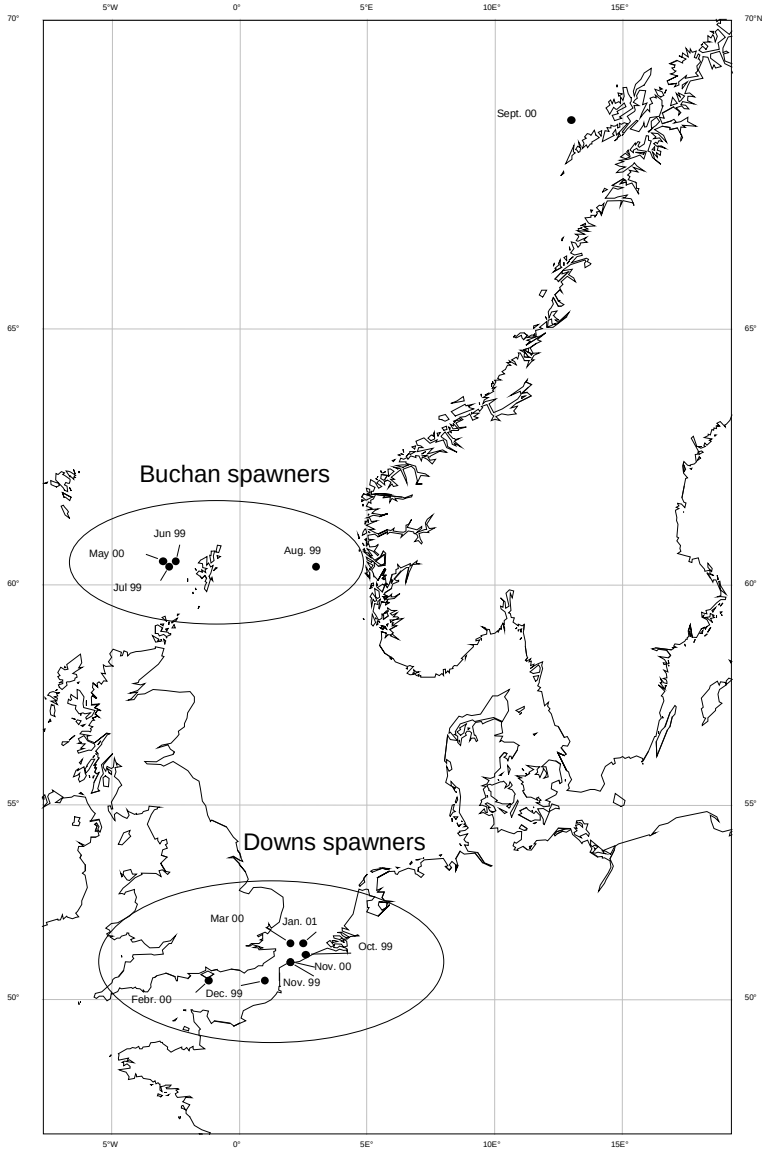


Figure 1- Periods and catching areas of the herring studied to evaluate seasonal changes over the year.

Before the herring was processed in a fish-processing company, some specimens were collected, and total length, weight, and maturity stage were recorded. According to the collected data, and using the maturity scale recommended by the herring committee of International Council for the Exploration of the Sea (ICES) (*63*) and by others (*64*), the maturity categories IV-V describe herring approaching spawning condition and categories VI-VII are related to those which are about to spawn or have recently spawned. Categories I-III and VIII concern fish unlikely to spawn for some time. In this study, fresh herring was used except for the months of June, July, and August; fish caught during these months have been deep-frozen before being processed.

Processed fillets, deboned and skinned, were collected and characterized for crude and fatty acids composition. Obtained byproducts from the filleting operation were also characterized and used for fish oil production. In this way, data available on fillets, byproducts, and oil could be directly compared because they originated from the same batch. The same procedure was performed over the year. The study was carried out for all months except April.

Each fish oil production run required ~ 1000 kg of mixed (heads, frames, skin, viscera, etc.) herring byproducts. Byproducts were minced, and immediately pumped to an insulated scraped-surface heat exchanger indirectly heated by steam (95 °C). Finally, the mixture was separated in a three-phase decanter into a phase containing solids (called the protein phase), a water phase (stickwater), and a lipid phase (oil). The same conditions and system as described earlier were used (*59*). On each occasion, samples of the intermediate mixture after the mincer and from the produced crude herring oil were collected. All samples were kept in the absence of light and oxygen and frozen at –80 °C until analyzed.

Fatty Acid Composition. Lipids from herring fillets and byproducts were extracted according to the method of Bligh and Dyer (*65*). Fatty acid methyl esters (FAMEs) of oil samples and of lipids extracts of fillets and byproducts were prepared according to AOCS (*66*) Official Method Ce 1b-89 and analyzed with regard to the content of individual fatty acids. On each occasion, three samples (n = 3) were analyzed once (a = 1). The different FAMEs were separated from each other by gas chromatography (GC) and identified using the conditions described previously (*59*). Results were expressed as a percent of the total fatty acids area.

Lipid Content. The total lipid content in the samples was determined gravimetrically after extraction according to the Bligh and Dyer (*65*) procedure (n = 3, a = 1). Results were expressed as grams of lipid per kilogram of samples.

Moisture. Moisture was determined as described earlier (*59*) (n = 3, a = 1). Results were expressed as percentage of wet weight.

Protein. Total nitrogen in the homogenized samples was determined using the Kjeldahl digestion method as described previously (*59*) (n = 3, a = 1). Results were expressed as percentage of wet weight.

Salt. Chloride content in all of the samples was titrated according to Volhard's method as described by Kolthoff and Sandell (*67*) (n = 3, a = 1). Results were expressed as percentage of wet weight.

In all cases, with the exception of the fatty acid composition analysis, internal reference materials were analyzed together with the samples.

Statistical Analysis

SPSS, version 10.0, software was used. The results were calculated by using General Linear Models (GLM), based on the least-squares method. Models of analysis of variance were used to evaluate any effect of fatty acids and month of the year. When using one-way ANOVA, Pearson's bivariate two-tailed correlation was used for the total saturated, MUFAs, and PUFAs to check any existent relationship for the total groups of fatty acids of the fillets, byproducts, and oil. The relationship of fatty acids between fillets and byproducts and between byproducts and oil from the grouped data was also subjected to an ANOVA test. For comparisons of the fatty acids composition, the months were placed in three groups based on the maturity stage and PUFAs content of fillets over the year. The groups were designated stage I, which contains the months of December to March, stage II (May to August) and stage III (September to November). The residuals were tested for normality using normality probability plots. Observed power was used

as a statistical tool to evaluate the strength of the drawn inferences related to the failure to reject the H_0 (*68*). In all cases p was set at 95%.

RESULTS AND DISCUSSION

In the first part of this section, the ecological (distribution/catching areas) and physiological (length, weight, and maturity stage) characteristics of herring used in this study are described. The crude composition from the processed fillets and the respective obtained byproducts after processing is also considered. In the second part, the changes of fatty acid composition of fillets, byproducts, and produced oils over the year are discussed. For this, first, the individual monthly composition is shown, followed by grouped studied periods for fillets versus byproducts and byproducts versus oil.

Ecological and Physiological Data

The total North East Atlantic herring stock is divided into two major groups: shelf and oceanic. This subdivision has been made from a series of observations on the morphological, physiological (spawning time, maturation cycle, pattern, and rate of growth, etc.), and ecological characteristics of herring spawning at different times and in different localities (*69*). In this study, the herring was caught at different locations throughout the year and their weight, length, and maturity stages were recorded. As can be observed in Figure 1, all of the herring belonged to the shelf group subdivision with the exception of the batch caught in September 2000, off the Norwegian Coast, which belonged to the oceanic group. These two groups present different biological characteristics concerning general distribution, growth, spawning place and time. As noted in the literature (*69, 70*), herring of the oceanic group are often larger and consequently heavier, as can be observed with our own collected data (Figure 2), where significantly higher values were found for the month of September.

The shelf group herring can be classified as Buchan, Dogger, and Down spawners according to spawning time and location. At the bottom part of the map (Figure 1) the group belonging to the Downs spawning group is shown. In this group, spawning occurs late autumn-winter, mainly from the third week of November to early January (*71*). Therefore, spent herring were caught from January to March (Figure 2, maturity stage). Herring shoals of the Downs population move north anticlockwise and reach the northern and central North Sea during the summer feeding season (*72*). The herring caught from May to August belongs to the Buchan group. This group is mainly composed of autumn-spawners herring, although spring spawners can also be found in high proportions on the Shetland Islands at various seasons (*72*).

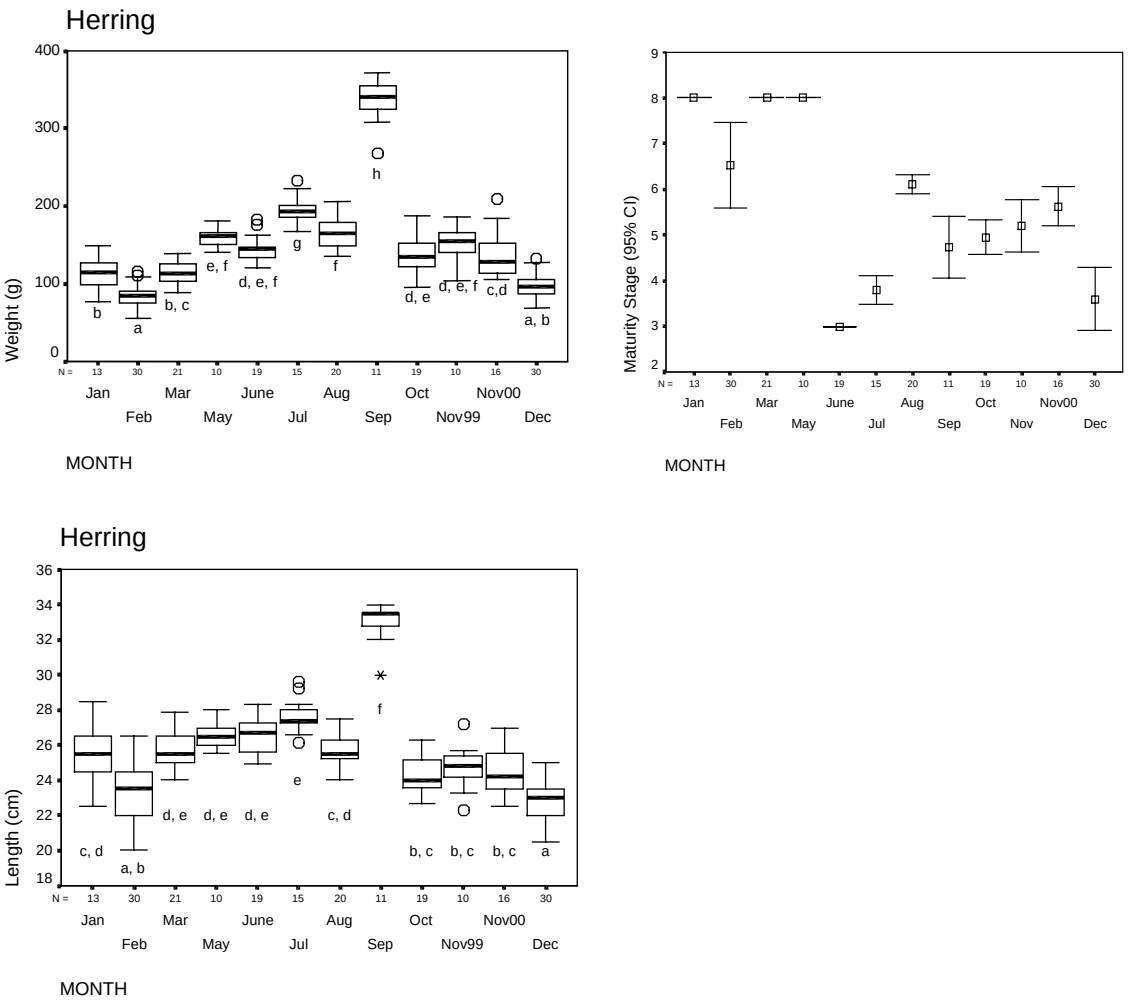


Figure 2- Box and whiskers plots of the weights and lengths of the caught herring. Different letters under the box plots represent significant differences ($P < 0.05$). □ Interquartile range, ▬ Median, I non-outlier (maximum and minimum), O outlier, ∗ extreme, n number of samples. Error bars with 95% confidence interval representing the range of variation for the maturity stage.

In general it can be seen that the weight increased from March until November and then decreased. The data also showed a quite good positive correlation between the length and the weight ($r = 0.911$). The month of November was sampled in the two consecutive years to see if yearly differences could be found. The results showed no significant difference between the samples. On the other hand, the month of April is missing in the study, because fish oil productions at the processing company never used fish from this month. With respect to the maturity stage it can be concluded that the investigated herring had a spawning period in autumn-winter with the exception of the fish caught in June, which were spring spawners. It is known that maturation is dependent on temperature and also on feeding conditions. Continuous recruitment of fish to the spawning population means

that maturation within the stock can be asynchronous (*54*). In several sampling areas there was a mixture of fish in different gonad stages (Figure 2). Fish sampled in the months of January to March and June were unlikely to spawn for some considerable time.

Crude Composition

The crude composition of herring fillets and byproducts was determined over the year and the results obtained are presented in Figures 3 and 4. The chemical composition of herring undergoes large fluctuations in response to a variety of factors. For example, the sexual maturity stage of the fish affects the lipid content due to increased consumption of fat reserves during the spawning period. Food availability and environmental water temperature are also important factors. Thus, fish will have various lipid contents, depending on the breeding cycle and time of year (*52, 73*). Herring generally is known for its high fat content, although fat storage usually increases in summer when food is more available and may decrease to lower values in winter. Generally, fat and moisture contents are inversely proportional, whereas other body components (e.g., protein) remain fairly constant (*70, 73, 74*). Considering the fillets and byproducts, the water contents ranged from 62 to 77% and from 61 to 80%, respectively. As expected, the highest lipid content occurred in the month of lowest water content (May and September). Comparing fillets and byproducts for fat content, it is interesting to notice that the fat level in the byproducts is generally more constant than in the fillets. It is known that accumulation and storage of fat in herring prior the maturation take place in the muscle–both within the fibers themselves and between the fibers (*75*). Although the fillets collected in September were richest in lipid content (19%), it is in May that higher amounts of lipids were found in the byproducts (22%). Even though these results are reported for herring belonging to different groups with different morphological characteristics, a possible explanation for the differences found might be that herring recovers after a feeding period and has tendency to accumulate fat in the dark muscle and skin rather than in the white muscle. Similarly, Ke and co-authors (*76*) found that white muscle of mackerel showed the largest seasonal variation in fat content. It is also known that dark muscle is the tissue used for continuous swimming and therefore is only used when lipids from the white muscle have been depleted. To support this theory, during the leaning period the byproducts (mainly composed of skin and dark muscle) presented also higher lipid values than the fillets (7.2 and 5.8%, respectively), suggesting that the reserves are used first from the white muscle that composes the fillets. During the winter period no food is available, and therefore the herring uses all of its fat resources, explaining the low values of fat content. The minimum value was reached in March, which coincides with the period of post-spawning

and starvation due to lack of food resources. A low fat value was obtained for the month of June. A very likely explanation is that the herring caught in that month belonged to a spring-spawning group (confirmed with the maturity stage III), being therefore recovering spent herring. There is a drop in fat content from January to March because of starvation and for the month of June after gonad maturation.

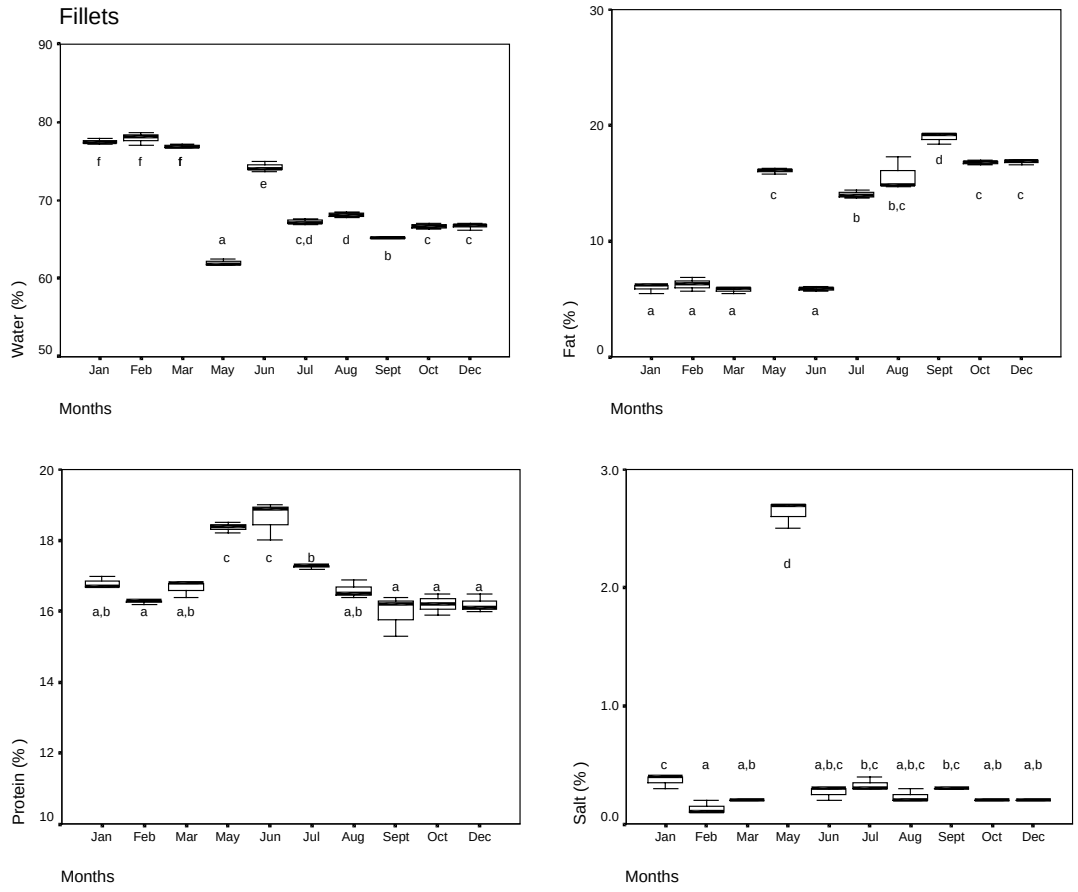


Figure 3- Box and whiskers plots of the crude composition over the year of the herring fillets originating the byproducts. Different letters under the box plots represent significant differences ($P < 0.05$), $n = 3$.

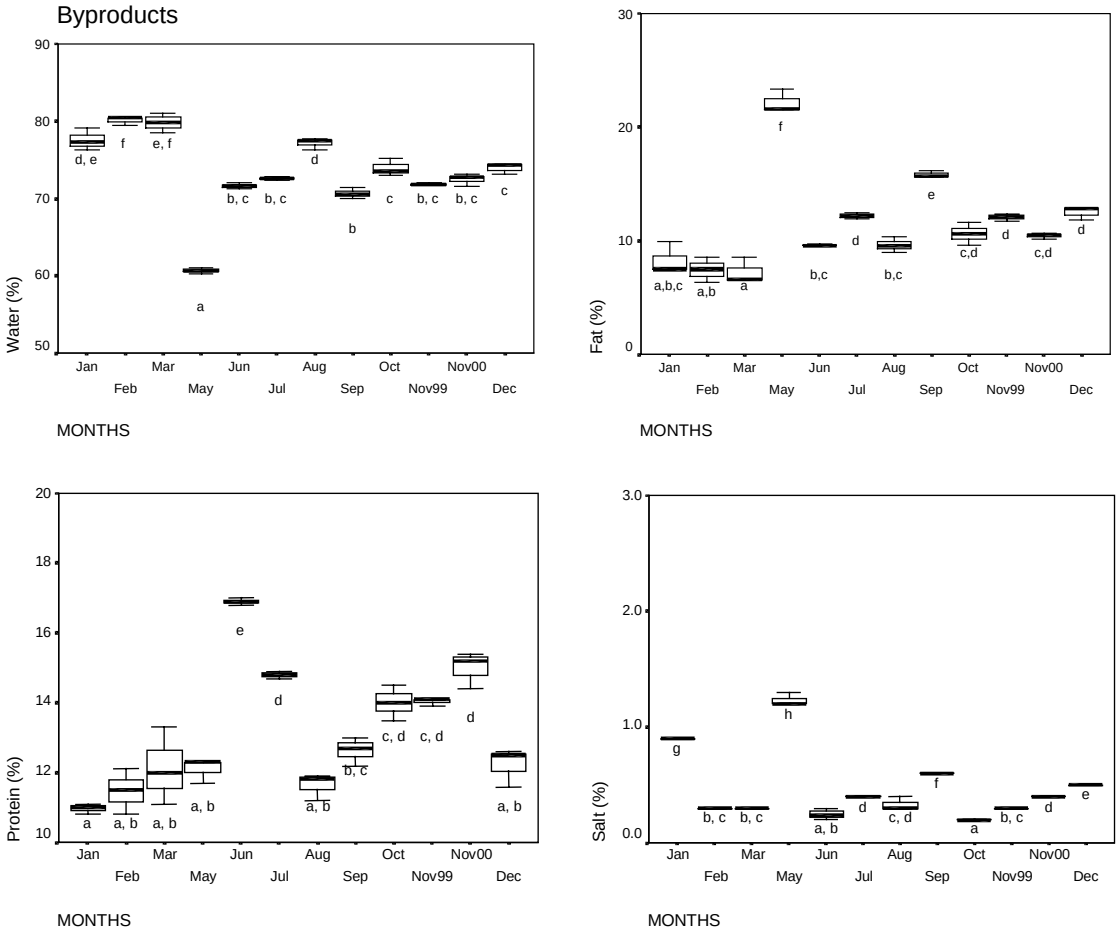
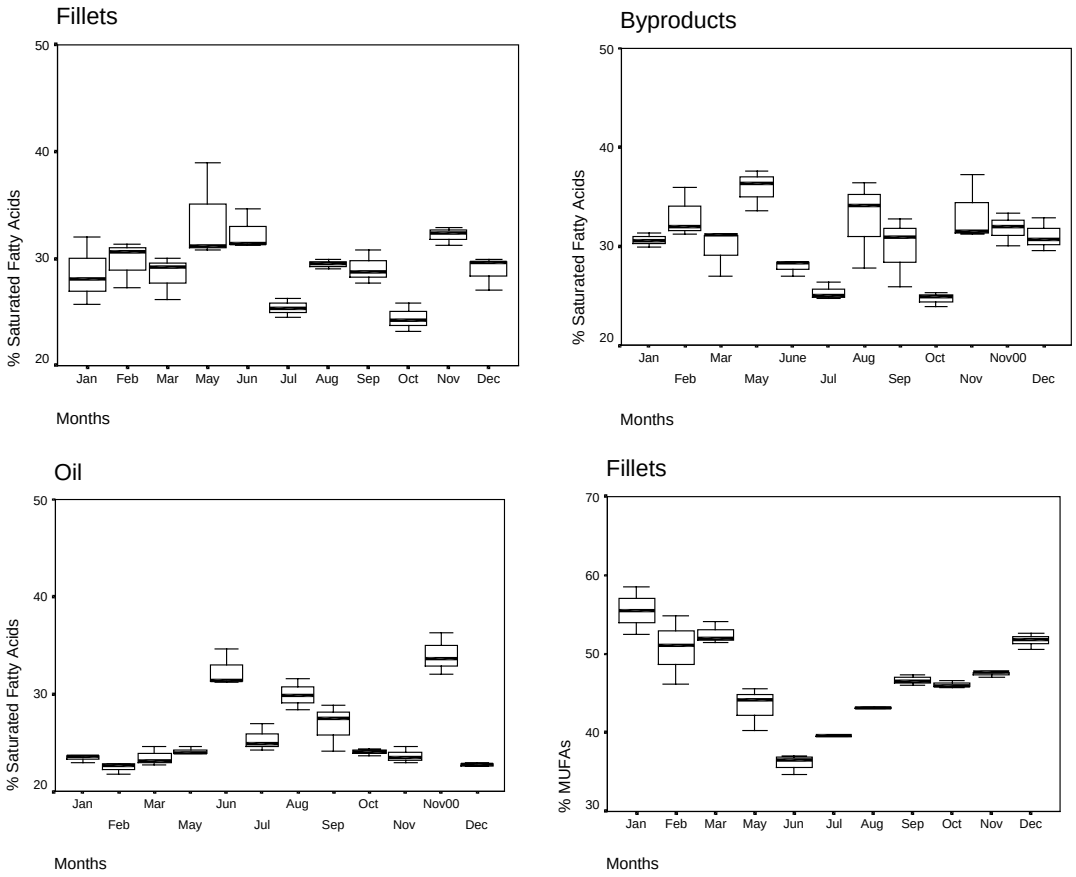


Figure 4- Box and whiskers plots of the crude composition over the year of the herring byproducts used for fish oil production. Different letters under the box plots represent significant differences ($P < 0.05$), $n = 3$.

From a comparison of the two figures, it is not surprising that the fillets contained a higher amount of protein than the byproducts. In general, the protein values ranged between 16.0 and 18.9% in the fillets (September and June, respectively), whereas for the byproducts a variation between 11.0 and 16.9% was found (February and June, respectively). The salt content presented in May an extremely high value for the fillets and byproducts (2.6 and 1.2%, respectively). This can be easily explained due to the production of a popular and characteristic Dutch product, maatjes herring, where brine is added to the fillets (*59*), thereby increasing the salt content significantly. It can be stated that in general the byproducts contained a higher content of moisture than the fillets, which is expected because less fat and protein is present.

Fatty Acid Composition

Details of individual fatty acids composition of fillets, byproducts, and processed oil studied over the year are presented in Appendix 1, and the measured total saturated, monounsaturated fatty acids (MUFAs), and PUFAs are shown in Figure 5. As can be observed, fatty acids composition showed seasonal changes during the year. However, regardless of the season and maturity stage, MUFAs constitute the majority of fatty acids in the three products studied, followed by the saturated and PUFAs. The saturated fraction ranged from 22.5 to 35.8%. Within this group the major fatty acid was palmitic acid (16:0). The total monoenes content ranged from 34.9 to 58.8%, with 22:1 being the prominent monounsaturated fatty acid (comprising between 14 and 28% of the total). The high level of this fatty acid is characteristic for herring and in accordance with values reported in the literature (*59*, *77-79*), being originated in the fatty alcohols common in copepods (*11*, *77*, *78*).



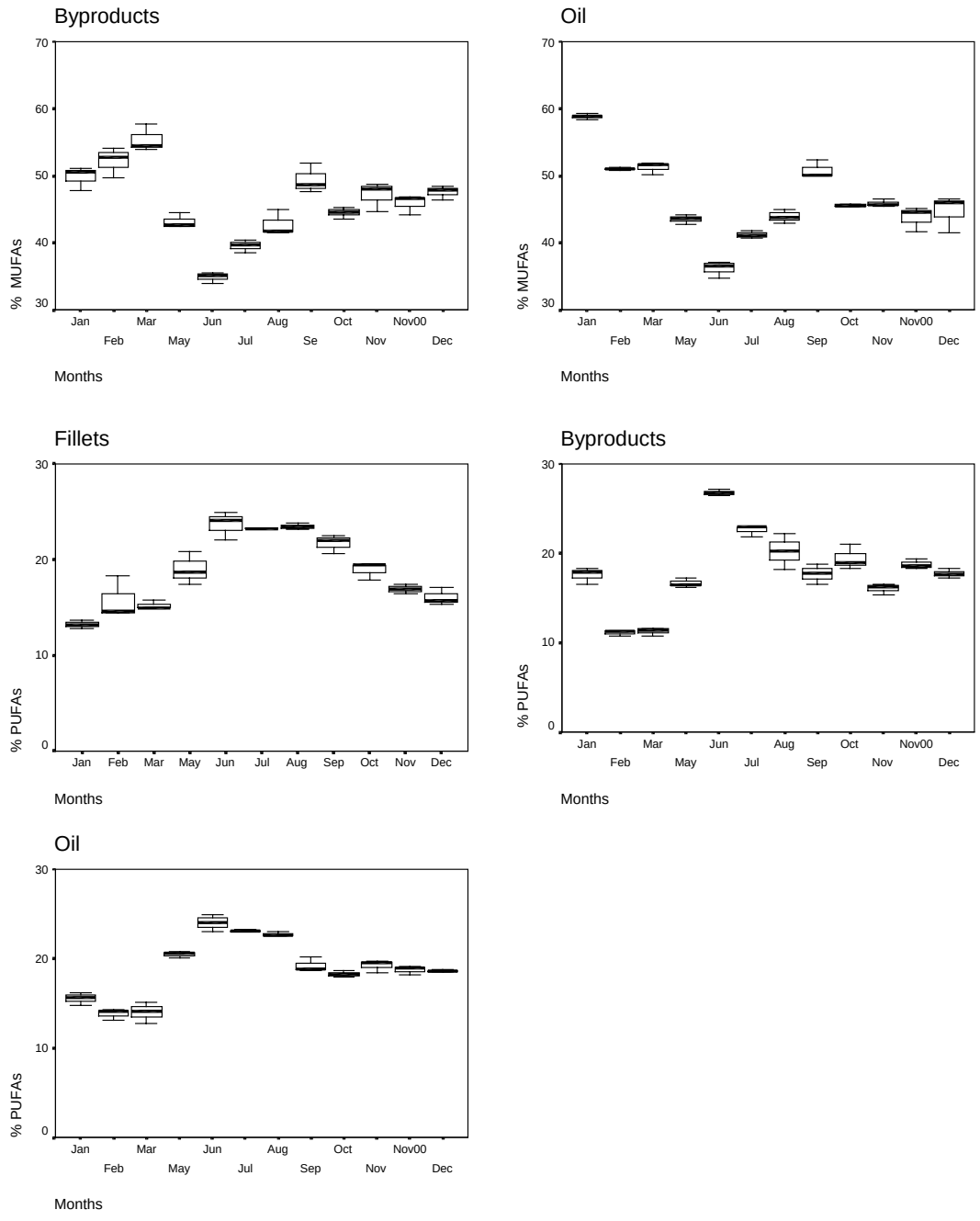


Figure 5- Box and whiskers plots representing the variation of the Saturated, MUFAs and PUFAs of the herring fillets, byproducts and produced oil over the year (n = 3).

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were the major PUFAs in the fillets, byproducts, and herring oils, and changes in percentage of these fatty acids occurred over the year. Except in May, DHA was found in higher values than EPA. It has been reported (*17*) that in sardine, accentuated reduction in DHA content with a proportional increase for EPA occurred in the month of April, which was attributed to

differences in diet. Because DHA is an important component of membrane structural lipids, its relative percentage can decrease during April, postspawning time, whereas EPA is a main fatty acid of plankton origin (*17*) and its level will therefore be more constant.

It has been suggested that the proportions of the different fatty acids varied with the lipid content (*52*); lean herring was reported to present significantly higher values of saturated fatty acids. This inverse relationship between lipid content and degree of unsaturation, as reported by other authors (*49*, *52*), was not observed. This implies, as previously suggested (*55*), the influence of food intake; that is, the composition of lipids in herring follows that in plankton.

The highest values for EPA, DHA, and total amount of PUFAs were found in general from May to August. The decrease in the percentage of PUFAs in herring fillets from September on may be an adaptation to spare these fatty acids for ovary construction, mainly using EPA and DHA (*51*). In gonadal herring lipids (*57*), it was observed that PUFAs were the major components, and it was assumed that a higher proportion of PUFAs than MUFAs was mobilized. In our case, increases of MUFAs, particularly 18:1 and 22:1, during maturation were observed.

To find whether any relationship existed between the total content of different unsaturation of fatty acids (Figure 5), Pearson's correlation was tested for the different individually fractions studied. Over the year, the amount of MUFAs in the fillets was consistently negatively correlated with PUFAs content ($r = -0.859$; $p < 0.01$; $n = 33$). In that respect the highest levels of PUFAs were found during the months of June, July, and August (~23 %) and the lowest in the month of January (~13%). It has been proposed (*51*) that the degree of unsaturation of pike neutral lipids reserves may influence the physical state (viscosity) of the same reserves and the speed with which they can be hydrolyzed to supply energy. This would explain the increase in the percentage of MUFAs during autumn and winter as an adaptation to compensate for reductions in PUFAs and maintain adequate viscosity at low water temperatures. Reciprocal changes in short- versus long-chain fatty acids also occurred. Declining food intake during winter may reduce the activity of elongases and desaturases enzymes (*80*, *81*) to such an extent that 18-carbon PUFAs are maintained while longer chain PUFAs decrease substantially. On the other hand, in the byproducts, the total amount of PUFAs was negatively correlated not only with the total MUFAs but also with the total saturated content ($r = -0.862$ and $r = -0.465$, respectively, with $p < 0.01$; $n = 36$, in both cases). For the produced oils, the PUFAs showed a significant positive correlation with the total saturated ($r = 0.518$; $p < 0.01$; $n = 36$) and a negative correlation to the content of MUFAs ($r = -0.785$; $p < 0.01$; $n = 36$). The total content of MUFAs, in the oil, correlated negatively over the year with the amount of

saturated and PUFAs content ($r = -0.534$ and $r = -0.785$, respectively, with $p < 0.01$and $n = 36$, in both cases). These results support previous statements (*82*) that fat is not metabolized continuously but in a stepwise manner; that is, fish build up energy reserves prior to spawning, during which the reserves are spent rapidly.

To organize the information and clearly perceive variations, the data were gathered into three stages. The stages were chosen on the basis of similarity of spawning period/maturity stage and total MUFAs and PUFAs content. Stage I corresponds to the beginning of the spawning and subsequent lean herring period, with a decrease and an increase of PUFAs and MUFAs, respectively, during the months from December to March; stage II from May to August is associated with a postfeeding period with an increase of fat and PUFAs content. Stage III, covering the remaining months, from September to November, is linked to the period that the herring is preparing to approach the spawning condition with the respective change in fatty acids content. To evaluate what influence of the time of year was on the type of herring product (whether fillets or byproducts, and byproducts or oil), concerning the fatty acids composition, all of the data were subjected to a Least Squares Estimates (LSE) test, which for each fatty acids group gave rise to the following parameters in the model:

$$Y_{(\text{fatty acids})} = \beta_0 + \beta_{1,\,t} + \beta_{2,\,\tau} + \beta_{3,\,t*\tau}$$

In this equation, t (class variable) is the effect of the period of time (stage) that the fish has been caught over the year, and τ (class variable) is the type of product studied (fillets, byproducts, or oil). $t*\tau$ represents the interaction term of the two class variables on the fatty acids studied. The estimation of the constant β_0 is given with the value determined for the intercept.

Fillets versus Byproducts. Appendix 2 shows that, with the exception of the total saturated fatty acids measurements, all models were highly significant ($P << 0.05$). Within the significant models, in all cases only the influence of the period of time that the herring was caught was shown to have a significant effect ($p = 0.000$ and power = 1.000 except for DHA, for which a power = 0.998 was found). Figure 6 represents schematically the statistical outcome for the variation in the total saturated, MUFAs, PUFA (PUFAs excluding EPA and DHA), EPA, and DHA fatty acids composition comparing herring fillets and byproducts over the year. The total amount of saturated fatty acids remained constant over the year (~ 31%), whereas the proportion of MUFAs and PUFAs changed seasonally. For the other groups of studied fatty acids, a consistently significant effect of the stages was observed. Herring caught in the first stage contained a significantly higher amount of MUFAs than the ones caught during the third stage, and

the lowest significant level was found for the second stage. The total unsaturation level of the fillets and byproducts increased from its lowest level during stage I to the highest level in stage II. These changes are mainly due to the changes in EPA and DHA content. A similar tendency was observed in the EPA content, while the amount of DHA showed that the highest significant value was found in stage II but no significant difference was detected between the stages III and I.

Byproducts versus Oil. It was notable that in byproducts and oils during the months of February and March, the lowest values of EPA and DHA were measured (Appendix 1). Interestingly, those months corresponded to the post-spawning period with spent or recovering spent herring. It is known that lipids are mobilized when fish starve or mature (*82*), because sexual maturation involves the mobilization of relatively large quantities of nutritive material for the developing offspring. A specific selection of fatty acids is mobilized into the developing roe (*57*). Love (*82*), summarizing the findings of several authors, suggests that DHA tends not to be mobilized from muscle, except when the lipid content of the muscle is very low, implying that PUFAs are broken down in the fish muscle only at a late stage of starvation. It is likely that it does not occur until the triglycerides have first been moved and the muscle cells have started to disintegrate (*82*).
A similar statistical procedure as described earlier was performed to study differences between byproducts and the respective produced oils. The fitted models (Appendix 3) were found to be all highly significant for the studied group of fatty acids. Concerning the group of MUFAs, PUFAs, and DHA, only the stage had a significant influence ($p << 0.05$, power = 1.000). Related to the total fraction of saturated fatty acids, the effect of the type of product (byproducts or oil) and the interaction effect between time and type were found to be significant ($p << 0.05$; power = 0.999 and power = 0.754, respectively). For the EPA content, not only time had a significant effect but also the type of product ($p << 0.05$, power = 1.000 and 0.842, respectively). Figure 6 shows the different groups of fatty acids with the significant differences within the byproduct and oil. Significantly lower values of total saturated fatty acids were found for the oil compared to the byproducts. To the other groups of fatty acids the influence of stages is important. As expected, the content of MUFAs reaches the maximum during stage I and the minimum during stage II. In contrast, the maximum contents of EPA, DHA, and total PUFAs are reached in stage II and the minimum levels during stage I. In all cases, significant differences among the three different stages of the year were found. Apparently, interconversion of those fatty acids occurred. In addition to the period of the year, for EPA also an interaction effect of time and type, either in byproducts or oil, was present.

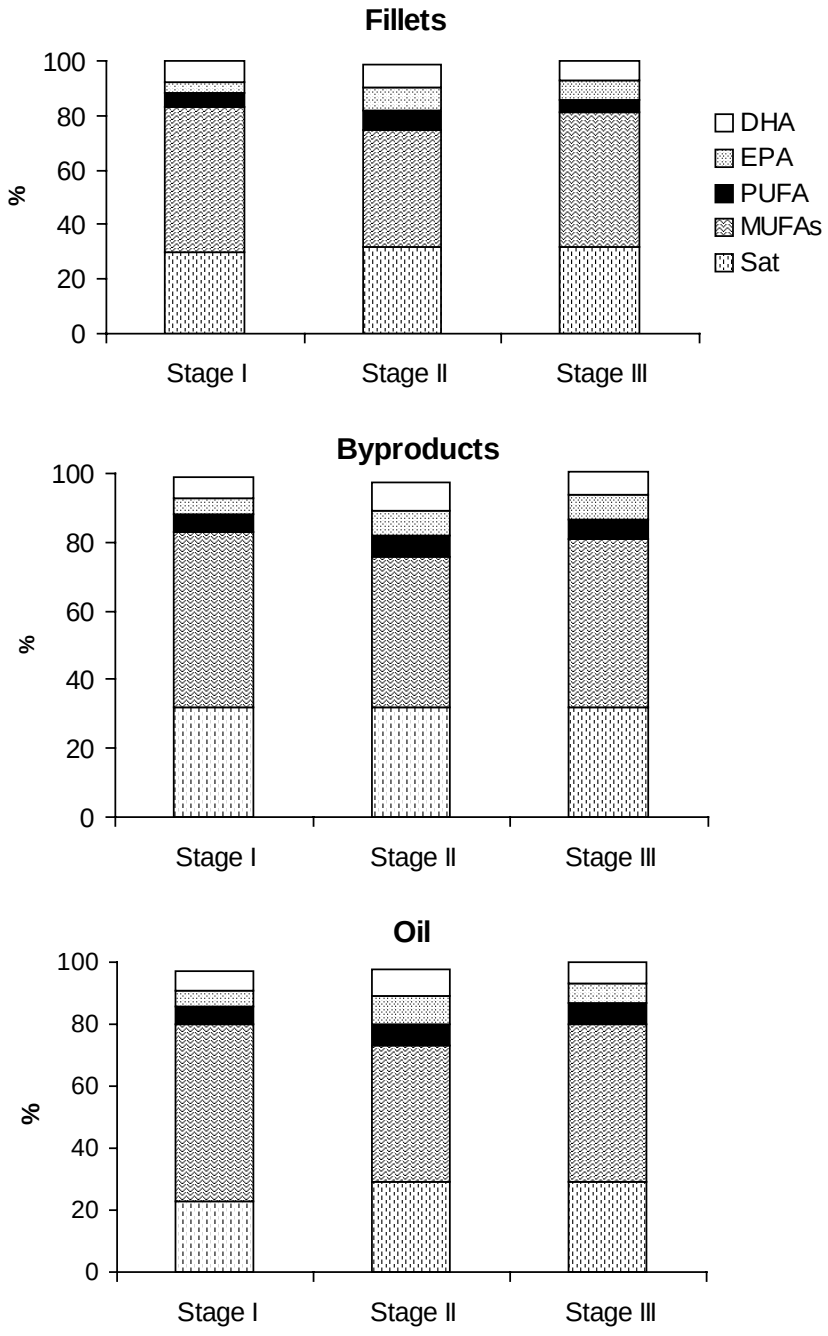


Figure 6- Seasonal variation on some groups of fatty acids influenced by the stages when comparing fillets versus byproducts and byproducts versus oil over the year.

In conclusion, it can be stated that the fillets contained a higher protein content than the byproducts, and a larger variation of the fat content over the year was observed. In the byproducts the fat content is less affected by seasonal variation, although certain variation

occurred over the year, with a maximum reached in May and a minimum value obtained from January to March. This paper also confirms that fatty acid composition in the fillets fluctuates in an annual cycle depending on the maturity stage of the herring and food availability. However, the fatty acid composition in the byproducts is relatively stable. Nevertheless, the highest amount of total PUFAs in the oil was reached during the months of June to August in contrast to January to March, when the lowest levels were found, although never less than 14%. The upgrading process also offers a good opportunity to contribute to an environmental solution by reducing the amount of waste. Moreover, this type of valorization is highly interesting because it confers an added value to the herring byproducts originating from the fish industry during the entire year.

Acknowledgements

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Appendix 1- Fatty acid profile (w/w % of total fatty acids) of fillets (I), byproducts (II) and respective oils (III) produced from herring (*Clupea harengus*) byproducts over the year. Mean values of three independent measurements. Values in rows followed by different superscript are significantly different ($P < 0.05$).

(I)

Fatty Acids	Fillets										
	Jan	**Feb**	**March**	**May**	**June**	**July**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
14:0	10.4 ^{ab}	11.4 ^{ab}	9.6 ^{a}	15.6 ^{c}	9.2 ^{a}	8.9 ^{a}	10.6 ^{ab}	11.1 ^{ab}	9.3 ^{a}	13.6 ^{bc}	11.1 ^{ab}
16:0	16.2 ^{ab}	16.6 ^{ab}	17.0 ^{b}	15.8 ^{ab}	20.3 ^{c}	14.5 ^{ab}	17.2 ^{b}	16.5 ^{ab}	13.7 ^{a}	17.0 ^{b}	16.2 ^{ab}
18:0	2.0 ^{b}	1.7 ^{ab}	1.9 ^{b}	2.3 ^{c}	2.9 ^{d}	2.0 ^{b}	1.7 ^{ab}	1.6 ^{a}	1.5 ^{a}	1.6 ^{a}	1.6 ^{a}
Σ ^{a}	28.6 ^{ab}	29.8 ^{ab}	28.5 ^{ab}	33.7 ^{b}	32.4 ^{b}	25.4 ^{a}	29.5 ^{ab}	29.1 ^{ab}	24.5 ^{a}	32.2 ^{b}	28.9 ^{ab}
16:1	5.1 ^{abc}	4.7 ^{a,b}	4.8 ^{abc}	5.6 ^{bc}	6.7 ^{d}	5.3 ^{abc}	5.7 ^{c}	5.1 ^{abc}	4.6 ^{a}	5.6 ^{c}	5.2 ^{abc}
18:1	11.8 ^{fg}	11.6 ^{fg}	12.8 ^{g}	5.8 ^{a}	7.9 ^{bc}	6.6 ^{ab}	8.5 ^{cd}	13.0 ^{g}	8.1 ^{bcd}	9.7 ^{de}	10.4 ^{ef}
20:1	15.8 ^{b}	10.9 ^{ab}	14.9 ^{b}	13.4 ^{ab}	7.8 ^{a}	9.9 ^{ab}	11.4 ^{ab}	12.4 ^{ab}	12.0 ^{ab}	13.3 ^{ab}	13.6 ^{ab}
22:1	22.8 ^{d}	23.4 ^{d}	20.1 ^{bcd}	18.6 ^{abcd}	13.8 ^{a}	17.8 ^{abc}	17.7 ^{abc}	16.2 ^{ab}	21.4 ^{cd}	18.8 ^{bcd}	22.5 ^{cd}
Σ ^{a}	55.5 ^{e}	50.7 ^{defg}	52.5 ^{fg}	43.3 ^{bc}	36.1 ^{a}	39.6 ^{ab}	43.2 ^{bc}	46.7 ^{cde}	46.1 ^{cd}	47.4 ^{cdef}	51.7 ^{efg}
18:2	1.7 ^{abc}	2.1 ^{c}	1.8 ^{bc}	1.1 ^{a}	1.7 ^{abc}	1.5 ^{abc}	1.6 ^{abc}	1.4 ^{ab}	1.9 ^{bc}	2.0 ^{c}	1.9 ^{bc}
18:3	0.7 ^{a}	0.9 ^{ab}	0.7 ^{a}	0.9 ^{ab}	1.2 ^{bcd}	1.3 ^{bcd}	1.3 ^{bcd}	1.2 ^{abcd}	1.5 ^{d}	1.5 ^{cd}	1.2 ^{abcd}
18:4	1.0 ^{a}	1.5 ^{a}	1.0 ^{a}	4.4 ^{d}	2.4 ^{b}	2.8 ^{bc}	3.9 ^{d}	4.3 ^{d}	2.8 ^{bc}	3.1 ^{c}	2.3 ^{b}
20:5	3.7 ^{a}	4.0 ^{a}	4.3 ^{ab}	6.4 ^{c}	8.0 ^{d}	8.0 ^{d}	7.6 ^{d}	6.4 ^{c}	5.3 ^{b}	4.5 ^{ab}	4.6 ^{ab}
22:6	6.1 ^{a}	7.2 ^{ab}	7.4 ^{ab}	6.1 ^{a}	9.7 ^{d}	9.6 ^{cd}	9.0 ^{bcd}	8.5 ^{bcd}	7.5 ^{abc}	5.8 ^{a}	6.0 ^{a}
Σ ^{a}	13.2 ^{a}	15.7 ^{abc}	15.2 ^{ab}	19.0 ^{de}	23.0 ^{f}	23.3 ^{f}	23.4 ^{f}	21.7 ^{ef}	18.9 ^{cde}	16.9 ^{bcd}	16.1 ^{abcd}

(II)

Fatty Acids	Byproducts											
	Jan	Feb	March	May	June	July	Aug	Sept	Oct	Nov	Nov 00	Dec
14:0	11.4 ^{abc}	13.2 ^{c}	11.0 ^{ab}	17.1 ^{d}	9.2 ^{ab}	8.6 ^{a}	11.2 ^{abc}	11.3 ^{abc}	8.4 ^{a}	12.2 ^{bc}	12.5 ^{bc}	12.0 ^{bc}
16:0	17.0 ^{ab}	17.9 ^{ab}	16.7 ^{ab}	16.3 ^{ab}	16.4 ^{ab}	14.8 ^{a}	19.4 ^{b}	16.9 ^{ab}	14.5 ^{a}	19.2 ^{b}	17.2 ^{ab}	17.2 ^{ab}
18:0	2.1 ^{cde}	2.0 ^{bc}	2.0 ^{bcd}	2.4 ^{f}	2.3 ^{ef}	2.0 ^{bcd}	2.2 ^{def}	1.7 ^{a}	1.8 ^{ab}	2.0 ^{bc}	2.2 ^{cde}	1.8 ^{ab}
Σ ^{a}	30.6 ^{abcd}	33.1 ^{cd}	29.7 ^{abc}	35.8 ^{d}	27.9 ^{ab}	25.4 ^{ab}	32.8 ^{cd}	29.8 ^{abcd}	24.7 ^{a}	33.3 ^{cd}	31.8 ^{bcd}	31.0 ^{abcd}
16:1	5.1 ^{ab}	6.0 ^{b}	5.2 ^{ab}	6.0 ^{ab}	5.7 ^{ab}	4.9 ^{ab}	5.8 ^{ab}	5.1 ^{ab}	4.3 ^{a}	5.9 ^{ab}	5.3 ^{ab}	5.6 ^{ab}
18:1	12.0 ^{ef}	11.2 ^{def}	10.8 ^{cdef}	6.0 ^{a}	6.3 ^{ab}	7.3 ^{ab}	9.3 ^{abcde}	13.8 ^{f}	7.6 ^{abc}	9.6 ^{bcde}	8.1 ^{abcd}	9.2 ^{abcde}
20:1	13.3 ^{de}	15.0 ^{fg}	15.8 ^{g}	13.6 ^{ef}	8.3 ^{a}	9.9 ^{ab}	11.2 ^{bc}	13.1 ^{de}	11.7 ^{cd}	12.9 ^{de}	13.4 ^{ef}	13.0 ^{de}
22:1	19.4 ^{abc}	19.9 ^{bc}	23.5 ^{c}	17.6 ^{ab}	14.6 ^{a}	17.5 ^{ab}	16.4 ^{ab}	17.4 ^{ab}	21.0 ^{bc}	18.7 ^{abc}	19.1 ^{abc}	19.7 ^{abc}
Σ ^{a}	49.9 ^{de}	52.2 ^{ef}	55.4 ^{f}	43.1 ^{bc}	34.9 ^{a}	39.6 ^{ab}	42.7 ^{bc}	49.4 ^{de}	44.5 ^{c}	47.2 ^{cd}	45.9 ^{cd}	47.5 ^{cde}
18:2	1.6 ^{abcd}	1.7 ^{abcd}	1.4 ^{abcd}	1.1 ^{a}	1.5 ^{abcd}	1.1 ^{ab}	1.6 ^{abcd}	1.3 ^{abc}	1.8 ^{cd}	1.7 ^{bcd}	1.4 ^{abcd}	2.0 ^{d}
18:3	1.0	1.0	0.9	0.9	1.3	1.2	1.1	1.2	0.9	1.2	1.1	1.4
18:4	2.6 ^{b}	1.6 ^{a}	1.7 ^{a}	3.8 ^{f}	3.5 ^{ef}	2.7 ^{bc}	3.6 ^{ef}	3.3 ^{de}	2.7 ^{bc}	2.4 ^{b}	3.0 ^{cd}	2.8 ^{bc}
20:5	5.9 ^{de}	3.3 ^{a}	3.4 ^{a}	6.0 ^{e}	8.7 ^{f}	8.2 ^{f}	6.5 ^{e}	5.1 ^{bcd}	5.7 ^{cde}	4.6 ^{b}	6.1 ^{e}	4.9 ^{bc}
22:6	6.5 ^{bc}	3.6 ^{a}	4.0 ^{a}	4.8 ^{ab}	11.9 ^{f}	9.3 ^{e}	7.4 ^{cd}	6.6 ^{cd}	8.3 ^{de}	6.0 ^{bc}	7.2 ^{cd}	6.6 ^{cd}
Σ^{a}	17.6 ^{bcd}	11.1 ^{a}	11.3 ^{a}	16.7 ^{bc}	26.8 ^{f}	22.6 ^{e}	20.2 ^{de}	17.7 ^{bcd}	19.4 ^{d}	16.0 ^{b}	18.8 ^{cd}	17.7 ^{bcd}

(III)

Fatty Acids	Oil											
	Jan	**Feb**	**March**	**May**	**June**	**July**	**Aug**	**Sept**	**Oct**	**Nov**	**Nov 00**	**Dec**
14:0	8.5 ^{a}	8.6 ^{ab}	9.3 ^{abcd}	10.6 ^{bcd}	9.2 ^{abcd}	9.0 ^{abc}	11.2 ^{d}	10.8 ^{cd}	9.1 ^{abc}	8.9 ^{abc}	14.7 ^{e}	8.7 ^{ab}
16:0	13.1 ^{abcd}	12.2 ^{ab}	12.6 ^{abc}	11.5 ^{a}	20.3 ^{f}	14.2 ^{cd}	16.8 ^{e}	14.5 ^{d}	13.3 ^{bcd}	13.1 ^{abcd}	17.2 ^{e}	12.5 ^{abc}
18:0	1.8 ^{abcd}	1.7 ^{ab}	1.6 ^{ab}	2.1 ^{cde}	2.9 ^{f}	2.2 ^{e}	1.9 ^{bcde}	1.5 ^{a}	1.7 ^{abc}	1.6 ^{ab}	2.1 ^{de}	1.6 ^{ab}
Σ ^{a}	23.4 ^{ab}	22.5 ^{a}	23.5 ^{ab}	24.2 ^{ab}	32.4 ^{de}	25.4 ^{ab}	29.9 ^{cd}	26.8 ^{bc}	24.1 ^{ab}	23.7 ^{ab}	34.0 ^{e}	22.7 ^{a}
16:1	5.2 ^{bcd}	4.4 ^{ab}	4.6 ^{abc}	4.3 ^{ab}	6.7 ^{d}	5.5 ^{bcd}	5.7 ^{bcd}	4.9 ^{abcd}	4.6 ^{abc}	4.6 ^{abc}	6.3 ^{cd}	3.0 ^{a}
18:1	10.0 ^{d}	8.5 ^{c}	8.6 ^{c}	5.2 ^{a}	7.9 ^{b}	7.5 ^{b}	8.7 ^{c}	13.4 ^{e}	7.4 ^{b}	7.8 ^{b}	8.5 ^{c}	7.7 ^{b}
20:1	15.7 ^{f}	13.1 ^{de}	13.3 ^{e}	13.1 ^{de}	7.8 ^{a}	10.3 ^{b}	11.7 ^{c}	13.6 ^{e}	12.0 ^{c}	12.1 ^{c}	12.4 ^{cd}	11.9 ^{c}
22:1	27.9 ^{g}	25.0 ^{f}	24.8 ^{f}	20.9 ^{de}	13.8 ^{a}	17.9 ^{bc}	17.8 ^{bc}	19.0 ^{cd}	21.5 ^{e}	21.4 ^{de}	16.5 ^{b}	22.1 ^{e}
Σ ^{a}	58.8 ^{e}	51.0 ^{d}	51.2 ^{d}	43.5 ^{bc}	36.1 ^{a}	41.2 ^{b}	43.9 ^{bc}	50.8 ^{d}	45.6 ^{c}	45.9 ^{c}	43.8 ^{bc}	44.7 ^{bc}
18:2	1.9	1.8	1.8	1.7	1.7	1.2	1.6	1.3	1.3	2.0	1.5	2.3
18:3	0.0 ^{a}	1.1 ^{b}	1.0 ^{b}	1.1 ^{b}	1.2 ^{b}	1.2 ^{b}	1.3 ^{b}	1.2 ^{b}	1.5 ^{b}	1.5 ^{b}	1.2 ^{b}	1.4 ^{b}
18:4	2.1 ^{ab}	1.6 ^{a}	2.1 ^{ab}	4.1 ^{ef}	2.4 ^{abc}	3.2 ^{cde}	4.5 ^{f}	4.1 ^{ef}	3.2 ^{cde}	3.3 ^{cde}	3.9 ^{def}	2.9 ^{bcd}
20:5	5.5 ^{bc}	4.5 ^{a}	4.3 ^{a}	7.3 ^{e}	8.0 ^{f}	9.0 ^{g}	7.7 ^{ef}	5.9 ^{cd}	5.8 ^{bc}	5.9 ^{cd}	6.4 ^{d}	5.3 ^{b}
22:6	6.1 ^{bc}	4.9 ^{a}	4.7 ^{a}	6.3 ^{bc}	10.7 ^{e}	8.5 ^{d}	7.7 ^{d}	6.8 ^{c}	6.5 ^{bc}	6.6 ^{bc}	5.8 ^{b}	6.7 ^{c}
Σ ^{a}	15.6 ^{a}	13.8 ^{a}	14.0 ^{a}	20.5 ^{c}	24.0 ^{d}	23.1 ^{d}	22.8 ^{d}	19.2 ^{bc}	18.3 ^{b}	19.2 ^{bc}	18.8 ^{bc}	18.6 ^{bc}

[a] The values do not add up to 100% because minor fatty acids are not reported.

Appendix 2- ANOVA table from the GLM of the different groups of fatty acids measured on fillets and byproducts data comparing the effect of time of the year (stages), type of products (fillets or byproducts) and interaction effect. MS= mean squares, df = degrees of freedom, F = F-test, P = probability level.

Measure	df	MS	F	P	Measure	df	MS	F	P
Saturated					**MUFAs**				
Model	5	10.628	0.813	0.545	Model	5	327.14	35.175	0.000
Intercept	1	57433.2	4394	0.000	Intercept	1	139334	14981	0.000
Error	60	13.072			Error	60	9.30		
Effects					Effects				
time	2	11.079	0.848	0.434	time	2	811.4	87.246	**0.000**
Type	1	17.156	1.312	0.257	Type	1	4.315	0.464	0.498
time*Type	2	6.208	0.475	0.624	time*Type	2	3.564	0.383	0.683
PUFAs					**EPA**				
Model	5	128.011	17.197	0.000	Model	5	25.324	32.8	0.000
Intercept	1	21903	2942.4	0.000	Intercept	1	2072.9	2684.6	0.000
Error	60	7.444			Error	60	0.772		
Effects					Effects				
time	2	312.10	41.927	**0.000**	time	2	62.998	81.58	**0.000**
Type	1	15.030	2.019	0.161	Type	1	0.052	0.067	0.797
time*Type	2	0.988	0.133	0.876	time*Type	2	0.298	0.387	0.681
DHA									
Model	5	19.378	6.519	0.000					
Intercept	1	3372.03	1134.4	0.000					
Error	60	2.973							
Effects									
time	2	40.951	13.78	**0.000**					
Type	1	8.617	2.899	0.094					
time*Type	12	2.602	0.875	0.105					

$Y_{(measurement)} = \beta_0 + \beta_{1,t} + \beta_{2,\tau} + \beta_{3,t*\tau}$

Where t (class variable) is the effect of the time of the year and τ (class variable) is the type of product studied. $t*\tau$ represents the interaction term of the two class variables on the measurements studied. The estimation of the constant β_0 is given with the value of the intercept.

Appendix 3- ANOVA table from the GLM of the different groups of fatty acids measured on byproducts and oil data comparing the effect of time of the year (stages), type of products (byproducts or oil) and interaction effect. MS= mean squares, df = degrees of freedom, F = F-test; P = probability level.

Measure	df	MS	F	P	Measure	df	MS	F	P
Saturated					**MUFAs**				
Model	5	106.619	8.094	0.000	Model	5	278.01	21.102	0.000
Intercept	1	57563.2	4370.1	0.000	Intercept	1	153692	11666	0.000
Error	66	13.172			Error	66	13.174		
Effects					Effects				
time	2	29.096	2.209	0.118	time	2	691.05	52.454	**0.000**
Type	1	355.31	26.974	**0.000**	Type	1	2.406	0.183	0.670
time*Type	2	59.796	4.54	**0.014**	time*Type	2	2.766	0.210	0.811
PUFAs					**EPA**				
Model	5	125.115	20.395	0.000	Model	5	24.209	36.20	0.000
Intercept	1	24620.3	4013.3	0.000	Intercept	1	2585.8	3866.6	0.000
Error	66	6.135			Error	66	0.669		
Effects					Effects				
time	2	303.701	49.505	**0.000**	time	2	57.482	85.953	**0.000**
Type	1	18.078	2.947	0.091	Type	1	6.052	9.050	**0.004**
time*Type	2	0.04874	0.008	0.992	time*Type	2	0.0150	0.022	0.978
DHA									
Model	5	21.251	8.585	0.000					
Intercept	1	3348.405	1352.8	0.000					
Error	66	2.475							
Effects									
time	2	51.380	20.758	**0.000**					
Type	1	0.154	0.062	0.804					
time*Type	12	1.671	0.675	0.513					

$Y_{(measurement)} = \beta_0 + \beta_{1,t} + \beta_{2,\tau} + \beta_{3,t*\tau}$

2.2 Upgrading of Maatjes Herring Byproducts: Production of Crude Fish Oil

ABSTRACT

Fish oil has been extracted from byproducts of the maatjes (salted) herring production using a pilot plant consisting of a mincer, heat exchanger and a three-phase decanter. The crude herring oil obtained had an initial peroxide value (PV), anisidine value (AV) and free fatty acids (FFA) level of only 3 meq perox./kg of lipid, 8.9, and 2.9%, respectively. 5, 8, 11, 14, 17- Eicosapentaenoic acid (EPA) and 4, 7, 10, 13, 16,19- docosahexaenoic acid (DHA) were present in considerable amounts (99 and 91 g/kg, respectively). During storage of the oil, no photooxidation could be detected. Storage at room temperature led to significant autoxidation over time, apparent from primary and tertiary oxidation products, measured by a decrease of hydroperoxides and an increase of fluorescent compounds (FC). Storage at 50 °C, resulted in significant increases in secondary (AV) and tertiary oxidation (FC) products. At all storage conditions, the FFA contents remained low (<3%) and the α-tocopherol content remained constant. These results open the possibility for fish oil production of good quality using salted herring byproducts.

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INTRODUCTION

There is a sizeable and growing world market demand for high-quality fishmeals and oil, and production can be quite profitable if suitable raw material is available. Clupeids (e.g., herring and sprat) provide the largest single source of raw material for production of fish meal and oil. They may be classified as fatty, although the fat content may vary from 2 to 30% depending on species and seasons (*5, 83*). The fish industry would be wise to handle the byproducts from gutting, filleting, and other fish-processing operations with care because these have proven to be a good raw material for fish meal and oil production. Skåra and Cripps (*6*) have found that byproducts from farmed salmon can be used to produce fish oil of a quality that is well suited for human consumption. Besides salmon, byproducts from other fatty fish species such as herring and mackerel might be used as a source of raw material.

In The Netherlands the main fatty fish species that is processed for human consumption is herring. At present, the Dutch fish industry sells their byproducts to fish meal plants in Germany and Denmark. The byproducts are transported to these countries and then converted into fish meal, fish oil and pet food of rather poor quality due to long transport times. For this reason it would be better to process the herring waste near the source where it is produced. If possible, the fish byproducts should be held under chilled conditions to minimize the effect of microbial and enzymatic attack on the fish tissue. This spoilage is responsible for increased free fatty acid contents and increased oxidative breakdown by, for example, peroxidases (*8, 9*).

Maatjes herring, a very popular product in The Netherlands and surrounding countries, is produced mainly from fresh herring landed and gibbed in Denmark and subsequently lightly salted (*84, 85*). Production is also possible from herring frozen in blocks. Gibbing is the removal of gills, liver, heart, intestines, and stomach but not the pyloric appendix. The gibbed, lightly salted herring is transported from Denmark to The Netherlands in barrels. After arrival, it is sorted and packed in smaller buckets. Brine is added, and the herring is frozen in order to kill nematodes and preserve the herring. Large processors produce maatjes herring fillets to be sold to retailers. In lightly salted herring the ripening effect by the enzymes from the pyloric appendix is mainly on the texture of maatjes herring and not on the taste or odor of the product (*30, 84, 85*).

The byproducts of maatjes herring processing consisting of heads, frames, skin, and fins could be used for the recovery of oil. The total amount of maatjes herring byproducts available from the major Dutch companies is estimated at ~2100 tons/year. However, these byproducts have been treated with salt, which may affect the quality and stability of

the oil (*86, 87*). Oxidation may be initiated and promoted by several organic and inorganic substances, such as copper and iron salts, often present in salt (*31, 88, 89*).

Fish oil, rich in polyunsaturated fatty acids (PUFAs), is very sensitive to oxidation. In general, three oxidation steps are recognized: initiation, propagation, and termination. The first stage of the oxidation process is characterized by the production of hydroperoxides, which is usually measured as the peroxide value (PV). The amount of hydroperoxides in oil usually increases with time to a certain maximum and then decomposes rapidly to the secondary oxidation products, leading to a subsequent decrease of the PV. The second stage is represented by the further degradation of lipids through a radical oxidation process initiated by the hydroperoxides. The resulting nonvolatile secondary end-products (high molecular weight saturated and unsaturated carbonyl compounds in triacylglycerols) can be measured by the anisidine value (AV). In many cases the AV is associated with the term “oxidative rancidity”: an objectionable level of off-odors and off-flavors (*90*). This type of deterioration can be serious in poorly processed oils containing n-3 PUFAs (*91*).

Lipid deterioration compounds (peroxides and carbonyls) cause the formation of interaction compounds with fluorescent properties, the fluorescent lipid oxidation products (FC). The production of these so-called tertiary oxidation products is among others a function of temperature (*92*). The measurement of FC has been successfully applied to marine oils (*93*). The FFA content is usually measured because this is still one reliable parameter for oil quality (*5*). Furthermore, high levels of FFA can be a presage for lipid oxidation development (*45*).

Another approach to monitor the progress of oxidation is to study the decrease in antioxidant content. For that reason, for example, the α-tocopherol content in the oil, a major antioxidant in these oils, can be followed.

The aim of this paper is to investigate the possibility of using the salted byproducts of the maatjes herring industry as a raw material source for fish oil production. Therefore, a pilot extraction facility was developed and used to upgrade the herring byproducts by the production of high-quality herring oil. The paper focuses on the oil quality change during processing and storage of the oil from maatjes herring byproducts. First, a characterization of the raw material has been made, and mass balances of the process and oil yields achieved have been calculated. In the second part, a stability study was performed following the expected loss of α-tocopherol, development of FFA, and formation of oxidation products in the oil when stored at different conditions. Linear regression models were used as a tool to evaluate whether any changes were occurring at

a statistically significant level during storage and to compare the different stability measurements.

MATERIALS AND METHODS

The raw material used in this study was herring byproducts from frozen herring (*Clupea harengus*) caught at the North Sea and used for maatjes herring production.

Equipment

The oil recovery process is schematically represented in Figure 1.

A batch of ~1000 kg of maatjes herring byproducts (heads, frames, skin, viscera, etc.) was minced (mincer type, SAB, 49-033.2). Immediately after mincing, the minced byproducts were pumped (monopump, SW 032, speed drive 50 Hz, representing a typical flow of 250 kg/h) to an insulated scraped-surface heat exchanger indirectly heated by steam. The heated suspension was then separated in a three-phase decanter (Alfa Laval, Denmark, NX 409S-11G, set at 5600 rpm) into a semisolid phase (called protein phase), a water phase (stickwater), and lipid phase (oil). All equipment parts exposed to the products, with the exception of the mincer, were made of stainless steel.

Sampling

Samples were collected from four consecutive stages of the process, that is the minced byproducts, the oil, the stickwater, and the protein phase, and these were analyzed.

Part of the recovered herring oil was divided into three containers and exposed to three different storage environments: at room temperature, in a closed dark container, flushed with nitrogen; at room temperature in closed transparent glass bottles placed in front of the window during the months of July and August 1999; in an oven at 50 °C in closed dark containers.

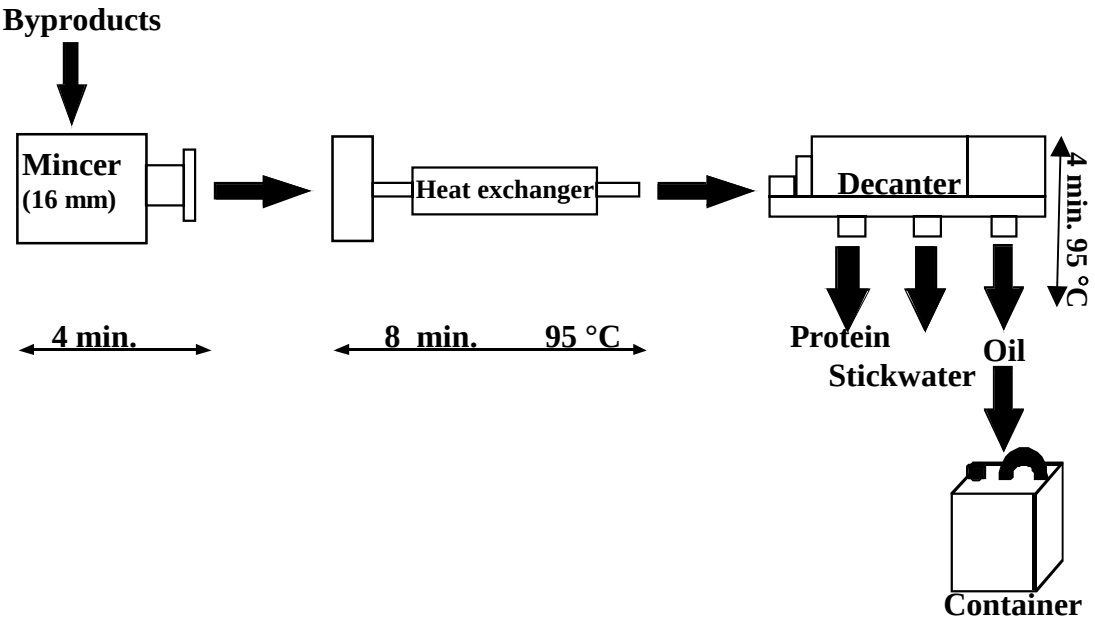

Figure 1- Scheme of the fish oil plant with processing time and temperature. Samples were taken after mincing and at the outlet of the decanter.

Two oil samples were taken, at regular intervals, from each storage condition. To avoid further oxidation, all of the samples were kept in an –80 °C freezer until being analyzed further. Prior to

the analysis, the oil samples were thawed at room temperature for 30 min. The samples were then analyzed for the level of oxidation products, FFA formation, and α-tocopherol content.

Analytical Methods

Fatty Acid Composition. Lipids from herring fillets and byproducts were extracted according to the method of Bligh and Dyer (*65*). Fatty acid methyl esters (FAMEs) from the lipid extractions and the oil samples were prepared according to the AOCS (*66*) Official Method Ce 1b-89 and analyzed with regard to the amount of content of individual fatty acids. In each occasion three samples of fillets, byproducts and oils (n = 3) have been analyzed once (a = 1). The different FAMEs were separated from each other with gas chromatography (GC) using a Fisons 8130 instrument, equipped with an autosampler (Carlo Erba A200S) and detected with a flame ionization detector (FID). A fused silica capillary column (0.25 mm i.d. x 50m) coated with CP Sil-88 for FAMEs (film thickness = 0.20 μm), from Chrompack (Middelburg, The Netherlands) was used. The chromatographic conditions applied were as follows: column oven temperature, 190 °C, injection port and detector temperatures, 250 °C; sample size, 0.1 μL; split flow, 60 mL/min. Helium was used as carrier gas with an inlet pressure of 145 kPa. FAMEs were identified by comparison of their retention times with those of chromatographic standards (from Alltech and Sigma). Quantification was performed, using TurboChrom software (version 4.2, Perkin-Elmer), by integrating peaks on the chromatogram. Results are expressed as g/kg of lipid.

Extraction of Lipids for Determination of Lipid Classes, Peroxide Value and Fluorescent Oxidation Products: Total lipids from the herring fillets and byproducts were extracted according to the method of Burton et al., (*94*) as modified by Undeland et al. (*95*).

Determination of Lipid Class Distribution. The contents of neutral lipids (NL), phospholipids (PL), and FFA existent in the total lipids from herring fillets, byproducts and in the fish oil were determined gravimetrically by solid phase extraction (SPE) according to the method of Kaluzny et al., (*96*) (n = 2, a = 2). Disposable extraction columns Bakerbond SPE™ Amino (NH_2-500 mg) were used (J. T. Baker, Deventer, the Netherlands). Results are expressed as g/kg of lipid.

α-Tocopherol. α-Tocopherol was analyzed by reversed phase HPLC (analytical column, 3 x 100 mm, inert Sil 3ODS3, Varian, The Netherlands) with fluorescence detection (excitation, 295 nm; emission, 330 nm), according to the slightly modified version of the method described by Lie et al., (*97*). Briefly, the homogenized samples were prepared for analyses by saponification in 5 mL of ethanol and 0.5 mL of 50% KOH and extraction of the unsaponifiable material in 2 x 2 mL hexane. Ascorbic acid was added before saponification, to prevent oxidation of the sample. Determination of the content was done by calibration with an external standard. On each occasion n = 2, a = 1. The repeatability of the method for analyzing α -tocopherol was 5.1% (n = 1, a = 6). Results are expressed as mg/100g of lipids. Internal reference oil materials were analyzed together with the samples.

Free Fatty Acids. The amount of FFA of the oil samples was determined by titration according to AOCS (*66*) Official Method Ca 5a-40 (n = 2, a = 1). The percentage of FFA was calculated as oleic acid. Internal reference materials were analyzed together with the samples.

Peroxide Value. The peroxide value (PV) of the herring oil samples was determined according to AOCS (*66*) Official Method Cd-8b-90 (n = 2, a = 1). The content is expressed in terms of meq of peroxides per kg of lipid. Internal reference materials were analyzed together with the samples.

Anisidine Value. The anisidine value (AV) of the herring oil was carried out according to AOCS (*66*) Official Method Cd 18-90 (n = 2, a = 1). Internal reference materials were analyzed together with the samples.

Fluorescence Products (FC). Total lipid soluble fluorescent lipid oxidation products with an excitation (ex) maximum at 367 nm and an emission (em) maximum at 420 nm were measured in the oil samples using FIA as described by Undeland et al., (*98*) (n = 2, a = 1). Results were expressed as peak area units per picogram of lipid. The repeatability was 7.0% (n = 1, a = 6).

Lipid Content. The total lipid content in the samples was determined gravimetrically after extraction according to the Bligh and Dyer (*65*) procedure (n = 2, a = 2). Results were expressed as grams of lipid per kilogram of samples. Internal reference materials were analyzed together with the samples.

Moisture. Moisture content of the oil samples was determined according to the Karl-Fischer method (n = 2, a = 2). In the other samples moisture was determined by weighing the samples, until a constant weight was achieved, after drying in an oven at 105°C (n = 2, a = 2). Results were expressed as percentage of wet weight. Internal reference materials were analyzed together with the samples.

Protein. Total nitrogen in the homogenized samples was determined using the Kjeldahl digestion method. Protein content was calculated as N x 6.25 (n = 2, a = 2). Results were expressed as percentage of wet weight. Internal reference materials were analyzed together with the samples.

Salt. Chloride content in all of the samples was titrated according to Volhard's method as described (*67*), (n = 2, a = 2). Results were expressed as percentage of wet weight. Internal reference materials were analyzed together with the samples.

Analysis of Copper (Cu). To make copper available for analysis, wet sample digestion was used. The samples (about 0.5 g of oil and 1.0 g of the other samples) were destructed with concentrated nitric acid in a microwave oven for 45 min in Teflon destruction vessels. After destruction, the acid was partially evaporated and the samples were diluted to 50 mL with Milli-Q water. The copper content in each sample was determined with a Perkin-Elmer 5100 graphite furnace atomic absorption spectrometer (AAS) with Zeeman background conditions (*99*) (n = 2, a = 2). Results are expressed as mg/kg of wet sample. The limit of detection was 0.1 mg/kg. The repeatability of the method was 6.0%. Certified reference materials were analyzed together with the samples.

Analysis of iron (Fe). About 1.0 g of each sample was ashed at 500 °C (*100*). The ash was dissolved in 5 mL of 6.0 M hydrochloric acid and diluted to 50 mL with double-deionized water. The concentration of iron was then measured with a flame atomic absorption spectrometer (Perkin-Elmer 5100, Norwalk, CT). In the case of the oil, samples were diluted with xylene and immediately measured with a graphite furnace atomic absorption spectrometer (Perkin-Elmer 5100, Norwalk, CT). In both cases, a deuterium background correction (*101*) was used (n = 2, a = 2). Results are expressed as mg/kg of wet sample. The limits of detection were 0.01 and 0.1 mg/kg, and the repeatabilities of the methods were 10.0 and 5.0% for the oil and the other samples, respectively. Internal or certified reference materials were analyzed together with the samples.

Statistical Analysis

Data from the α-tocopherol and stability measurements were subjected to regression analyses to fit a model describing whether there was a significant increase or decrease over time. Lack of fit tests showed that linear models ($y = \beta t + \alpha$) could be used and therefore were tested. This technique also provided the possibility to compare the overall rates at which the different oxidation products are produced. Analysis of variance for testing the hypothesis H_0: $\beta = 0$ against H_A: $\beta \neq 0$ was used. Confidence intervals for the parameter β (regression coefficient) and α of different measurements were estimated. The p value was set at 95%.

RESULTS AND DISCUSSION

In the first part of this section the composition of the raw materials and produced oil is presented. In the second part, the stability of the oil during storage is discussed.

Compositional Data

Crude Composition

The herring byproducts contain a high moisture content (68.6%), whereas the oil and protein contents were 16 and 11.7%, respectively (Table 1).

The relatively high oil content can be explained by the fact that the maatjes herring production uses catches only from the end of May/beginning of June, when it is known that the herring has a high fat content. Note that the total lipid contents in the herring fillets and the byproducts are very similar (16%).

Table 1- Compositional data of frozen raw material used for production of maatjes herring (*Clupea harengus*): fillets, byproducts, oil, protein and stickwater phase.

	Fillets	Byproducts	Herring oil	Protein phase	Stickwater
Crude composition					
Protein (% w/w)[a]	16.7 ± 0.6	11.7 ± 0.3	-[c]	24.9 ± 0.9	9.9 ± 1.0
Moisture (% w/w)[a]	64.6 ± 0.6	68.6 ± 1.4	0.09 ± 0.01[b]	64.2 ± 0.8	77.8 ± 0.3
Salt (% w/w)[a]	1.67 ± 0.05	2.94 ± 0.07	-	3.48 ± 0.05	4.07 ± 0.01
Lipid components					
Total lipids (g/kg wet sample)[a]	164 ± 7	162 ± 0.5	-	56.2 ± 0.5	78.5 ± 12.5
Neutral lipids (g/kg lipid)[a]	937 ± 20	901 ± 1	971 ± 4	-	-
Phospholipids (g/kg lipid)[a]	13 ± 2	8 ± 4	7 ± 3	-	-
Free Fatty acids (g/kg lipid)[a]	38 ± 11	71 ± 5	31 ± 4	-	-
α-tocopherol (mg/100g lipid)[b]	1.4 ± 0.4	4.2 ± 0.4	2.8 ± 0.1	-	-
Trace elements					
Fe (mg/kg wet sample)[a]	9.2 ± 0.3	26 ± 3	0.8 ± 0.1	51± 1	17.1 ± 0.2
Cu (mg/kg wet sample)[a]	0.66 ± 0.08	0.45 ± 0.06	<0.1	1.06 ± 0.04	0.41 ± 0.02

[a] : n = 2, a = 2, results are given as: mean value ± (max-min)/2. For each of the two samples, a = 2. Mean values from these two analyses were used to establish the sample variation.
[b] : n = 3, a = 1, mean ± sd.
[c] -: Not measured.

After processing, the protein and stickwater fraction contain 5.6 and 7.9% of oil, respectively. The relative distribution of NL, PL and FFA from fillets, byproducts and oil

shows that in all cases the NL are the major lipid constituents, followed by FFA and PL. The higher FFA levels indicated more hydrolysis on the herring byproducts than in the fillets (7.1 versus 3.8%). As expected, a higher protein content was determined in the fillets comparatively to the byproducts (16.7 versus 11.7%). The produced herring oil shows a moisture content of <0.1%. This value meets the quality assurance specification given by Young (*35*) who reported for a crude fish oil a maximum of 0.3%, whereas according to Bimbo (*88*) it should range on a usual basis from 0.5 to 1% maximum.

An overall mass balance was calculated on the basis of the crude composition of the raw material (100 kg of byproducts) and the end products, giving 9.3, 17.6, and 73.1 kg of oil, protein, and water phase, respectively. The total yield of the oil was therefore 59%. Optimizing the decanter can probably further increase this value. However, it should be emphasized that our aim was not to optimize high yields, and thereby stress the oil, but to isolate oil of high quality. The low yield of the protein phase indicates a good protein/water separation.

Fatty Acid Composition

It is known that within the same fish species the fatty acid composition can vary widely due to geographical location, fishing season, and parts of the body analyzed. Table 2 shows the fatty acid composition of the fat from the three different stages of the process: herring fillets, byproducts and obtained oil.

High concentrations of C20:1, C22:1, C20:5, and C22:6 fatty acids were measured. It is likely that these fatty acids originated from the feed of the herring, mainly copepods (natural marine zooplankton) from relatively high latitudes (*11, 102, 103*). The fillets and byproducts fractions showed the following ranking order for the amount of saturated, monounsaturated, and polyunsaturated fatty acids of their fat: monoenes > saturates > polyunsaturates; whereas for the oil fraction this order was monoenes > polyunsaturates > saturates. The absolute amounts of saturates are quite similar, showing that palmitic (C16:0) acid, as in most of animals (*104*), is the principal saturated fatty acid (~14% of the total fatty acids). The byproducts and the oil had nearly the same concentration of monoenes, whereas oil from fillets had a somewhat higher concentration. Most of the difference can be explained by the content of C22:1, with the fillets having up to 18% higher levels. The total content of polyunsaturated fatty acids of the fat from fillets and byproducts was very similar but lower than in the fish oil fractions. The large differences regarding concentrations of individual polyunsaturates, especially C20:5 and C22:6, may possibly come from some oxidation during lipid isolation from the fillets and byproducts. This reveals that the fish oil extraction method used for a large-scale process seems to be

more suitable for recovery of PUFAs. Higher values were found, implying that there were fewer losses.

Table 2- Fatty acid profile (g/kg of lipid)[a] in total lipids from fillets, byproducts and oil produced from Maatjes Herring (*Clupea harengus*) byproducts.

Fatty acids	Fillets	Byproducts	Oil
Saturated			
C14:0	90 ± 4	89 ± 3	98 ± 6
C16:0	143 ± 7	138 ± 2	147 ± 8
C18:0	18 ± 1	18 ± 0	21 ± 2
Total	252 ± 11	245 ± 5	266 ± 16
Monoenes			
C16:1	50 ± 2	46 ± 1	52 ± 4
C18:1	60 ± 6	59 ± 1	72 ± 1
C20:1	139 ± 11	127 ± 3	130 ± 15
C22:1	202 ± 2	182 ± 3	166 ± 12
Total	451 ± 20	414 ± 8	369 ± 29
Polyunsaturated			
C18:2	13 ± 3	15 ± 1	13 ± 2
C18:3	15 ± 2	13 ± 0	13 ± 2
C18:4	46 ± 2	47 ± 0	61 ± 5
C20:5	68 ± 2	71 ± 0	99 ± 13
C22:6	70 ± 3	72 ± 1	91 ± 11
Total	212 ± 12	217 ± 3	277 ± 33

[a] Mean value ± SD, (n = 3, a = 1).

Trace Elements (Fe and Cu)

As shown in Table 1, the iron and copper contents varied substantially among the different fractions analyzed. Maatjes herring byproducts contained almost a three times higher iron content compared to the fillets, whereas concentrations of copper were higher (by ~32%) in the fillets than the byproducts. These results reflect the high levels of various hemoproteins (*74*, *82*) and low molecular weight iron (*105*) in byproducts. Concentrations of copper were much lower than iron concentrations in all fractions. The protein fraction contained the higher metal content, whereas the oil had the lowest. The herring protein contained ~64 times as much iron and at least three times as much copper as the corresponding oil. Values of 0.8 and <0.1 mg/kg of wet weight have been determined in the present crude herring oil for iron and copper, respectively. For herring oil, Notevarp and Chahine (*106*) reported values ranging between 10.2 and 0.5 mg/kg for

iron and from 0.12 to 0.03 mg/kg for copper. Young (*89*) recommended as acceptable levels 1.5 and 0.2 mg/kg maximum for iron and copper content, respectively.
Bimbo (*88*) proposed values of 0.5-7.0 mg/kg for iron and <0.3 mg/kg for copper. This implies that the oil obtained is of acceptable quality. Oxidative stability of the capelin oil showed no correlation with the content of iron but some tendency to correlation with copper (*106*). In addition, Young (*89*) stated the importance of metals content, in particular, copper as a catalyst for the oxidation of oils and fats. Therefore, it can be concluded that our low oxidation levels (initial and developed over time) might be partly due to the low content of copper present in the crude herring oil.

α-Tocopherol

The α-tocopherol content showed large distribution variations. However, it should be pointed out that in some previous studies a large individual difference has been found (*107*). Lipids from herring byproducts contain three times as much α-tocopherol as the fillets. These results are in accordance with the results reported for mackerel dark muscle versus light muscle lipids (*74, 82, 108*). According to these authors, a possible explanation would be the abundance of mitochondria in dark muscle. The amount of α-tocopherol present in the oil, 2.8 ± 0.1 mg/100 g of lipids, is lower than the values reported in the literature for herring fish oil, for which values of 13, 21, and 14 mg/100 g of lipids have been described (*106, 109*). Following the α-tocopherol content for the three different storage conditions has shown that there was hardly any loss of this antioxidant. This is confirmed by the fact that no significant losses or gains were found over time as is shown in Table 3.

Table 3- Statistics describing the outcome of the linear regression analysis of the data obtained at three different storage conditions. For all the cases average values, obtained from duplicated measurements, were used. α and β represent the intercepts and slopes respectively with the correspondent confidence intervals; r^2 are the correlation and *P* the significance levels found. Values given in bold represent significant differences.

Measure		Dark (n = 20)	Light (n = 13)	50 °C (n = 13)
α-Tocop.	α	3.62 ± 0.41	3.62 ± 0.56	3.65 ± 0.70
	β	0.003 ± 0.009	0.013 ± 0.025	0.00207 ± 0.040
	P	0.477	0.272	0.912
	r^2	0.0285	0.108	0.0012
FFA	α	2.73 ± 0.06	2.78 ± 0.09	2.62 ± 0.14
	β	0.0013 ± 0.0014	-0.0003 ± 0.0040	0.0062 ± 0.0080
	P	0.074	0.868	0.115
	r^2	0.167	0.0026	0.2097
PV	α	1.27 ± 0.407	2.50 ± 1.48	2.388 ± 1.41
	β	-0.0106 ± 0.0088	0.038 ± 0.0666	0.023 ± 0.080
	P	**0.021**	0.234	0.542
	r^2	0.261	0.126	0.035
AV	α	7.26 ± 0.35	7.59 ± 0.97	7.36 ± 1.87
	β	-0.0073 ± 0.0077	-0.019 ± 0.044	0.17 ± 0.107
	P	0.059	0.349	**0.004**
	r^2	0.184	0.080	0.539
FC	α	19.05 ± 1.55	13.27 ± 3.74	13.98 ± 4.94
	β	0.165 ± 0.034	0.031 ± 0.168	1.678 ± 0.272
	P	<< **0.05**	0.691	<< **0.05**
	r^2	0.856	0.015	0.949

Oxidative Stability

The value of FFA in oil is an important quality parameter, not only because usually the FFA value is checked due to contractual reasons (between the retailer and purchaser of the oil) (*5*) but also because the FFA are more susceptible to oxidation than esterified fatty acids (*45, 110*). As quality specifications for crude fish oil, Bimbo (*88*) suggested that the FFA content should range between 1 and 7% but usually ranges between 2 and 5%, whereas Young (*35*) suggested maximum acceptable values of 4.0%. The results (Figure 2) show that the amount of FFA present in the maatjes herring oil was low (2.9%) and remained almost constant during storage time for all three different storage conditions. In fact, no significant change over time was indeed revealed for the referred studied storage conditions (Table 3). This suggests that significant hydrolysis of the oil did not occur during the storage period, possibly due to the low moisture content present in the oil (Table 1). Nambudiry (*86*), showed that with an increase in the salt content, the rate of FFA production in sardine muscle tissue decreases. In addition, in crude capelin oil

(*106*), a positive correlation between iron content and FFA was found: oils with low iron contents tend to have low FFA values. Addison et al., (*111*) reported consistent results showing that the FFA of several herring oils had arisen primarily through hydrolysis of phospholipids, which occurs before or during oil production. In our case, low values of iron and phospholipids were determined, and it could be that this reduces FFA production.

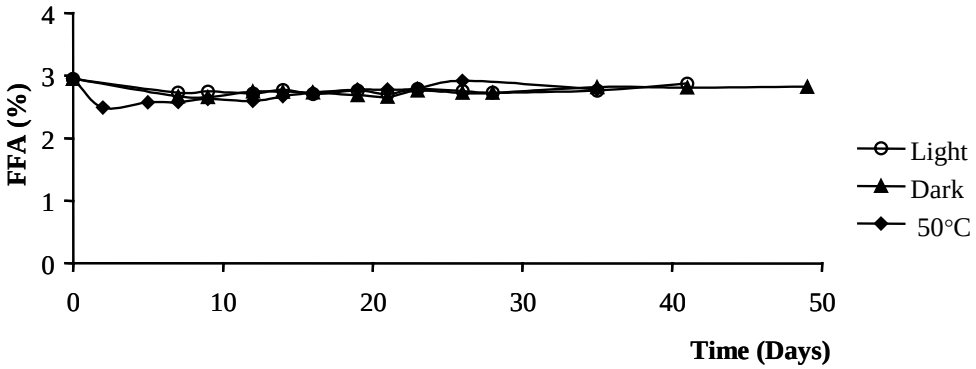

Figure 2- Free fatty acids of maatjes herring oil during storage.

The primary oxidation products were measured as the hydroperoxides, presented by the peroxide value (PV). The herring byproducts had a PV of 10.2 ± 2.1, whereas the oil presented a value of only 3.0 ± 0.3 meq. perox./kg of lipid. The decreased PV measured may be due to rapid hydroperoxide breakdown during the heating step of the process (*47, 87*). During storage of the maatjes oil, two reproducible peaks of peroxide values are shown for the conditions at 50 °C and placed under light (Figure 3). This phenomenon can be caused by two different oxidation reactions with different speeds or initiation times (autoxidation and photooxidation, for the two studied conditions, respectively). It is known that the oxidation of unsaturated lipids is accelerated by exposure to light, there being two types of sensitizers recognized for photosensitized oxidation (*91*). Autoxidation and photooxidation produce hydroperoxides. However, no significant increase in time was found for PV at these storage conditions (Table 3). In the oil stored under dark and nitrogen conditions, the peroxide value remains very low and decreases significantly over time (β = -0.0106; P = 0.021).

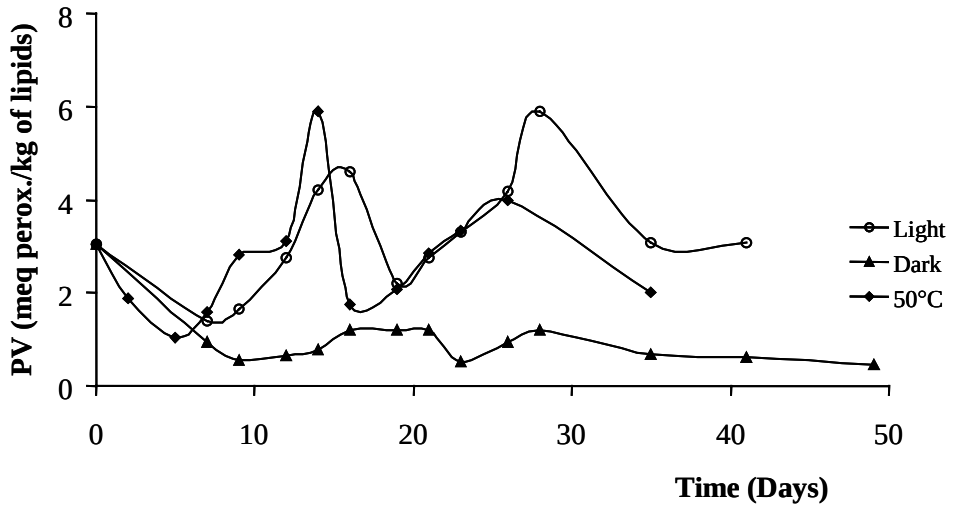

Figure 3- Peroxide value of maatjes herring oil during storage.

This is unexpected, taking into account that fish oil is rich in polyunsaturated fatty acids and consequently susceptible to oxidation. A possible explanation for the low PV measured is the rapid degradation of hydroperoxides during the thermal treatment, as stated previously. The result is that the rate of degradation in the oil is much higher than the rate of formation, and low hydroperoxides as result, which become even lower in time due to the propagation reactions.

The hydroperoxides are very unstable and decompose into secondary oxidation products (volatile and nonvolatile end products). The nonvolatile secondary oxidation products can be followed with the anisidine value (AV). The byproducts presented an AV of 7.5 ± 0.4, whereas the oil had an AV of 8.9 ± 0.5. Figure 4 shows the AVs measured in the oil stored over time. In the oil kept at 50 °C, the development of the secondary oxidation products initiates within a few days after storage. This implies that the primary oxidation products are rapidly decomposed to secondary oxidation products. In this case a significant, positive slope was found for the development of AV with storage time (β = 0.17). Although the correlation was not very high (r^2 = 0.539), the change was significant (P = 0.004). The two maxima present for peroxide value at 50 °C (Figure 3) are also observed in the anisidine value at 50 °C (Figure 4), which explains the low r^2. In the oil samples stored under light conditions, only two small peaks are present.

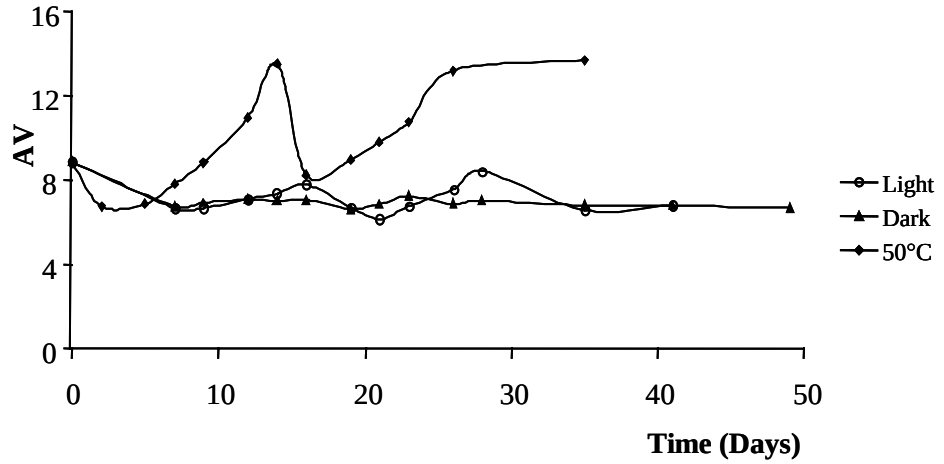

Figure 4- Anisidine value of maatjes herring oil during storage.

Possibly, for the photooxidation process, a termination reaction is favored above a propagation reaction, under the conditions studied. There is hardly any change in the AV for the oil kept in dark conditions. The oil under this condition is stable. In both cases (light and dark, room temperature), slightly negative slopes were obtained with poor correlation values. The slopes were not significantly different from zero (Table 3). Therefore, it can be concluded that in the presence of light and at room temperature the low amounts of secondary oxidation products are caused by a lack of oxygen. The tertiary oxidation products are shown in Figure 5 as fluorescent compounds (FC). An initial value of 17.7 area units/pg for the oil was determined. The maatjes herring oil stored at 50 °C shows an increase of FC values over time. This fact is confirmed with a positive slope, high correlation value, and significant increase over time (β = 1.678; r^2 = 0.949, and $P << 0.05$, respectively).

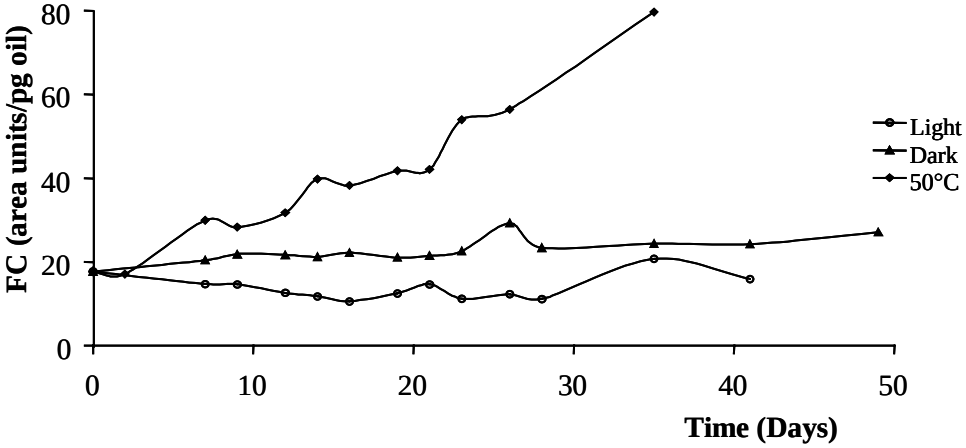

Figure 5- Fluorescent compounds of maatjes herring oil during storage.

The reaction from secondary to tertiary oxidation products clearly takes place. The oil stored under the light condition show a very low FC value, with a nonsignificant change over time. It is obvious that the low FC is due to low AV formed. Apparently, the primary oxidation products, formed in this case via photooxidation reaction, are only partly transformed into secondary oxidation products. A negative, although nonsignificant, slope found for the AV over time for this condition corroborates this hypothesis. The oil stored under dark conditions show a stable value for the FC, which persuades us that the high amounts of tertiary oxidation products are inhibited by a lack of oxygen and light when the oil is stored at room temperature. During storage of the oil under dark conditions a positive slope and a good correlation were found for the development of fluorescent lipid oxidation compounds with storage time (β = 0.165, r^2 = 0.856), and the change was significant ($P << 0.05$). For 50 °C the formation of fluorescent compounds progresses 10 times more quickly than at room temperature (β = 1.678 versus β = 0.165). Consequently, it can be concluded that there is a significant effect of temperature. Assuming Arrhenius kinetics, the activation energy has been estimated as ~74 kJ/mol. Such values are expected for chemical reactions.

Concluding Remarks

The results show that it is possible to produce good-quality fish oil from salted (maatjes) herring byproducts. This oil, like the oils from fatty fish species, contains a high percentage of the valuable polyunsaturated n-3 fatty acids, mainly EPA and DHA. The oxidation values found reveal that the crude herring oil obtained has low initial values of FFA, PV, AV, and FC. Low values of copper and iron were determined in the herring oil, explaining partly the low oxidation values.

From the herring oil stability study, it can be emphasized that linear models were not good to describe the effect of light, because for this condition all r^2 values were below 0.15.

For the studied oil, α-tocopherol and FFA contents did not change over time for the applied storage conditions. The oil kept under dark conditions at room temperature shows hardly any oxidation with a very slow decrease of PV and a slight increase of FC. For the dark conditions at 50 °C the AV and FC increase rapidly over time.

Only a significant increase over time of fluorescent lipid oxidation products was found both for the dark conditions at room temperature and at 50 °C. The formation of FC was under the last condition 10 times faster than under the former condition. Therefore, it can be concluded that temperature has a strong effect on preservation of the studied oil.

Further FC can be used as a valuable rapid method to evaluate the progress of lipid oxidation in oil. The oil presents low oxidation products over time when it kept under dark conditions, in the absence of oxygen, and at ambient temperatures. This shows that usage of maatjes herring byproducts is very promising for fish oil production of good quality.

Acknowledgements

We thank Maurice Boonman, Eelco Scherpenisse, and Gerda Booy for their assistance with part of the analytical work.

2.3 Chemical and sensory evaluation of crude oil extracted from herring byproducts from different processing operations

ABSTRACT

Three herring oils, extracted from byproducts from different herring processing (salted or unsalted) and storage (fresh or frozen) were evaluated on their chemical and sensory properties. The obtained crude oils had very low content of copper (< 0.1 mg/kg oil) and iron values ranged between 0.8, 0.1 and 0.03 mg/kg oil to maatjes, frozen and fresh oil, respectively. For the maatjes oil, a much lower value was found for α-tocopherol compared to the other oils. Storage stability results showed that the oils behave differently. The secondary oxidation stage was reached for fresh oil, while for the maatjes and frozen oil tertiary oxidation products were detected. Over storage time, the maatjes and frozen oils became more intense in odor, correlating positively at the end with sensory attributes of train-oil, acidic, marine and fishy. The best correlation between overall quality rating by sensory and chemical analyses was found for FFA and fishy off-odor ($r = 0.781$).

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INTRODUCTION

Fish and fish oils contain high concentrations of ω-3 polyunsaturated fatty acids (PUFAs) (*11, 29, 112, 113*). These PUFAs receive intense interest in the scientific and industrial communities because of their positive role in human health (*24-26, 114, 115*). As awareness of their nutritional importance has risen, attention for their supply has increased. Presently, most of the fish oil available on the market is fish body oil which results from the pressing/heating of whole pelagic fish to release the oil mainly concentrated in the body of the fish (*5, 36, 37*). However, fish oil can also be produced from byproducts from the processing industry (*6, 41, 59, 61*).

In the Netherlands the main fatty fish species that is processed for human consumption is herring (*Clupea harengus*) mostly caught at the North Sea. It is estimated that the three major Dutch fish processing companies produced an amount of about 27,000 tons/year of herring byproducts. These byproducts derived from the production of two popular products: maatjes and marinated herring. Marinating herring has traditionally been applied as a conservation method, using fresh or frozen herring as raw material. For this process, brines are added after the filleting operation; therefore the salts do not come in contact with the byproducts itself. This is in contrast to the maatjes production, where brine is added to the whole fish, as described earlier (*59*). This is relevant to whether it is possible to keep the stability and quality of oils extracted from herring byproducts constant when the byproducts vary according to processing operations and storage of the raw material. Marine lipids, which contain higher quantities of ω-3 PUFAs are susceptible to oxidation following successive degradation (*116*). It is known that lipid oxidation takes place in fatty fish species during processing and storage (*47, 117, 118*). To follow the degree of oxidation, use can be made of different chemical measurements. The oxidation products formed affect sensory properties of the oil adversely. Therefore, sensory assessment remains the most direct quality criteria for edible oil and its shelf life.

The aim of this study is to investigate the quality and stability of crude herring oil produced from byproducts obtained with different processing routes as well as to provide a comparison between the use of fresh and frozen herring as raw material. To follow lipid oxidation progress, several methods were used. Attention was focused on the development of primary, secondary and tertiary oxidation products. The loss of α-tocopherol and the change in the free fatty acids content were also followed in time. Sensory assessment of the oil was evaluated with six aroma attributes for freshly produced oils as well as for various storage times of the oils. Multivariate data analysis

was used to detect chemically and sensorial changes and to identify correlations between the measured parameters.

MATERIAL AND METHODS

Experimental Procedures

Fish oil was extracted from fresh unsalted herring byproducts obtained from frozen and from fresh herring (*Clupea harengus)* used for marinated production obtained after the filleting. The herring from the frozen and fresh production were respectively caught in June and October 1999, off of 60.50 N 02.50 W and 51.20 N 02.60 E, with average weight of 144 ± 1 and 138 ± 23 g, length of 26 ± 1 and 24 ± 1 cm, and maturity stage III and V (in both cases, n = 19). The oil produced from byproducts coming from the frozen herring used in the marinated process is referred to as "frozen oil", whereas the oil produced from byproducts of fresh herring was called "fresh oil". For the production of the maatjes oil the herring was caught in May 99 and was frozen until processing. This type of oil (further referred as "maatjes oil") was extracted from salted herring byproducts as described earlier (*59*).

Equipment

Three production runs of ~ 1000 kg each of herring byproducts (heads, frames, skin, viscera, etc.) generated from the different processing and storage of herring were minced. Immediately, they were pumped to an insulated scraped-surface heat exchanger indirectly heated by steam and separated in a three phase decanter into a high solids phase (referred to as protein phase), a water phase (stickwater), and lipid phase (oil) using the same conditions and system as described earlier (*59*).

Sampling set-up

In all storage experiments, the oil was blanketed with nitrogen, and kept in closed dark containers at room temperature (~ 20 °C). Two oil samples were taken, at regular intervals, from the different oils and analyzed for levels of oxidation products, FFA formation, α-tocopherol content, and sensory evaluation. Sampling was discontinued when the oils developed a strong off-odor, therefore different maximum storage times resulted (155, 92, and 57 days for fresh, maatjes and frozen oil, respectively). All samples were kept at –80 °C freezer until being analyzed. Prior to the analysis, the oil samples were thawed at room temperature for 30 min. Averages of the two oil samples measurements were used for further interpretation.

Chemical analyses. The level of free fatty acids (FFA) was determined by titration according to the AOCS (*66*) Official Method Ca 5a-40. The peroxide value (PV) was determined according to the AOCS (*66*) Official Method Cd-8b-90. Determination of the anisidine value (AV) was carried out according to the AOCS (*66*) Official Method Cd 18-90. α-Tocopherol was analyzed according to the slightly modified method of Lie et al. (*97*) as described earlier (*59*) using reversed phase HPLC and fluorescence detection. The UV absorbance at 270 nm (conjugated trienes, CT) of the oil samples were measured using flow injection analysis (FIA) as described by Undeland et al. (*98*). The results were expressed as peak area units per microgram of lipid. The repeatability was 4.3% (n = 1, a = 6). Total lipid soluble fluorescent lipid oxidation products (FP) with an excitation (ex) maximum at 367 nm and an emission (em) maximum at 420 nm were measured in the oil samples using FIA as described by Undeland et al. (*98*). Results were expressed as peak area units per picogram of lipid. The

repeatability was 7.0% (n = 1, a = 6). On each occasion, two oil samples (n = 2) have been analyzed once (a = 1).

Sensory Analysis. Eight assessors were selected for the panel. The assessors were trained during 17 sessions on profiling fish oil (*119*) prior to evaluation of our herring oil samples. Measuring odor rather than flavor is viewed as a sensory task less likely to fatigue panelists considering that oil samples are difficult to clear from the mouth (*120*). For each profiling of fish oil the following attributes were evaluated: fishy, train-oil, musty, green/vegetable, acidic/sour, and marine. Prior to serving, the samples (blanketed in nitrogen) were heated in a water bath at 50 °C, for 6 min. Before each session, the panel was calibrated by presenting freshly prepared reference oil to each assessor. The reference sample contained refined menhaden oil. Fish oil (0.5 mL, dispersed in glass pearls, ∅ 2 mm) was presented to the panelists in small opaque, glass flasks (120 mL) with a black lid. The panelists evaluated six different samples per session. Tap water was provided for oral rinsing at the beginning of the sessions and between oil samples. A complete block design and triplicate samples were used for sensory assessment. The order of presentation of samples to the panelist was balanced to minimize possible carry-over effects between the samples. The panel rated all attributes for each sample on separate 10-cm unstructured scales using the Compusense ® Five Program (Compusense Inc., Canada), where 0 indicated no intensity or presence and 10 a high intensity or presence of the attribute to the oil.

Fatty Acid Composition. Fatty acid methyl esters (FAMEs) of oil samples were prepared according to the AOCS (*66*) Official method Ce 1b-89 and analyzed with regard to the amount of individual fatty acids. On each occasion, n = 3, a = 1. The different FAMEs were separated from each other with gas chromatography (GC) and identified using the conditions described previously (*59*).

Analysis of copper (Cu). The copper content present in the oil samples was determined with a Perkin-Elmer 5100 graphite furnace atomic absorption spectrometer (AAS) with Zeeman background conditions (*99*) as described previously (*59*) (n = 2, a= 2). Results are expressed as mg/kg of oil. The limits of detection were 0.1 mg/kg. The repeatability of the method was 6.0%.

Analysis of iron (Fe). The oil samples were diluted with xylene and immediately measured with a graphite furnace atomic absorption spectrometer (Perkin-Elmer 5100; Norwalk, CT) using a deuterium background correction (*101*) (n = 2, a = 2). Results are expressed as mg/kg of oil. The limits of detection were 0.01 mg/kg and the repeatability of the method was 10.0%.

Internal or certified reference materials were analyzed together with the samples, except for the fatty acids analysis.

Statistical Analysis. Data from the α-tocopherol, stability measurements and sensory analysis were subjected to multivariate data analyses. The chemical and sensory data sets were evaluated separately by Principal Component Analysis (PCA). The differences in the sensory score levels of the different assessors and each individual attribute were firstly evaluated by PCA. Differences in how assessors scale sensory scores are a recognized problem in sensory assessment, and the problem occurs despite intensive training of panelists (*121*). Partial Least Squares (PLS) regression was performed on the data in order to relate chemical and sensory measurements: chemical data was used as X variables and sensory data as Y variables. In all analyses, all variables were weighed by 1/standard deviation, full cross validation and the Jack-knifing principle were employed.

RESULTS AND DISCUSSION

In the first part of this section, a characterization of the initial crude oils is presented. In the second part, the stability of the different oils during storage is compared and discussed.

Crude Composition

The data shown in Table 1 indicates the properties of the different extracted crude herring oils. In all cases, the concentrations of iron were much higher than the copper concentrations (the latter below the detection limit). The metal contents, in particular copper, are known to be important as a catalyst for the oxidation of oils and fats (*88*, *89*). For the iron content, it can be observed that the maatjes oil presented a value eight and almost twenty seven times higher than the frozen and fresh herring oil, respectively. This is in accordance with what should be expected, since for the maatjes production salt is used in the brine before the filleting operation and byproducts' collection. It is known that salt may contain inorganic substances such as copper and iron that can initiate and promote the oxidative process (*31*, *89*, *122*). Nevertheless, the values determined for the studied oils fulfil the quality guidelines as reported in the literature (*88*, *89*) for crude fish oils.

The maatjes oil presented the highest initial oxidation values as well as FFA content. In contrast, fresh herring oil had the lowest values. It is worth noticing that: oil produced from fresh raw material presented lower oxidation values, iron and FFA levels than the oil from frozen raw material. Within the use of frozen herring, the frozen oil presented lower iron, FFA levels and initial oxidation products than the maatjes oil. This may be related to the absence of salt in the byproducts.

Table 1 – Initial compositional data of the three herring oils extracted from herring (*Clupea harengus*) byproducts originated from different processing methods.

	Maatjes	Frozen	Fresh
Trace elements			
Fe (mg/kg of oil)[a]	0.8 ± 0.1	0.1 ± 0.0	0.030 ± 0.006
Cu (mg/kg of oil)[a]	< 0.1	< 0.1	< 0.1
Oxidation Status			
PV (meq. perox./kg of lipids)[b]	3.0 ± 0.3	3.0 ± 0.2	0.65 ± 0.17
AV [b]	8.9 ± 0.5	6.2 ± 0.3	0.36 ±0.06
FFA (%)[b]	3.1 ± 0.4	2.0 ± 0.3	0.60 ± 0.01
Fatty acids			
EPA (g/kg of lipid) [b]	99 ± 13	99 ± 5	58 ± 1
DHA (g/kg of lipid) [b]	91 ± 11	110 ± 2	65 ± 1
Σ Polyunsaturated [b]	277 ± 33	271 ± 12	183 ± 5
α-Tocopherol (mg/100g lipid) [b]	2.8 ± 0.1	9.8 ± 0.0	8.1 ± 0.4

[a] : n = 2, a= 2, results are given as: mean value ± (max-min)/2. For each of the two samples, a = 2. Mean values from these two analyses were used to establish sample variation.
[b] : n = 3, a = 1, results are given as: mean value ± std.

Different amounts of EPA, DHA and total PUFAs were determined in the oils (Table 1). It can be observed that the fresh oil presented the lowest value in all cases. However, the differences in fatty acids content are more likely to be related to the seasonal cycle (*18, 41, 70*) than to the herring processing method employed or to the prior storage of the herring. Table 1 also shows that the initial α-tocopherol content found in the maatjes oil is much lower (~ one third) than the content of the frozen and fresh oil. Clearly, the reduced levels of this endogenous antioxidant resulted from the oxidation of the oils. That means that the consumption of α-tocopherol occurred in an early stage probably already in the raw material itself.

Oxidative Stability

For evaluation of the oxidative stability of the oils, firstly the chemical changes inherent in the stages that can be discerned in the oxidation process are described and assessed. Secondly, the sensory changes are presented and discussed. Finally the relation between chemical and sensory data is evaluated.

Chemical analyses

The chemical variables measured during autoxidation in time for the three studied oils stored at ~ 20 °C are shown in Figure 1.

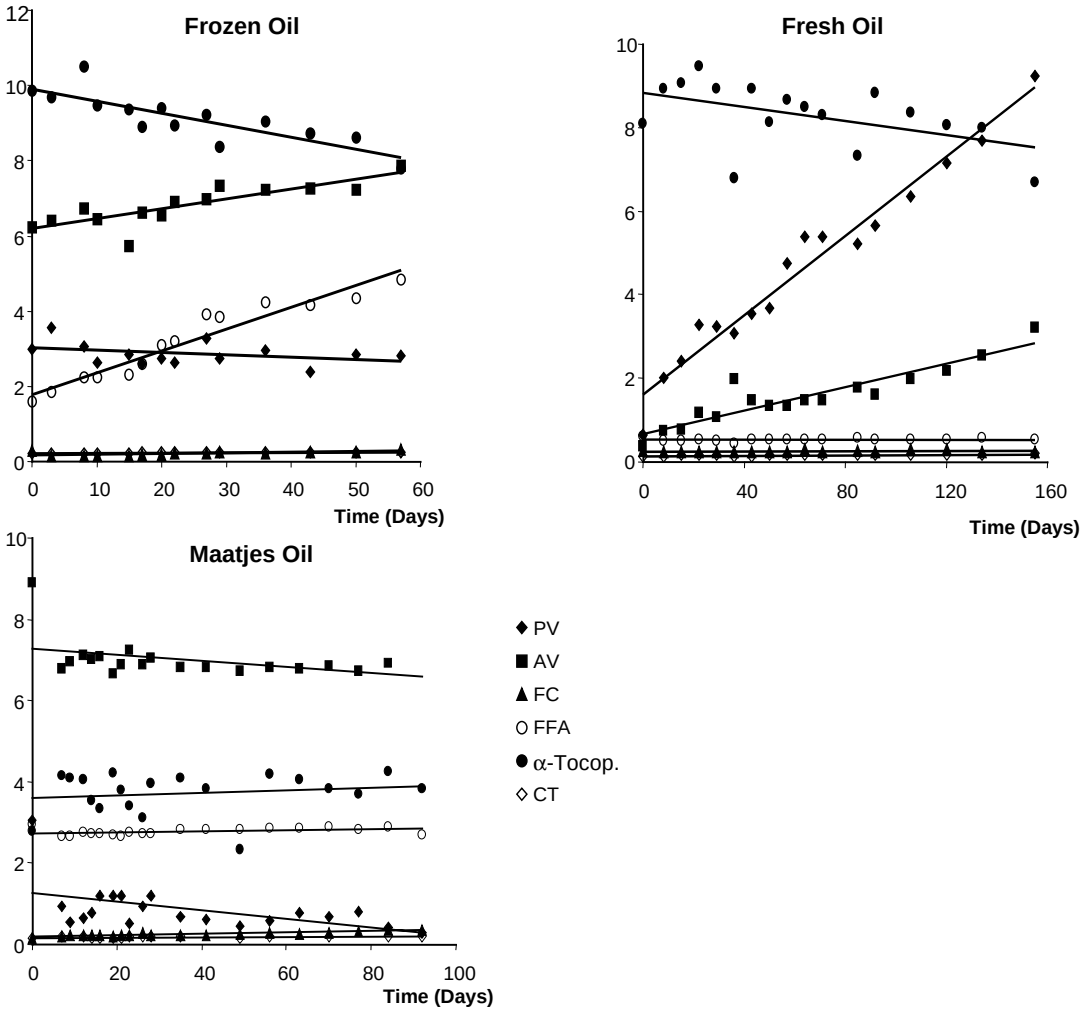

Figure 1- Trend lines and values determined for crude oil extracted from frozen, fresh and maatjes herring byproducts, stored under nitrogen and dark condition (20 °C). α-Tocopherol is expressed as mg/100 g of lipids; PV is expressed as meq perox/kg of oil, CT is expressed as area units per nanogram of lipids, FC is expressed as area units per picogram; and FFA is expressed in percentage as oleic acid.

To evaluate whether fresh, frozen or maatjes oil had the greatest effect on the oxidative changes, all of the data were subjected to a multivariate data analysis. Figure 2 shows three distinct clusters (maatjes, frozen and fresh) obtained from the PCA evaluation. The first two PCs, PC1 and PC2 described 52% of variance in X and 30% variance in Y. The bi-plot of PC1 and PC2 showed that oil produced from frozen and maatjes herring had positive values for PC1, whereas oil samples from fresh herring had negative values for PC1. This means that PC1 describes the difference between oil produced from frozen and fresh herring. On the other hand, samples produced from unsalted byproducts (frozen) had negative values for PC2, whereas oil samples with salt had positive value for PC2

(maatjes). Thus, PC2 mainly describes differences between oil samples obtained from byproducts processed with or without salt.

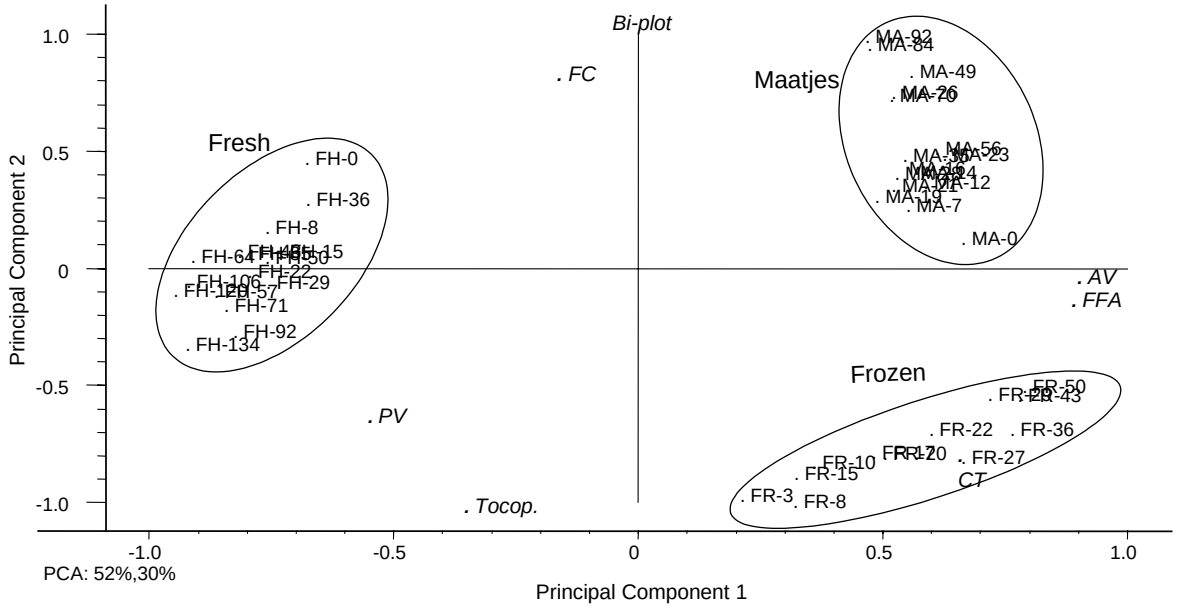

Figure 2- Scores and loading plots from Principal Component Analysis (PCA) for the data from the chemical analyses. The number after the hyphen in the variable name refers to the storage time in days.

In the same plot (Figure 2), the variance of the different chemical variables is shown. During storage of the oils different behaviors were found. The variables describing AV, FFA, and CT presented a positive value of PC1, whereas FC had a positive value for PC2. The AV and FFA measured variables were close to each other and were located far to the right. Hence these variables correlate with each other to a high degree (r = 0.914) and were of high significance. The correlation found between FFA measurements with lipid oxidation products such as AV is in accordance with the theory of the influence of lipid hydrolysis on lipid oxidation (*123*). AV and FFA are positively related to frozen and negatively related to fresh oil. The frozen oil moved from left to right with progressive storage time. Thus, frozen oil stored for 50 days was located far to the right indicating that this oil presented the highest levels of AV and FFA. Furthermore, CT seemed to correlate quite well with the frozen oil. The FC increased during storage time of maatjes and frozen oil. α-Tocopherol decreased during storage of frozen and to some extent with fresh oil.

α-Tocopherol

It is known that antioxidants act by being oxidized in preference to the oils (*124, 125*), therefore the level of α-tocopherol as a whole can provide useful information. The value is dependent on the handling and storage of the raw materials and the recovered oils as

well. One approach is to regard the decrease in α-tocopherol content as oxidation progress. The α-tocopherol was consumed significantly in fresh and frozen oil and this consumption occurs faster in the frozen than in the fresh oil. Surprisingly, no significant change was found in the maatjes oil. The reduced amount of α-tocopherol present in this oil can not prevent the progress of the autoxidation process. Therefore permitting a rapid degradation of the primary oxidation products favoring the formation of secondary and tertiary oxidation products.

FFA

The results show that the level of FFA presented in the maatjes and fresh oil was low and remained almost constant during storage for these oils, in particular for the fresh oil. For the frozen oil, a significant and consistent increase in time was determined for the FFA formation. This suggests that significant hydrolysis of the oil occurred during the storage period, probably due to the iron content. Nambudiry (*86*) and Hsieh and Kinsella (*122*), showed that with an increase in the salt content, the rate of FFA production in sardine muscle tissue decreases. In addition, in crude capelin oil, Notevarp and Chahine (*106*) found a positive correlation between iron content and FFA: oils with low iron contents tend to have low FFA values. Therefore, the salt content present in the flesh and byproducts of the maatjes production and the very low initial iron content for the fresh oil would explain, as suggested previously (*59*), the non-increase of FFA over time in contrast to the increase trend developed for the frozen oil.

Primary Oxidation Products

The primary oxidation products were measured as hydroperoxides presented by the PV and by the conjugated trienes analyses. During the autoxidation process hydroperoxides are produced. This is shown for the fresh oil, where PV increased significantly over time, as can be seen in Figure 1 and 2. In contrast, for the maatjes oil, the PV remained low; in fact a significant decrease was detected. This suggests that the degradation of hydroperoxides was faster than its generation. Degradation of hydroperoxides of the maatjes oil however did not increase the formation of a higher level CT. However, for the fresh oil, a significant development of CT was found. It has been reported that a 270 nm not only CT hydroperoxides but also various bifunctional oxidation products, such as ethylenic diketones and oxodienes, are detected (*107*, *123*, *126*). The results confirm our previous findings that the CT measured are related to the formation of secondary oxidation products containing a conjugated triene system (*59*).

Secondary Oxidation Products

The secondary oxidation stage is characterized by the further degradation of lipids through a radical oxidation process initiated by the hydroperoxides, which generates the level of off-flavors and off-odors (*90*). A possible way to quantify the oxidation process is by measuring the AV. Following the same tendency as the CT formation, the AV measurements increased significantly for the frozen and fresh oils. In fact, for frozen oil, the formation of secondary oxidation products was faster than for the fresh herring. Slightly lower values were obtained for the maatjes oil.

Tertiary Oxidation Products

The measurement of FC has been successfully applied to marine oils (*59, 60, 93, 127*). As can be seen in Figure 1 and 2, the reaction from secondary to tertiary oxidation products clearly takes place for the maatjes and for the frozen oils. In both cases, FC presented a significant positive increase over time. This indicates that the tertiary oxidation stage was reached. In contrast, the fresh oil presented a stable value for the FC, which showed that the formation of tertiary oxidation products was not yet important. Possibly, a termination reaction is favored above a propagation reaction, resulting in a stable value of the FC measurements.

These results suggest that the FC analysis can be successfully applied for oils in a later stage of degradation and/or produced from frozen raw material while for the fresh oil early oxidation quality parameters such as PV, CT and AV should be employed.

Sensory Analysis

In each of the experiments, seven trained panelists sniffed fresh and stored oil samples and then rated their odor intensity of various attributes on the intensity scale. During evaluation of the data, the attribute green/vegetable was removed from further calculations, because there was disagreement between the panelists in the score of this attribute. The results were first analyzed by PCA in order to find the main quality variation among the oil samples as well as to find the relation between the attributes. Triplicate samples were used but for matter of clarity, only average values are shown in Figure 3. Middle and final storage times of the frozen and maatjes oils presented positive PC1 values, whereas initial frozen and maatjes oil had negative values. Therefore, it can be concluded that PC1, explaining 83% of the data variance, is related to storage time of the oil. The odor of the fresh oil was evaluated as strongly musty when initially extracted, but surprisingly, during storage, distinct differences from musty in odor intensity were observed. It is likely that the musty attribute is associated with the natural odor of the

fresh oil, which may intensify at early storage periods. This attribute diminished rapidly and was largely replaced by other type of odors. The typical sensory attributes identified at later storage times of the fresh oil were identical to the attributes for initial maatjes and frozen oil. These latter oils were negatively correlated with train-oil, acidic, marine, and fishy sensory attributes. In contrast, the odors of maatjes and frozen oils became increasingly more intense over storage period; both oils correlated positively with the train, acidic, marine, and fishy attributes.

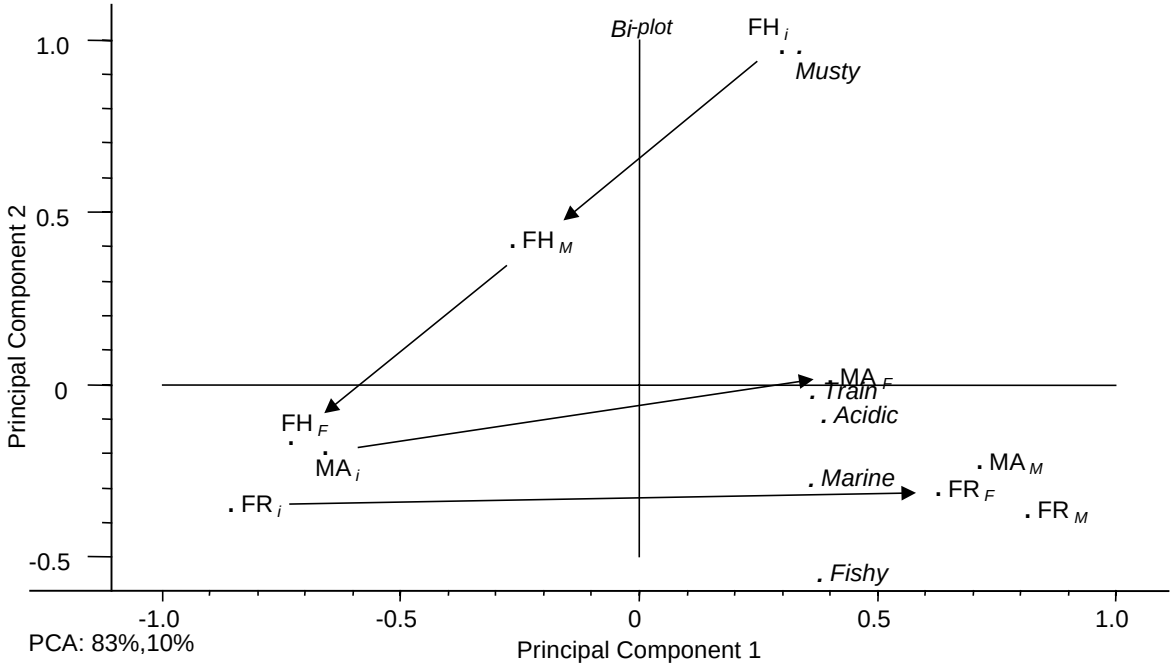

Figure 3- Scores and loading plots from PCA for the data from the sensory analysis. Abbreviations used are the following: FH, fresh oil; MA, maatjes oil; FR, frozen oil. The letters *i*, *M*, and *F* refers to initial, middle, and final storage time, whereas 0, 71, 155; 0, 49, 92; and 0, 27, 57 days for fresh, maatjes and frozen oil, respectively.

In frozen fatty fish species the principal changes in odor resulted from alteration in lipid components (*128*). The panelists could clearly distinguish between fresh and stored frozen and maatjes oil samples, since they were placed in different quadrants. However, discrimination between middle and final storage time could not be well performed. Nevertheless, the final maatjes oil's samples correlated strongly with train and acidic off-odors attributes. The fresh oil samples moved from the first to the third quadrant during storage time. The samples from the initial frozen and maatjes oils appeared also in this quadrant. During storage time, the maatjes and frozen oils moved further to the fourth quadrant. Flavor and odor deterioration has been attributed mainly to the formation of secondary oxidation products from the polyunsaturated fatty acids (*90*) that make up 18 to

28% of the total fatty acids in herring oil. The results confirmed that because of the higher proportion and degree of unsaturation of the fatty acids (Table 1), the more prone the oil is to oxidation.

During storage, maatjes and frozen oils, both processed from frozen herring, were found to be less stable than fresh oil. The differences should be explained by the effects of variations found of the starting raw material quality, since the conditions during production of the oils were identical.

Chemical versus sensory data

Data from analyses of PV and AV did not indicate that the maatjes were oxidized further than the frozen oil samples. However, the sensory data revealed a stronger off-odor for the maatjes than for the frozen oil.

For clear comparison between the chemical and sensory data, Partial Least Square (PLS) regression was performed (Figure 4 and 5). PC1 explained 42% and PC2 21% of the variance in the X variables and 32 and 18% of the variance in the Y variables, respectively. Hence the two components explained 63% of the variance in the X variables and 50% of the variance in the Y variables.

The score plots (Figure 4) supply information about the relationship between the objects, whereas the loadings plot (Figure 5) gives information over the relationship between the original variables. The fresh oil presented a negative PC1 value. In contrast, all samples from the maatjes and frozen oils presented positive PC1 values, increasing over storage time. In the loading plot, it is clear that the longer the oils were stored, the higher the FFA levels and to a lesser extent, the development of acidity, fishy, and marine off-odors. This is in accordance with the PCA results obtained for the chemical and sensory data.

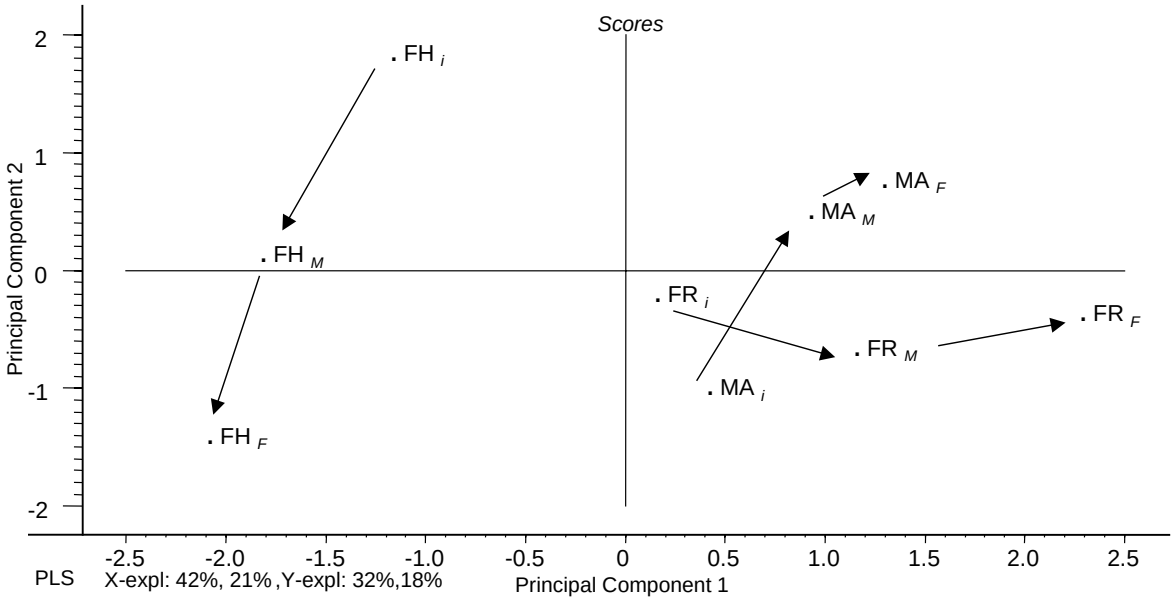

Figure 4- Correlation scores plot from the PLS regression on the studied chemical and sensory measurements of the different oils.

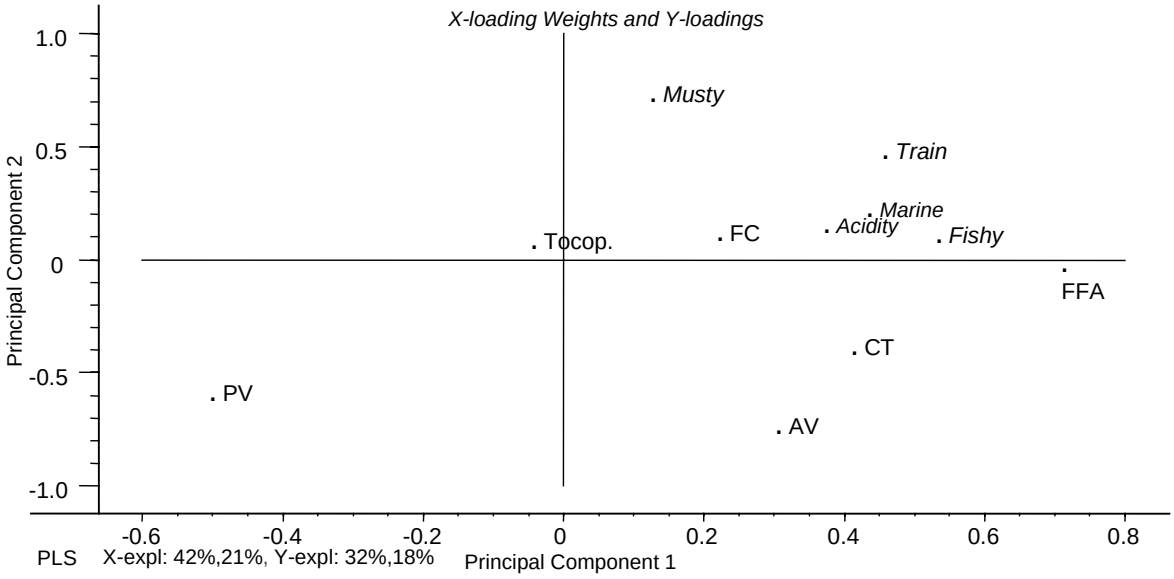

Figure 5- Correlation loading plot from the PLS regression on the studied chemical and sensory measurements of the different oils. The designed chemical variables were used as X-data and the sensory variables were used as Y-data.

Apart from the musty attribute, all the other attributes were located close to each other. This indicates a certain degree of interdependency (in all cases r >0.88). The FFA measurement correlates well with fishy off-odor (r = 0.781). On the other hand, the PV measurement showed a negative correlation to all the sensory attributes, being stronger to

the train off-odor (r = -0.664). Apparently, low PV are related to the development of off-odors. This implies that the low PV measured were not related to a low degrees of oxidation but to the reverse situation: the oxidation process progressed further towards secondary and probably tertiary oxidation products. This hypothesis is supported with the development of off-odors, CT, AV and FC measurements over time, in particular for the maatjes oil. Predictors projected close to the center, such as α-tocopherol, are not very well represented in the model. Although the α-tocopherol level decreased over time (Figure 1), this can not be related directly to any of the off-odors studied. In other studies, the FC measurement show a high correlation with sensory measurements for salted dried sardines and herring fillets (*129, 130*), however in our study this was not observed. Storage promotes gradual lipid oxidation with decrease in shelf stability due to continuing chemical processes. The results suggested that FFA evaluation was the chemical method that correlated best with off-odors such as fishy, acidity, marine and train presented in the oil and therefore interesting to be followed.

Concluding Remarks

The whole organization of the supply and processing chain has a large effect on the properties of fish oil, and the way it develops over time. Using freshly caught herring yielded oil that was in the beginning of the oxidation process, since only primary and secondary oxidation products were detected, and a much lower decrease of α-tocopherol level occurred. Freezing the herring immediately after the catch caused the extracted oil to reach the secondary and tertiary oxidation stage, favoring over time the development of off-odors such as train oil, fishy, acidic and marine. FFA enhances the odor changes in the oils. When the byproducts were in contact with transition metals ions, oil with more oxidation products and lower α-tocopherol levels was extracted. The facts suggest that the oil obtained from byproducts of fresh herring production is different from the ones produced from frozen raw material. Furthermore, within the frozen raw material, different oils are obtained depending of the *prior* treatment of the herring. As expected, the use of salt accelerates the oxidation process. This leads to the conclusion that the best oil is obtained when extraction is made from fresh and unsalted herring byproducts. Nevertheless, all oils present a relatively good chemical and even sensory stability over time, which suggests that it is possible to upgrade the byproducts from the different processes of the herring industry into fish oil.

Acknowledgements

We thank Maurice Boonman, Sónia Lourenço and Carlos Farinha for their valuable help with part of the analytical work. Furthermore, we are grateful to the sensory panel at the Netherlands Institute for Fisheries Research for their skillful sensory work.

Chapter 3

Processing raw material into High-Quality Fish Oil

3.1 Quality of Crude Fish Oil Extracted from Herring Byproducts: Relation to Byproducts Freshness

ABSTRACT

Herring byproducts were stored at 2 and 15 °C for up to 72 hours. Over time, significant increases of total volatile bases (TVB), histamine, putrescine, cadaverine, and tyramine were detected. However, only tyramine and TVB levels were temperature-dependent. The level of total polyunsaturated fatty acids (PUFAs) was constant. A longer storage time of the byproducts gave rise to an oil with higher levels of free fatty acids, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and total PUFAs, while fluorescent compounds were lower. A higher storage temperature led to oil higher in α-tocopherol and EPA levels and lower in anisidine value. Storage of the produced oils showed that conjugated trienes and fluorescent compounds changed significantly over time. Surprisingly, the oil with the highest content of PUFAs was not produced from the freshest byproducts, and oil with low oxidation products can be extracted from stored byproducts.

This sub-chapter has been accepted for publication with minor modifications as: Aidos I, van der Padt A, Boom RM, Luten JB. **2002**. Quality of Crude Fish Oil Extracted from Herring Byproducts of Varying States of Freshness. *J. Food Sci.*

INTRODUCTION

The quality and freshness of the raw material is a factor of great importance in the preparation of premium-quality fish oil and fish meal (*5*). Enzymatic and bacteriologic activity in the fish and fish offal can rapidly increase (*131*), which in turn can substantially decrease the content and quality of the protein and oil. Protein decomposes to amines and ammonia, and both reduce the protein value and recovery. High content of free fatty acids (FFA) in the fish oil reduces the commercial value due to an established direct relationship between oil quality and FFA levels (*5*). It is well known that cooling and icing of the raw material will normally slow down the biological decomposition (*132-134*). The byproducts from the fish industry have especially low storage stability if not frozen or preserved (*135*). Biogenic amine levels have been used as a quality index in fish (*136-138*).

Immediately after capture, the concentration of endogenous antioxidant substances such as ascorbic acid, glutathione peroxidase, and tocopherol in muscle starts to decrease and continues with storage time (*139*). Simultaneously, there is an increase in levels of oxidative or catalytic substances such as hemin and low-molecular-weight iron with increasing storage time (*105*). During processing and storage, fish quality may thus decline as a result of several factors, for example, oxidation is promoted by blood (*140*). Fatty fish species contain polyunsaturated fatty acids (PUFAs) (*11, 29, 141, 142*), which are prone to oxidation, producing off-flavors and odors (*73, 122, 143*). Their generation represents a significant quality loss in PUFA-containing foods (*104*).

In the present work, the influence of temperature (2 and 15 °C) and time (up to 72 hours) on lipid deterioration during storage of herring byproducts to be used for processing into fish oil was studied. The study reports changes that took place during the storage of the byproducts, focusing on the quality parameters that could be used to monitor the quality of herring byproducts and the resulting fish oil. Therefore, the microbial content, total volatile bases (TVB), and levels of four biogenic amines (cadaverine, histamine, putrescine, and tyramine) in the byproducts were determined. Primary, secondary, tertiary lipid oxidation products, and lipid hydrolysis (FFA) were determined in the oils. Fatty acid compositions of the herring byproducts and crude oil were also evaluated. A comparison between storage stability of the different produced oils was undertaken to study the effect that freshness and storage temperature of the byproducts may have on the lipid oxidation progress. General Linear Models (GLM) and multivariate data analysis were used for statistical evaluation.

MATERIALS AND METHODS

The raw material used in this study was fresh byproducts obtained from fresh herring (*Clupea harengus)* caught in December 1999 off of 50.50 N 01.00 E (North Sea), with an average weight of 96 ± 16 g, length 23 ± 1 cm and found to be in maturity stage III (n = 30).

Sampling set-up

During filleting and processing of the herring, approximately nine tons of byproducts was collected. Immediately, fish oil was extracted using fresh byproducts. The remaining byproducts were divided into two parts and stored in closed tubs, at ~ 2 ºC (cooling room) and at ~15 °C (processing hall). After 8, 24, 48 and 72 hours of storage, the fish oil was processed. The design of the experiment is schematically presented in Figure 1.

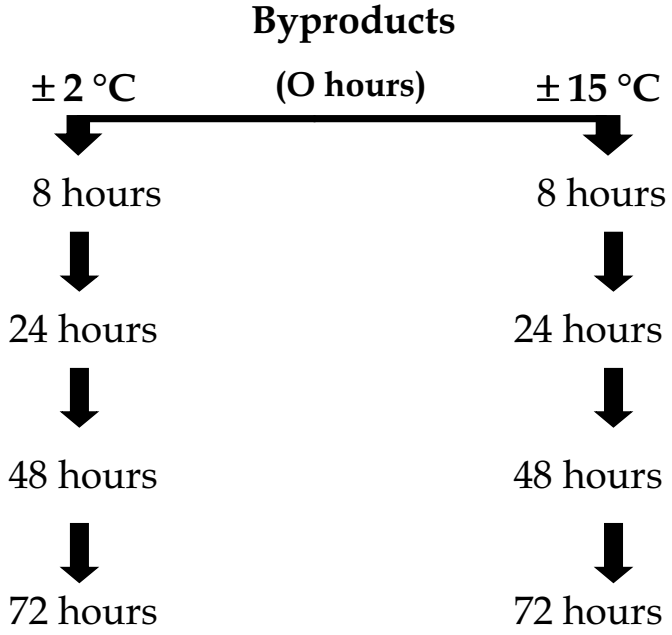

Figure 1- Scheme of the design of the experiment including temperature and storage time of the herring byproducts.

A portion of the produced oils was used for a storage stability experiment, in which the samples were in all cases blanketed with an inert atmosphere (nitrogen) and kept in closed dark containers at room temperature (~20 °C). Three oil samples were taken (~50 mL) at regular intervals from the different oils and analyzed for the concentration of oxidation products, which were measured as the amount of conjugated dienes (CD), trienes (CT), tetraenes, and the amount of fluorescence compounds (FC). In order to avoid further oxidation, all the samples were kept at –80 °C until analyzed. Prior to the analysis, the oil samples were thawed at room temperature for 30 min.

Equipment

Batches of ~ 1000 kg herring byproducts were processed, in each occasion. Immediately after being minced, the byproducts were pumped to an insulated scraped-surface heat exchanger indirectly heated by steam (95°C) and fractionated in a three-phase decanter into a solid phase (called protein phase), a water phase (stickwater) and a lipid phase (oil) using the same system and conditions as described earlier (*59*).

Chemical Methods

Fatty Acid Composition. Lipids from herring byproducts were extracted according to the method of Bligh and Dyer (*65*). Fatty acid methyl esters (FAMEs) of oil samples were

prepared according to the AOCS (*66*) Official Method Ce 1b-89 and analyzed with regard to the amount of individual fatty acids. In each occasion, three oil samples (n = 3) were analyzed once (a = 1). The different FAMEs were separated from each other with gas chromatography (GC) and identified using the conditions described previously (*59*). Results were expressed as g/kg of lipid.

α-Tocopherol. α-Tocopherol was analyzed according to the slightly modified method of Lie et al. (*97*) as described earlier (*59*) using reversed-phase HPLC and fluorescence detection. The repeatability of the method was 5.1% (n = 1, a = 6). Results were expressed as mg/100 g of lipids.

Extraction of lipids for determination of peroxide value, anisidine value, and free fatty acids. Total lipids from the different stored herring byproducts were extracted according to the method of Burton et al. (*94*) as modified by Undeland et al. (*95*).

Free Fatty Acids. The amount of free fatty acids (FFA) of the oil samples was determined by titration according to AOCS (*66*) Official Method Ca 5a-40 (n = 3, a = 1). The percentage of FFA was expressed as oleic acid.

Peroxide Value. The peroxide value (PV) of the samples was determined according to AOCS (*66*) Official Method Cd-8b-90 (n = 3, a = 1). The content was expressed in terms of meq of peroxides per kg of lipid.

Anisidine Value. The anisidine value (AV) of the oil was carried out according to AOCS (*66*) Official Method Cd 18-90 (n = 3, a = 1).

Absorbance at 235nm (A_{235}), 270 nm (A_{270}), and 300 nm (A_{300}). A_{235} (CD), A_{270} (CT) and A_{300} nm absorbances of the oil samples were measured using flow injection analysis (FIA) as described by Undeland et al. (*98*) (n = 3, a = 1). Results were expressed as peak area units per nanogram of lipid. The repeatabilities were 2.0, 4.3, and 7.0 % (n = 1, a = 6) for A_{235}, A_{270}, A_{300}, respectively.

Fluorescence Compounds (FC). Total lipid soluble fluorescent lipid oxidation products with an excitation (ex) maximum at 367 nm and an emission (em) maximum at 420 nm were measured in the oil samples using FIA as described by Undeland et al. (*98*) (n = 3, a = 1). Results were expressed as peak area units per picogram of lipid. The repeatability was 7.0% (n = 1, a = 6).

Lipid content. The total lipid content in the samples was determined gravimetrically after extraction according to the Bligh and Dyer (*65*) procedure (n = 2, a = 2). Results were expressed as grams of lipid per kg of samples.

Moisture. Moisture was determined as described earlier (*59*) (n = 2, a = 2). Results were expressed as percentage of wet weight.

Protein. Total nitrogen in the homogenized samples was determined using the Kjeldahl digestion method as described previously (*59*)(n = 2, a = 2). Results were expressed as percentage of wet weight.

Salt. Chloride content in all the samples was titrated according to Volhard's method as described by Kolthoff and Sandell (*67*) (n = 2, a = 2). Results were expressed as percentage of wet weight.

pH. The pH was measured using a personal pH meter, model PH 81 (Yokogawa Electric Corporation, Japan).

TVB. The total volatile bases (TVB) was determined by titration according to the method of Vyncke et al. (*144*) (n = 2, a = 2). Results were expressed in mg/100 g. The repeatability was 3.2%.

Biogenic amines. Cadaverine, histamine, putrescine, and tyramine were determined according to the method described by Veciana-Nogues et al. (*145*) using a HPLC (Perkin-Elmer series 410 pump), an autosampler ISS 200 (Perkin-Elmer), LC column (Hypersil 5 ODS, 100 x 3 mm), and a HPLC series 410 pump (Perkin-Elmer) for the derivatization. A

spectrofluorometric detector (TSP, FL2000) was set at excitation and emission wavelength of 354 and 430 nm, respectively. Quantification was performed, using TurboChrom software (version 6.1.2.0.1:1019, Perkin-Elmer), by integrating peaks on the chromatogram (n = 2, a = 2). The referred biogenic amines were identified using external standards. Results were expressed in mg/kg. The repeatability was 7.4, 7.1, 2.4, and 1.7% for tyramine, putrescine, cadaverine, and histamine, respectively.

In all cases, with exception of the fatty acid composition and lipid extractions, internal reference materials were analyzed together with the samples.

Microbiological examinations

PCA and L&H. Plate count agar (PCA) for aerobic microorganisms was used following the ISO (*146*) method (n = 2, a = 1). The Long and Hammer's (L&H) medium for *Pseudomonas putrefaciens* was used according to the method described previously (*147*). Results were expressed as CFU/g (colony forming units/g).

Statistical analysis

The data were subjected to the ANOVA univariate method ($P<0.05$) using SPSS software (version 10.0); comparison of means after the ANOVA test was performed using the Tukey HSD test. General Linear Models (GLM) were tested against differently obtained data in order to describe whether there was a significant effect of the temperature, storage hours, or storage days. For the byproducts and initially produced oils, the following model was tested:

$$Y_{(Measurement)} = \beta_0 + \beta_{1\,S} + \beta_{2\,T} + \beta_{3\,S.T}$$

For the oxidative stability of the oil the following model was tested:

$$Y_{(Measurement)} = \beta_0 + \beta_{1\,T} + \beta_{2\,S} + \beta_3 t + \beta_4 t._{S} + \beta_5 t._{T} + \beta_6 t._{S.T}$$

where, in both models, T (class variable) is the effect of the storage temperature of the byproducts, S (class variable) is the storage time, in hours, that the byproducts were kept before being processed into oil, and t (covariate) is the storage time of the oil, in days, at room temperature. Interaction terms of the two class variables on the measurements studied were taken into consideration. The residuals were tested for normality using normality probability plots. Observed power was used as a statistical tool to evaluate the strength of the drawn inferences related to the failure to reject the H_0 (*68*). Pearson's correlation coefficient (r) was calculated in order to study the correlation between various measures. The level of significance for all tests was set at $p = 0.05$. The assessment of byproducts and initial oil data from the freshness experiment was also evaluated by multivariate data analysis. The assessment of byproducts and initial oil data from the freshness experiment was also evaluated by multivariate data analysis. All Principal Component Analyses were done using the Unscrambler version 7.5 (CAMO, Oslo, Norway). All variables were standardized by 1/standard deviation. Jack-knife facility, and full cross validation were employed.

RESULTS AND DISCUSSION

In the first part of this section the characterization of the byproducts with respect to freshness parameters (microbial count, TVB, biogenic amines) and fatty acid composition is discussed. In the second part, the relationship between the processed herring oils and the respective storage stability towards oxidation is evaluated.

Quality of the byproducts used for fish oil production

The herring byproducts had contents of fat, protein and moisture of 12.5 ± 0.6, 12.2 ± 0.6, and 73.9 ± 0.7%, respectively. The byproducts were found to have the following initial oxidation values: 8.5 ± 0.4 meq peroxides/kg of lipids, 4.3 ± 0.2 for AV, 0.7 ±

0.0% of FFA, and an α-tocopherol content of 0.43 ± 0.12 mg/100 g. The pH of the byproducts was 6.20 ± 0.02 and did not change at the two studied temperatures during storage time. These results are in accordance with results obtained by El Marrakchi and co-authors (*134*) who concluded that pH measurement was not suitable for the evaluation of sardine quality.

Figure 2 shows, for the different measurements, the outcome results and significant differences found after analysis of variance and Tukey HSD test. Concerning the microbiological parameters, no significant change occurred over time for the studied temperatures. A study performed with different parts of Spanish mackerel stored at 0, 15, and 30 °C showed that the maximum counts never exceeded 10^6 CFU/g of flesh (*148*) and that similar levels were found after nine days for sardine stored in ice (*134*). This was the initial range of values determined in our study. The facts led us to the conclusion that the maximum microbial counts had already been reached in the herring byproducts.

As expected, the L&H values were slightly higher than the PCA. The reason is that the latter is counting the total number of mesophilic organisms (which have a maximum growth temperature of 35 °C) while L&H counts the total number of psychrophilic (maximum growth temperature of 15 °C). Since 15 °C was the temperature used during the trial, the growth of psychrophilic organisms was promoted.

The changes in content of total volatile bases (TVB) were found to be highly dependent on the storage temperature ($p = 0.014$, power = 0.713) as well as of the storage time ($p << 0.05$, power = 1.000). The release of ammonia by bacteria, the principal component of TVB, indicates that at higher temperatures and longer storage period, spoilage of the byproducts occurred faster, as expected (Figure 2). Similar results were reported for the assessment of sardine quality during storage (*92*, *134*). It is appropriate to conclude that the TVB value is useful in assessing the degree of deterioration of herring byproducts and in evaluating the changes occurring during storage. It might be of interest to be followed further in the protein fraction.

The biogenic amines were found useful as quality indices for fish decomposition in the scombroid and clupeia family and, therefore, their determination is of interest. Taylor (*149*) showed that among biogenic amines, histamine is considered as the principal toxic agent causing histamine poisoning syndrome, although other biogenic amines possibly potentiate histamine toxicity. This biologically active amine significantly increased during storage time (Figure 2) ($p << 0.05$, power = 1.000). The first eight hours showed much lower values (~3 mg/kg) comparative to the remaining time (~154 mg/kg). Contrary to data by Middlebrooks et al. (*148*), in our study constant total microbial counts were found, despite the increasing histamine levels. This could be

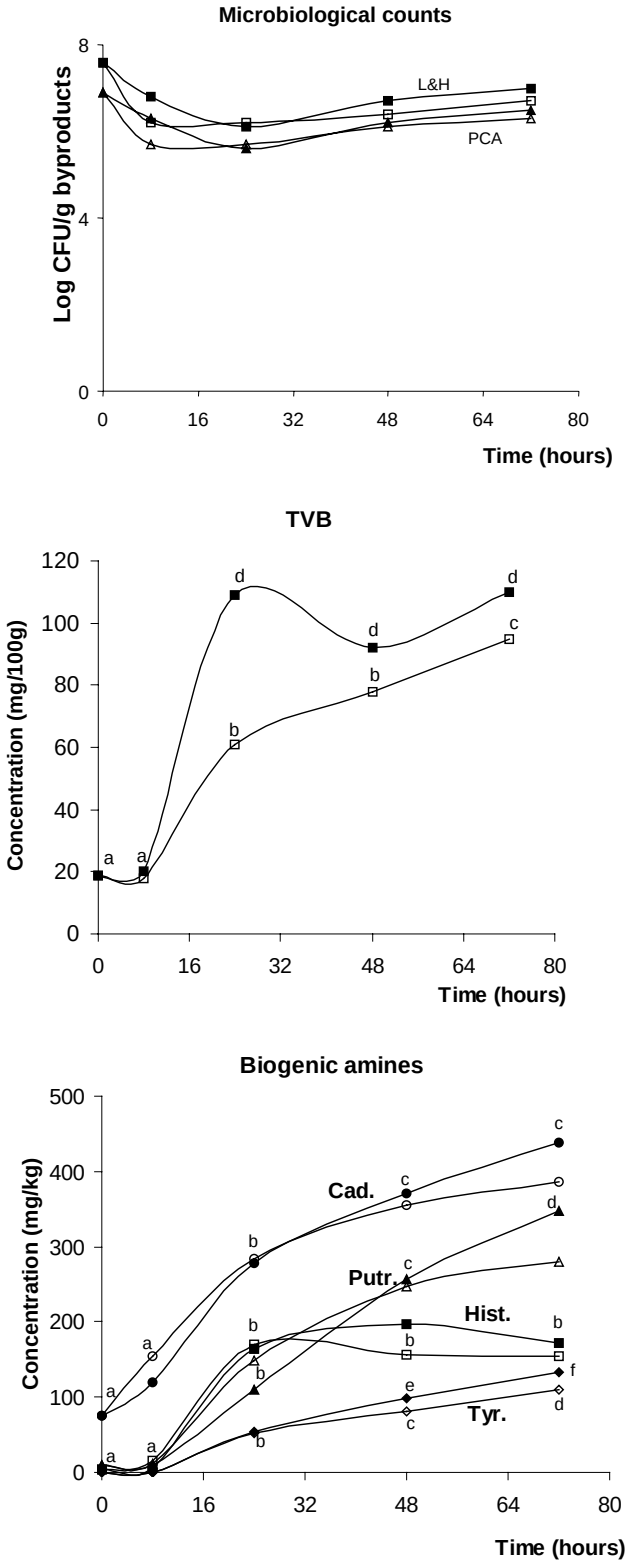

Figure 2- Development of microbiology parameters, TVB, and biogenic amines of the herring byproducts stored at 2 °C (open symbols) and 15 °C (filled symbols) for a maximum period of 72 hours. Data are given as mean values and different letters for each analysis represent significant differences ($P < 0.05$).

related to the ongoing decomposition of the proteins after the maximum growth level had been reached.

Other biogenic amines such as cadaverine and putrescine are considered to be histamine promotors (*138*). It was shown that the levels of cadaverine and putrescine in the byproducts increased as decomposition progressed over time ($p << 0.05$, power = 1.000) regardless of the temperature.

For tyramine, a significant effect was found not only for the storage time ($p << 0.05$, power = 1.000) but also for the storage temperature ($p << 0.05$, power = 0.619). Again, the lowest values were detected in the first eight hours of storage and increased significantly over time. After an eight hours period at both temperatures, the tyramine levels remained below the detection limit (< 1 mg/kg, Figure 2). Beyond 24 hours, the levels rose significantly at each storage time. Lower temperatures reduced the level of microorganisms with amino acid decarboxylase activity, since significant lower values were detected in the byproducts stored at 2 °C compared to 15 °C. These results are in agreement with those of Veciana-Nogues et al. (*137*). Table 1 describes the correlation coefficient between the different analytical parameters followed during the storage of the byproducts and the initial oxidation values of the oils. Within the chemical tests used to monitor freshness of herring byproducts, good correlations between the biogenic amines were found, in particular between cadaverine and putrescine and between putrescine and tyramine ($r = 0.933$ and $r = 0.958$, respectively). TVB measurements also correlated quite well with all biogenic amines, especially with histamine ($r = 0.915$), although these measurements are assessing different products. As the degradation process proceeded, each individual biogenic amine was produced, contributing to a higher amount of the non volatile products. At the same time, TVB increase was due to increases in trimethylamine and ammonia levels, which are both volatile products.

Table 1- Correlation as described by Pearson's correlation coefficient (r) between the different measures used to follow quality changes in herring byproducts and produced oil during storage of byproducts experiment. N = 30. Abbreviations used are as follows: cad, cadaverine; hist, histamine; put, putrescine; tyr, tyramine; tocop., α-tocopherol.

Measure	Cad	Hist	Putr	Tyr	TVB	Log PCA	Log LH	AV	FFA	PV	FC	CD	CT	EPA	DHA	PUFAs	Tocop.
Cad		0.895	0.933	0.897	0.866	-	-0.459	-	0.924	-	-0.609	0.502	0.416	0.524	0.650	0.503	-
Hist			0.860	0.818	0.915	-	-0.487	-	0.853	-0.473	-0.519	0.556	-	0.578	0.702	0.596	-
Putr				0.958	0.865	-	-	-	0.989	-	-0.515	0.530	-	0.471	0.744	0.516	-
Tyr					0.876	-	-	-	0.962	-	-0.480	0.494	-	0.458	0.668	0.424	-
TVB						-	-	-	0.865	-0.487	-0.560	0.513	-	0.555	0.690	0.564	-
Log PCA							0.975	-	-	0.520	-	-	-	-	-	-	-
Log LH								-	-	0.512	0.462	-	-	-	-	-	-
AV									-	0.762	-	-	-	-	-	-	-0.610
FFA										-	-0.526	0.536	-	0.450	0.750	0.500	-
PV											0.440	-	-	-	-	-0.595	-0.386
FC												-	-	-	-	-	-
CD													-	-	0.392	0.389	-
CT														-	-	-	-
EPA															0.555	0.642	-
DHA																0.753	-
PUFAs																	-
Tocoph																	

Correlation is significant at the 0.05 level (two-tailed). -: not significant

Fatty acids

In general, monounsaturated fatty acids are the major constituent class of fatty acids present in herring byproducts, with docosenoic acid (C22:1) and eicosenoic acid (C20:1) being the most important among these (Table 2). No changes were detected with either a higher storage temperature or a longer storage time for the 5, 8, 11, 14, 17-eicosapentaenoic acid (EPA, C20:5), 4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA, C22:6), or total PUFAs content. These results showed that herring byproducts could be kept at 2 and 15 °C for up to 72 hours period with no losses of EPA, DHA, and PUFAs.

Table 2- Fatty acid composition of the herring byproducts stored at 2 and 15 °C for different periods of time. Data are the mean ± standard deviations of three measurements. Results expressed as g/kg of lipids. Values within a row having different letters are significantly different ($P < 0.05$).

		2 ºC				**15 ºC**			
Fatty acids	0 hours	8 hours	24 hours	48 hours	72 hours	8 hours	24 hours	48 hours	72 hours
Saturated									
14:0	120 ± 8	141 ± 17	126 ± 2	124 ± 6	133 ± 6	114 ± 4	118 ± 19	138 ± 1	130 ± 5
16:0	172 ± 9	195 ± 21	177 ± 1	183 ± 5	183 ± 10	176 ± 2	174 ± 13	188 ± 2	179 ± 2
18:0	18 ± 0	19 ± 1	18 ± 0	19 ± 0	18 ± 1	18 ± 0	18 ± 0	19 ± 0	19 ± 1
Σ *	310 ± 17	355 ± 39	321 ± 2	326 ± 11	334 ± 16	308 ± 6	309 ± 32	345 ± 1	328 ± 6
Monoenes									
16:1	56 ± 2	64 ± 6	58 ± 0	59 ± 2	61 ± 2	55 ± 1	54 ± 6	63 ± 1	60 ± 0
18:1	92 ± 2	96 ± 4	94 ± 1	96 ± 1	96 ± 5	101 ± 3	97 ± 6	93 ± 3	95 ± 3
20:1	130 ± 2 a	123 ± 9 ab	129 ± 2 a	129 ± 3 ab	122 ± 2 b	131 ± 1 ab	131 ± 7 a	121 ± 2 ab	121 ± 1 b
22:1	197 ± 14	164 ± 26	188 ± 5	186 ± 6	175 ± 11	205 ± 7	202 ± 18	168 ± 5	173 ± 6
Σ *	475 ± 11	447 ± 25	470 ± 6	470 ± 7	455 ± 7	491 ± 8	484 ± 20	445 ± 6	450 ± 3
PUFAs									
18:2	20 ± 0	20 ± 0	20 ± 1	18 ± 3	20 ± 1	17 ± 2	20 ± 2	20 ± 0	20 ± 0
18:3	14 ± 0	15 ± 2	14 ± 1	13 ± 0	14 ± 1	13 ± 0	13 ± 1	15 ± 1	15 ± 1
18:4	28 ± 2 ab	28 ± 2 a	27 ± 3 a	28 ± 0 ab	30 ± 1 b	25 ± 2 a	25 ± 2 a	31 ± 1 ab	31 ± 1 b
20:5 (EPA)	49 ± 2	45 ± 6	48 ± 1	34 ± 29	51 ± 3	48 ± 2	47 ± 1	50 ± 1	53 ± 2
22:6 (DHA)	66 ± 6	56 ± 11	62 ± 2	63 ± 4	64 ± 6	65 ± 3	62 ± 5	62 ± 3	71 ± 4
Σ *	177 ± 5	165 ± 16	172± 7	156 ± 35	178 ± 9	167 ± 3	166 ± 5	179 ± 6	189 ± 6

* The values do not add up to 1000 because minor fatty acids are not reported.

Quality of the extracted Oil

Fatty acids

Table 3 shows the fatty acid composition of the extracted oils. It is worth noticing that, relative to the byproducts, the produced fish oils had higher levels of PUFAs. It is likely that this difference is related to the method used to extract the fat before analysis; a solvent extraction for the byproducts, and a heat treatment method for the produced oil.

Table 3- Fatty acid composition of the herring oil extracted from the stored byproducts. The mean values of three independent measurements and standard deviations are given. Results expressed as g/kg of lipids. Values in the same row followed by different letters are significantly different ($P < 0.05$).

		2 ºC				15 ºC			
Fatty acids	0 hours	8 hours	24 hours	48 hours	72 hours	8 hours	24 hours	48 hours	72 hours
Saturated									
14:0	87±1	92±5	91±1	88±1	96±5	94±7	93±4	88±2	89±1
16:0	125±1	131±3	131±2	129±3	133±4	131±4	133±3	129±1	129±1
18:0	16±0	15±0	16±1	16±2	16±1	16±1	16±0	16±1	15±0
Σ *	227±2	238±9	238±4	232±5	244±10	240±11	242±7	234±4	234±3
Monoenes									
16:1	30±21	45±1	44±0	43±1	44±2	46±3	45±1	43±1	43±0
18:1	77±1 ab	78±1 a	77±1 a	76±1 a	76±1 a	81±3 b	78±1 b	80±4 b	77±3 b
20:1	119±1	84±62	118±2	117±1	116±0	117±2	118±1	117±1	116±1
22:1	221±4 a	224±4 ab	217±3 ab	216±2 ab	212±4 b	213±6 ab	213±2 ab	216±5 ab	212±0 b
Σ *	447±28	431±69	456±6	453±4	449±7	456±14	452±5	455±10	449±5
PUFAs									
18:2	23±0	17±5	24±1	23±0	24±1	24±1	24±0	23±0	20±5
18:3	14±3	15±1	17±2	16±1	16±1	17±2	16±1	12±8	17±1
18:4	29±0	31±0	30±1	29±1	30±0	31±1	31±1	30±1	30±0
20:5 (EPA)	53±1 a	54±1 abc	55±0 bc	55±1 bc	54±2 bc	56±1ade	57±2 de	57±1 de	57±0 de
22:6 (DHA)	67±1 a	65±2 a	71±3 b	73±1 b	71±3 b	69±1 a	70±0 b	72±0 b	73±2 b
Σ *	186±4 a	182±9 ab	197±7 ab	196±3 ab	195±7 b	196±7 ab	197±4 ab	194±11 ab	197±9 b

* The values do not add up to 1000 because minor fatty acids are not reported.

As reported earlier (*41, 59, 60*), the latter achieves higher PUFAs and lower saturated values. Significant effect of temperature and storage time was detected for EPA ($p = 0.001$, power = 0.947, and $p = 0.001$, power = 0.989, respectively). Surprisingly, significantly higher amounts of EPA were measured for the oil produced from byproducts kept at 15 °C for longer than eight hours of storage (57 g/kg). The DHA was shown to be highly dependent on the storage time ($p << 0.05$, power = 1.000). DHA content was lowest at 0 and 8 hours (67 g/kg) and significantly increased

afterwards (up to 73 g/kg). Due to the cumulative influence of EPA and DHA contents, an effect of storage time ($p = 0.008$, power = 0.792) was detected for the total amount of PUFAs. These results point out that higher contents of PUFAs can be achieved in the herring oil produced after at least eight hours of storage of byproducts. Higher amounts of EPA and DHA were also obtained when the byproducts were stored longer than eight hours, while storage temperature was only positively related to the EPA content. Positive correlations were found between the contents of EPA, DHA, total PUFAs, and biogenic amines and TVB. The best correlation was found between DHA and putrescine and histamine ($r = 0.744$ and $r = 0.702$, respectively). Apparently after some degradation of the herring byproducts, higher amounts of these essential fatty acids were liberated achieving higher extraction efficiency. It is known that, in animals, the most important role of lipids is to form the structural unit for all cell membranes, mainly phospholipids and cholesterol (*150*). Since DHA is usually concentrated in cell membranes, it can be assumed that with degradation of the byproducts, rupture of the cells occurred rendering a higher yield of DHA content. For the EPA content, besides time, higher temperatures also promoted liberation of the triglycerides (present mainly in fat deposits) resulting in a higher concentration of EPA.

Oxidative status

Initial oxidation status of the different herring oils extracted is shown in Table 4.

Table 4– Characterization of the crude fish oil extracted from herring byproducts kept at two temperatures and stored for different times. The mean values of three independent measurements and standard deviations are given. Values in the same row followed by different letters are significantly different ($P < 0.05$). PV is expressed as meq. perox./kg of lipid; CD and CT as area units/ng of lipids; FC as area units/pg of lipids; FFA in % as oleic acid; and α-tocopherol as mg/100 g lipids.

		2 ºC				**15 ºC**			
	0 hours	8 hours	24 hours	48 hours	72 hours	8 hours	24 hours	48 hours	72 hours
PV	4.4±0.1	5.3± 0.2	0.5± 0.1	3.2± 0.4	2.5± 0.3	2.1± 0.3	0.5± 0.1	3.9± 0.1	2.9± 0.1
CD	39.0±5.6	34.1±6.5	42.9±6.6	46.9±8.9	47.6±8.7	39.0±5.6	45.7±6.0	45.8±16.3	56.3±17.2
CT	1.6±0.6	1.5±0.4	1.7±0.4	1.6±0.1	1.6±0.0	1.5±0.1	1.6±0.0	2.0±0.0	1.9±0.1
AV	2.6±0.1 ab	4.0±0.1a	1.4±0a	3.9±0.2a	2.7±0.6a	1.5±0.1b	1.5±0.1b	3.0±0.2b	2.8±0.1b
FC	28.0±10.6 a	22.7±7.6ab	17.3±7.6b	17.3±1.2 ab	12.7±1.2b	22.0±3.5ab	16.7±2.3b	22.0±3.5 ab	16.0±2.0 b
FFA	0.6±0 a	0.7±0 b	1.7±0 c	2.7±0 d	3.0±0.1 e	0.8±0 b	1.6±0.1 c	2.8±0 d	3.2±0.1 e
α-Tocop.	6.1± 0.4 ab	4.4± 0.4 a	5.3± 0.8 a	5.2± 1.0 a	5.8± 0.3 a	6.8± 0.5 b	7.0± 0.3 b	5.8± 0.1 b	5.9± 0.1 b

The PV, conjugated dienes (CD), and conjugated trienes (CT) in the produced oils were neither dependent on temperature nor on storage time of the byproducts and these

oxidation values remained relatively low. The low oxidation values measured could be related to the breakdown of hydroperoxides during the heating step involved in the process (*47, 59*). However, secondary oxidation products measured as AV were significantly lower in the oil produced from the byproducts stored at 15 °C compared to 2 °C ($p = 0.046$, power = 0.521). The development of tertiary oxidation products (FC) was found to be temperature-independent but decreased as a result of the storage time of the byproducts ($p = 0.004$, power = 0.930). It is known that oxidation products are unstable and tend to react with biological amino constituents (proteins, peptides, free amino acids) and phospholipids, leading to interaction compounds (*48, 151, 152*) that can be assessed by their fluorescent properties (*92, 153, 154*). Evidence has demonstrated that fluorescent substances formed from oxidized membrane lipids remain attached to the amino constituents (*155, 156*). Apparently, at the beginning of storage, fluorescent compounds were mostly lipid-soluble and could be measured with the FC system used. However, as lipid and protein damage increased with storage, these compounds became progressively more soluble in the water phase, explaining the decreasing value measured over time in the lipid phase. Similar results were reported in stored frozen sardine (*154*). Another possible explanation could be that accelerated by the storage time, fluorescent compounds formed in the first stages of interaction led to the formation of other fluorescent compounds with different wavelengths than theirs precursors, as suggested elsewhere (*154*). According to our results, with the conditions used and only analyzing the lipid phase, fluorescent compounds can not provide an accurate assessment of the fluorescent properties of the interaction compounds formed as a result of storage of the herring byproducts.

At the same time, the FFA proved to be very sensitive to the time of storage of the byproducts ($p << 0.05$, power = 1.000), increasing significantly over time at both temperatures (0.6% vs. 3.2%). However, this effect was not temperature dependent. These facts mean that, probably due to the action of lipases and phospholipases, lipid hydrolysis in the byproducts proceeded during storage. Koning and co-workers (*157*) also reported that besides a storage time dependency, the FFA content in the oil was lower than that of the anchovy itself. Obviously FFA, due to the polarity of the compounds, accumulated preferentially in the protein fraction rather than in the oil fraction as suggested elsewhere (*157*).

The α-tocopherol content did not change significantly over time, but varied significantly with the storage temperature of the byproducts ($p = 0.001$, power =0.952). α-Tocopherol content of the extracted oil was higher for the byproducts stored at 15 °C than at 2 °C (average values of 6.3 vs. 5.4 mg/100g). These results led us to the conclusion that, similarly to what had been reported previously in the herring oil (*60*), α-tocopherol was consumed more rapidly at 2 than at 15 °C. Higher levels of

secondary oxidation products (measured as AV) at 2 °C, enhancing the consumption of α-tocopherol, supported the fact that indeed these two measurements were negatively correlated (Table 1 and Figure 3).

Between the different oxidation measurements, correlations were found, the strongest being between PV and AV measurements (r = 0.762). The results showed that FFA had the strongest correlation with the freshness measurements in the byproducts such as putrescine, tyramine, and cadaverine (r = 0.989, r = 0.62, and r = 0.924, respectively). In contrast, measurement of the FC showed negative correlations with all four biogenic amines and TVB.

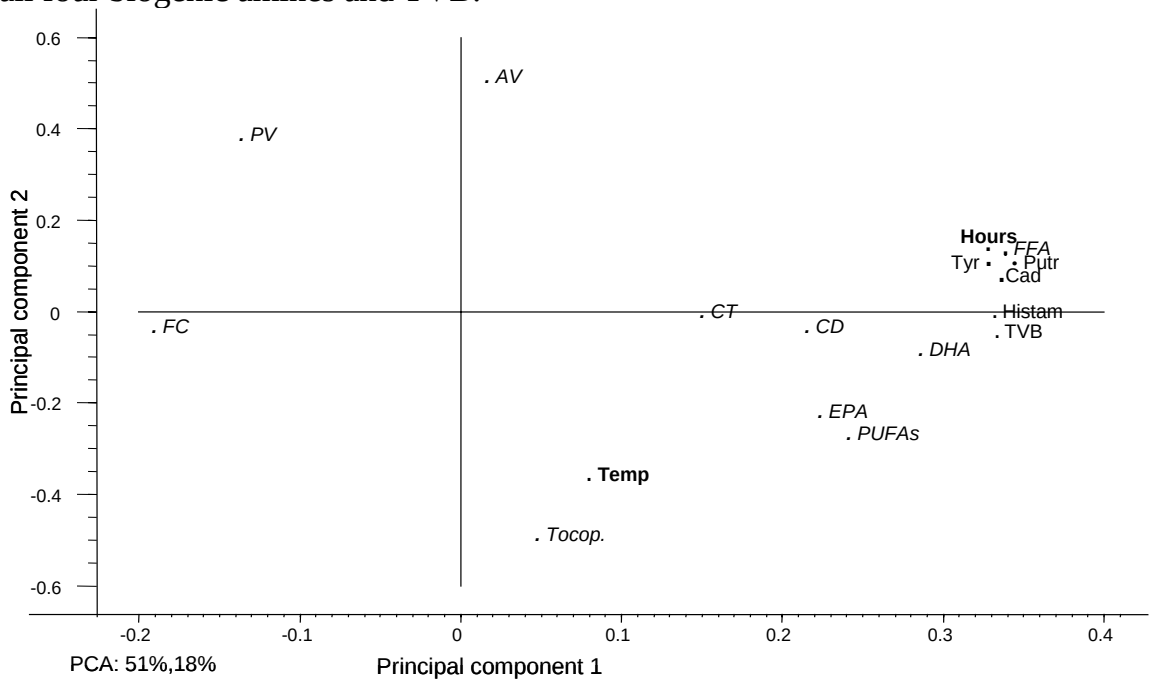

Figure 3- Loadings plot from the Principal Component Analysis (PCA) relating the freshness of herring byproducts and initial produced oil. For exact concentration levels, refer to Table 2, 3 and Figure 2. Abbreviations used are as follows: cad, cadaverine; hist, histamine; putres, putrescine; tyr, tyramine; tocop., α-tocopherol.

Principal component analysis showed that 69% of the variability of the data could be explained by two components. Since no changes occurred for the microbiological data of the byproducts, the measurements of PCA and L&H were not considered in order to strengthen the model. In the loadings plot (Figure 3), it was evident that the later the byproducts were processed (in hours), the higher the biogenic amines and TVB content of byproducts and FFA in the oil; and to a lesser extent, the more DHA, PUFAs, and EPA levels were present in the oil. Biogenic amines, TVB, and FFA were all located far to the right, showing that these variables were of high significance. Putrescine, FFA, and tyramine were located close to each other. Hence, these variables have a high degree of positive correlation, in contrast to the FC values, which is also in accordance with our previous conclusions and the Pearson's correlation coefficients

(Table 1). α-Tocopherol and temperature appeared close to each other. Finally, PV, AV, and FC levels were isolated.

It should be emphasized that, apart from an increase in the FFA levels, the oil retained low oxidation values at all storage temperatures and time. The results suggest that by determining the putrescine content of the herring byproducts it is possible to predict the value of FFA present in the produced crude oil, thus making possible an evaluation of the oil quality. However, since FFA determination is easier and simpler than any biogenic amine determination, it can be suggested to measure the level of FFA directly in the byproducts. It can be expected, based on previous research (*157*) that the FFA levels in the herring byproducts should be higher than in the oil.

Oxidative Stability

In addition to the characterization of the initially extracted oils, a storage trial at room temperature was carried out with the extracted oils. Conjugated oxidation products are produced by the conversion of PUFAs in oxidized lipids, they can be measured by the ultraviolet absorbance (*91*). Therefore, the amount of CD, CT, tetraenes and FC were determined. General Linear Models were tested for each oxidation measurement. The full fitted ANOVA models showed that CT and FC were important parameters. In contrast, it was concluded that the CD and tetraenes content were not suitable as quality measurements (data not shown), possibly because of the relatively unstable nature of these latter compounds (*92, 158, 159*). Concerning the measurement of CT, the initial model only showed a significant effect for the storage days of the oils ($p << 0.05$, power = 0.999). Related to the tertiary oxidation products, highly significant effects were detected for the storage days of the oils and for the storage time of the byproducts ($p << 0.05$, power = 1.000, and $p << 0.05$, power = 0.994, respectively).

By omitting stepwise non-significant effects (final models given in Table 5), for the CT and FC, the following mathematical equations were obtained:

$CT = 13.843 + \beta_{1.\text{Storage time}} + 0.07243$ days

$FC = 23.696 + \beta_{1.\text{ Storage time}} - 0.211$ days

In spite of the fact that the storage time of the byproducts did not have a significant effect in the original CT full model, a positive effect was detected for this parameter when the interactions terms were omitted, due to their insignificance. Thus, in the final equation, the contribution of the parameters was strengthened with the significant value of the initial intercept β_0 ($p << 0.05$, power = 1.000), to the storage time ($p = 0.034$, power = 0.735), and of oil storage ($p << 0.05$, power = 0.999). The parameter estimates and respective confidence intervals for the influence of the storage time (β_1) of the byproducts can be seen in Figure 4. The oil storage time of 72 hours was set to zero because it is redundant to the model. For the storage time, all the obtained

parameter estimates were within one group, meaning that there was not a significant difference among them. Nevertheless, an increase of CT occurred over time.

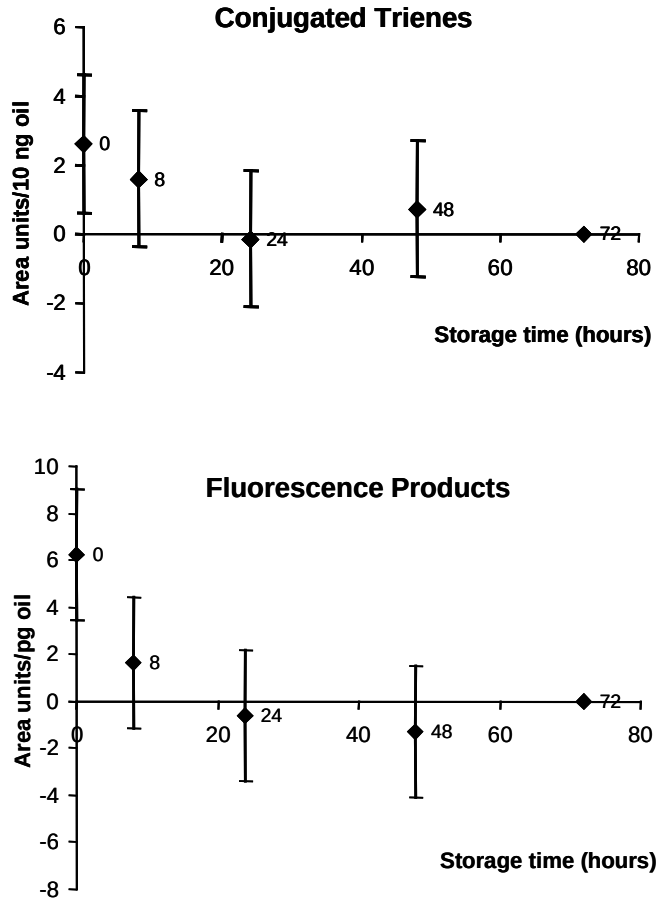

Figure 4- Parameters estimates and the respective confidence intervals for the measurement of the CT and FC of the oil stored at room temperature produced after different storage time (hours) of the herring byproducts, refer to text for more information.

In relation to the FC measurements, the contributors' terms of the final equation presented a high level of significance: intercept ($p << 0.05$, observed power = 1.000), storage time ($p << 0.05$, power = 0.999), and days ($p << 0.05$, power = 1.000). The parameter estimates of the storage time of the byproducts (Figure 4), related to the FC, showed that these are divided into two significant groups: the oil produced from byproducts stored at 0 hours is significantly higher in FC than the oil produced after 24 and 48 hours of storage.

The estimates progressively decreased as the herring byproducts were decomposed. In both equations, a positive and significant intercept was found indicating the pre-existence of these oxidation compounds. Earlier results reported that FC could be used as an oxidation measure for fish oil when produced from fresh herring byproducts (*59-61*) or in heat-treated oil (*93*). However, positive values are apparently achieved when the oil is produced within the first eight hours of storage. The negative slope of the

days implies that during storage of the oil, the development of the tertiary oxidation products are decreasing, keeping the same tendency observed for the initially produced oils.

In summary, for the stored byproducts, all the biogenic amines, and TVB increased over time. The levels were clearly correlated. Temperature only had a significant influence for tyramine and TVB. The microbial count was constant, indicating that the maximum level had already been reached. EPA, DHA and PUFAs content remained constant in the stored byproducts. EPA content, which was determined in the produced oil, was found to be time and temperature dependent. Significantly higher values were obtained in oil processed from byproducts stored at 15 °C and for a period longer than eight hours. On the other hand, the DHA level was only time-dependent. The total amount of PUFAs was shown to be time-dependent with a lowest value determined for the oil produced from fresh (0 hours) byproducts, in contrast to the highest value verified for oil produced from byproducts stored for 72 hours. This indicates that the higher degree of tissue disruption promotes recovery of higher levels of DHA and EPA. The produced oils presented quite stable and low oxidation values, except for FFA levels that increased significantly over time. During storage of the oil, the levels of CT and FC changed sharply. FC from the lipid phase seems to be more suitable to be used as a quality measurement of the oil when it is produced from fresh byproducts. It can also be stated that, regardless of all the other quality implications that it might have, herring oil richer in PUFAs content is produced from byproducts stored for longer periods at 15 °C.

CONCLUSIONS

Herring byproducts have good storage stability and crude oil with low oxidation products and richer in EPA, DHA and PUFAs content can be produced from stored byproducts.

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3.2 Composition and Stability of Herring Oil Recovered from Sorted Byproducts Compared to Oil from Mixed Byproducts

ABSTRACT

Herring oils produced from three different types of byproducts; only heads, mixed, and headless byproducts were compared. Heads byproducts and its oil presented the highest oxidation levels and the lowest α-tocopherol content. Heads contained the lowest polyunsaturated fatty acids (PUFAs) content and the highest amount of saturated fatty acids. No significant differences were found between the fatty acid composition of the mixed and the headless either in byproducts or in its oil. The oil was stored at two different temperatures (20 and 50 °C). Testing General Linear Models (GLM) showed that oxidation was related to the peroxide value with a positive significant effect of the temperature, while the free fatty acids' model was more complex, with significant contribution of all of the effects studied. Fluorescence measurement was the one that correlated best with the oxidation progress.

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INTRODUCTION

From several investigations, it has been suggested that the ω-3 polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (C20:5, EPA) and docosahexaenoic acid (C22:6, DHA) are beneficial to the human body (*24, 25*). It is known that fish, in particular fatty fish species, is one of the best sources of these types of fatty acids (*11, 142*). The ω-3 PUFAs are highly susceptible to oxidation due to their high degree of unsaturation (*160, 161*). This is one of the main difficulties during storage and shelf life of products containing considerable amounts of these fatty acids (*44, 45, 162*).

There are great differences in lipid storage between different fish species depending upon the organism constitution. Fat storage in fatty fish species, like herring, is dispersed, and the type of fat differs in various parts of the fish and organs (*107, 163*). Lohne (*164*) found that the head and belly cavity of mackerel and capelin contain extensive lipid deposits. Tuna heads are known to be rich in DHA content (*39*). Nair and Gopakumar (*165*) reported that skin lipids from the oil sardine (*Sardinella longiceps*) had a higher proportion of monoenoic acids and lower proportions of PUFAs than muscle lipids. Similar results were found in the skin and muscle lipids of Atlantic mackerel (*Scomber scombrus*) by Ke et al. (*76*). German and Kinsella (*166*), reported that skin enzymes may constitute a significant source of initiating radicals leading to oxidation of fish lipids.

After successful and promising initial results in the valorization of herring byproducts reported earlier (*59, 60*), the separation of the byproducts in to its different constituents, became of interest. In this way, it may be possible to influence the fatty acid composition and the lipid oxidation/stability of the resulting oil. The type of process used at the factory, after the filleting operation, gives the possibility of producing not only the normal mixed byproducts but also only the heads from the byproducts, and in this way, a headless byproducts variety also created.

This paper compares the composition and storage stability from the oil obtained from different sorted byproducts (only heads and the headless byproducts) to the oil obtained using mixed byproducts: heads, skin, frames and fins, and visceras. In the first part of the study, a characterization of the different raw materials and the crude oils is made. In the second part, the stability of the oils was investigated. To follow the lipid oxidation process, several methods were used. Attention was given to the development of primary, secondary, and tertiary oxidation products. The peroxide value (PV), absorbance at 235 nm (measuring conjugated dienes, CD) and 270 nm (measuring conjugated trienes, CT), anisidine value (AV), and fluorescence compounds (FC) were measured. The loss of α-tocopherol and the change in the free fatty acids (FFA) content were also followed in

time. General Linear Models (GLM) were used as a tool to evaluate whether any changes occurred on a statistically significant level during storage and to compare the different autoxidation process at the different temperatures and type of oil produced.

MATERIALS AND METHODS

The raw material used in this study was fresh byproducts obtained from frozen herring (*Clupea harengus)* caught in the North Sea in July 1999 off of 60.40 N 02.75 W. The fish were processed in October 1999, and had an average weight of 199 ± 15 g, length 28 ± 1 cm and were found to be in maturity stage IV (n = 15).

Equipment

Each production run required ~ 1000 kg of mixed (heads, frames, skin, viscera, etc.), heads, and headless byproducts. First, the byproducts were minced, and immediately after this stage, they were pumped to an insulated scraped-surface heat exchanger indirectly heated by steam (95 °C). Finally, it was separated in a three-phase decanter into a solid phase (called protein phase), a water phase (stickwater), and a lipid phase (oil). The same conditions and system as described earlier were used (*59*).

Sampling set-up

A portion of the recovered herring oils was used for the storage stability experiment. In all cases, the oil was blanketed with an inert atmosphere (nitrogen), kept in closed, light impermeable containers at room temperature (≈ 20 °C) and in an oven (5 0°C).

Two oil samples were taken, at regular intervals, from the different oils and analyzed for level of oxidation products, FFA formation and α-tocopherol content. To avoid further oxidation, all of the samples were kept at –80 °C until analyzed. Prior to the analysis, the oil samples were thawed at room temperature for 30 min. The average of the two sample measurements was used to calculate the final results.

Fatty Acid Composition. Lipids from herring fillets and byproducts were extracted according to the method of Bligh and Dyer (*65*). Fatty acid methyl esters (FAMEs) of oil samples were prepared according to the AOCS (*66*) Official Method Ce 1b-89 and analyzed with regard to the amount of content of individual fatty acids. In each occasion, three oil samples (n = 3) were analyzed once (a = 1). The different FAMEs were separated from each other with gas chromatography (GC) and identified using the conditions described previously (*59*). Results are expressed as g/kg of lipid.

α-Tocopherol. α-Tocopherol was analyzed according to the slightly modified method of Lie et al. (*97*) as described earlier (*59*) using reversed phase HPLC and fluorescence detection. On each occasion n = 2, a = 1. The repeatability of the method was 5.1% (n = 1, a = 6). Results are expressed as mg/100 g of lipids.

Extraction of lipids for determination of PV, AV, FFA and FC. Total lipids from the different types of herring byproducts were extracted according to the method of Burton et al. (*94*) as modified by Undeland et al. (*95*).

Free Fatty Acids. The amount of FFA of the oil samples was determined by titration according to AOCS (*66*) Official Method Ca 5a-40 (n = 2, a = 1). The percentage of FFA was calculated as oleic acid.

Peroxide Value. The peroxide value (PV) of the samples was determined according to the official AOCS (*66*) method Cd-8b-90 (n = 2, a = 1). The content is expressed in terms of meq of peroxides per kg of lipid.
Anisidine Value. The anisidine value (AV) of the oil was carried out according to AOCS (*66*) Official Method Cd 18-90 (n = 2, a = 1).
Absorbance at 235nm (A_{235}) and 270 nm (A_{270}). A_{235} and A_{270} nm of the oil samples were measured using flow injection analysis (FIA) as described by Undeland et al. (*98*) (n = 2, a = 1). Results were expressed as peak area units per nanogram of lipid. The relative standard deviation percentes were 2.0 and 4.3 (n = 1, a = 6) for A_{235} and A_{270}, respectively.
Fluorescence Compounds (FC). Total lipid soluble fluorescent lipid oxidation products with an excitation (ex) maximum at 367 nm and an emission (em) maximum at 420 nm were measured in the oil samples using FIA as described by Undeland et al. (*98*) (n = 2, a = 1). Results were expressed as peak area units per picogram of lipid. The repeatability was 7.0% (n = 1, a = 6).
Lipid content. The total lipid content in the samples was determined gravimetrically after extraction according to the Bligh and Dyer (*65*) procedure (n = 2, a = 2). Results were expressed as grams of lipid per kilogram of samples.
Moisture. Moisture was determined as described earlier (*60*) (n = 2, a = 2). Results were expressed as percentage of wet weight.
Protein. Total nitrogen in the homogenized samples was determined using the Kjeldahl digestion method as described previously (*60*). Results were expressed as percentage of wet weight.
Salt. Chloride content in all of the samples was titrated according to Volhard's method as described by Kolthoff and Sandell (*67*) (n = 2, a = 2). Results were expressed as percentage of wet weight.
In all cases, with the exception of fatty acid composition and lipid extractions, internal reference materials were analyzed together with the samples.
Statistical analysis: The data were subjected to the analysis of variance (ANOVA) univariate method ($P < 0.05$) using SPSS software (version 10.0). Comparison of means after the ANOVA test was performed using the Tukey HSD test. The relation of fatty acid composition in raw material to that in the produced oils was also subjected to an ANOVA test. For comparisons, the type of byproducts was placed in three groups and the products were placed in two groups according to composition of the product: byproducts and oil. General Linear Models (GLM) were tested to the stored oil data in order to describe whether there was a significant effect of the temperature, storage time or oil type. The residuals were tested for normality using probability plots followed by one sample Kolmogorov-Smirnov test.

RESULTS AND DISCUSSION

In the first part of this section, the composition of the different raw materials and produced oils is presented. In the second part, the stability during storage of the two produced oils is discussed. Complications during processing enabled us to produce only a limited amount of heads' oil, allowing for the characterization but not for the storage trial.

Compositional data

Crude composition

The results of the crude composition analysis in the three types of byproducts are shown in Table 1.

Table 1- Composition of the three types of herring byproducts used for fish oil production. Mean values of three independent measurements and standard deviation are given. Values in the same row followed by different letters are significantly different ($P < 0.05$).

	Mixed byproducts	Headless byproducts	Heads byproducts
Crude Composition			
Protein (% w/w)	14.8 ± 0.1 a	14.3 ± 0.1 a	14.3 ± 0.6 a
Moisture (% w/w)	72.6 ± 0.3 a	71.3 ± 0.4 b	66.9 ± 0.4 c
Salt (% w/w)	0.4 ± 0.01 a	0.3 ± 0.03 a	0.7 ± 0.1 b
Total lipids(g/kg wet sample)	122 ± 3 a	132 ± 6 a	129 ± 4 a
FFA (%)	0.7 ± 0.02 a	0.7 ± 0.01 a	0.8 ± 0.1 a
α-Tocopherol (mg/100g lipid)	0.5 ± 0.1 a	0.4 ± 0.1 a	0.0 ± 0.1 b
Oxidation Status			
PV (meq per./kg oil)	30.1 ± 2.8 a	47.8 ± 4.0 b	112.6 ± 6.3 c
AV	22.2 ± 0.2 a	33.9 ± 1.0 b	37.0 ± 3.7 b
FC (area units/ pg oil)	198 ± 128 a	164 ± 32 a	230 ± 36 a
A235 (area units/μg oil)	51.7 ± 4.5 a	67.1 ± 13.3 ab	80.3 ± 10.7 b
A270 (area units/μg oil)	6.4 ± 1.7 a	7.6 ± 1.9 a	19.5 ± 1.7 b

As can be seen, the composition is similar among the parts studied. In all cases, the highest value was found for the moisture content followed by protein and fat content (≈70 and 14% and 125 g/kg wet sample, respectively). No significant changes among the byproducts composition were found for the protein and fat content. On the other hand, related to moisture content, it was found that the three fractions were significantly different among each other; the mixed byproducts were the ones that contained higher amounts of moisture in opposition to the heads that presented the lowest value (72.6 versus 66.9%, respectively). It is usual that the moisture content increases as the content of fat decreases (*70*). It is likely that the statistical difference found for moisture content in the fractions is related to the standard deviations of the measurements (larger for fat than for protein and moisture content determination). A significantly higher salt content was determined for the heads byproducts as compared to the other fractions (0.7%). The fact can be explained due to the heads, which are mainly composed of bones. No significant differences in FFA content were observed, implying that the hydrolysis occurred in a similar way to the different analyzed byproducts. No α-tocopherol was

present in the heads byproducts contrary to the other two types of byproducts in which 0.4-0.5 mg/100g lipids were found. The difference found on the α-tocopherol contents between heads versus mixed and headless byproducts was significant. The fact could be explained due to the herring heads, which are mainly composed of bones and skin and not so much of muscle tissue. Studies performed comparing α-tocopherol levels in lipids from skin and muscle of mackerel and herring showed that skin had the lowest α-tocopherol content (*107, 167*) and also that skins oxidize much faster than the other parts of the fish (*107, 168*).

Regarding the oxidation parameters, the amount of primary, secondary, and tertiary oxidation products in the heads byproducts was, clearly, the highest. This implies that the oxidation path progresses much faster in the heads than in the other byproducts processed. This agrees with the fact that the amount of α-tocopherol in this fraction was almost zero. It is known that this antioxidant can act as a protective tool against oxidation (*130, 169*); hence, at lower α-tocopherol contents, higher values of oxidation products such as hydroperoxides, measured as PV, should arise, which was indeed observed. Consequently, those hydroperoxides compounds are gradually decomposed to the respective further oxidation products measured as CD, CT, and AV.

Endogenous enzymes liberated from the fish tissue itself can be a potential source of initiation of the peroxidation (*129*). In particular, peroxidases and lipoxygenases catalyze the formation of highly reactive hydroperoxides that can propagate the lipid oxidative chain reaction. Lipoxygenase activity has been reported in some fish species, and evidence supports the presence of different types of these enzymes in fish with varying distribution, activity, and stability (*166, 170*) predominantly concentrated in the skin tissue of herring (*168*). Ke et al. (*76*) studied differential lipid oxidation in various parts of frozen mackerel. They reported an unusual difference in reaction rates between skin and muscle lipids, as observed by the difference in induction period and in the overall accumulation of oxidation products. Studies of oxidation *in vitro* show that the faster oxidation of skin lipids is probably related to some fat-soluble enzyme in the mackerel skin lipids that catalyzes their oxidation (*76*). Apparently, heads herring byproducts liberate endogenous enzymes that associated to a greater surface/mass ratio of skin present in the heads promoted the lipid oxidation process.

Surprisingly, the lowest oxidation values were found for the mixed byproducts and not for headless byproducts as would be expected. The fact might be explained by the fractions collected of the headless byproducts that contain a large amount of viscera. These could be responsible for promoting the oxidative degradation. It is known that endogenous

enzymes from internal organs of herring are of prime importance for the proteolysis, especially concerning the ripening process (*171, 172*). Stoknes and co-workers (*173*) showed that the proteolytic activity of the intestinal and liver fractions dominated in herring. A strong and rapid development of rancid odor in very fresh unwashed mackerel fillets indicated that blood had a high pro-oxidative effect (*140*).

Fatty Acid Composition

The fatty acids pattern for each of the studied types of byproducts considered before and after respective fish oil production is shown in Table 2. In all of the cases, the fatty acids content showed the following ranking order: monounsaturated > saturated > polyunsaturated. The most abundant saturated and monounsaturated fatty acids in the different types of byproducts and oils were docosenoic acid (22:1) and palmitic acid (16:0). Among the PUFAs, eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) were the major components.

Table 2- Fatty acid profile (g/kg of lipid) in total lipids from different types of byproducts and respective oils produced from herring (*Clupea harengus*) byproducts. Mean values of three independent measurements and standard deviations are given. Values in the same row followed by different letters are significantly different ($P < 0.05$). * Implies that an interaction effect was found between byproducts and produced oil.

	Mixed		Headless		Heads	
Fatty acids	**Byprod.**	**Oil**	**Byprod.**	**Oil**	**Byprod.**	**Oil**
Saturated						
14:0	86 ± 4 a	90 ± 6 b	83 ± 1 a	88 ± 1 b	87 ± 8 a	94 ± 4 b
16:0	148 ± 5 a	142 ± 5 a	139 ± 1 ab	138 ± 0 ab	152 ± 8 b	145 ± 7 b
18:0	20 ± 0 a	22 ± 4 a	20 ± 0 a	20 ± 1 a	23 ± 1 a	23 ± 13 a
Total	254 ± 9 ab	254 ± 15 ab	242 ± 2 a	246 ± 2 a	262 ± 17 b	262 ± 14 b
Monounsaturated						
16:1	49 ± 1 a	55 ± 3 c	49 ± 1 a	54 ± 0 c	54 ± 1 b	56 ± 4 d
18:1	73 ± 3 a	75 ± 2 a	68 ± 2 a	72 ± 2 a	71 ± 3 a	71 ± 3 a
20:1	99 ± 3 a	103 ± 1 b	100 ± 2 a	103 ± 1 b	99 ± 2 a	99 ± 1 b
22:1 *	175 ± 5 ab	179 ± 3 ab	175 ± 2 a	177 ± 2 a	189 ± 6 b	178 ± 3 b
Total *	396 ± 12 a	412 ± 8 b	392 ± 7 a	406 ± 5 b	413 ± 12 a	404 ± 11 b
Polyunsaturated						
18:2	11 ± 3 a	12 ± 4 a	13 ± 0 a	13 ± 3 a	12 ± 0 a	14 ± 1 a
18:3 *	12 ± 2 a	12 ± 0 b	12 ± 1 a	14 ± 1 b	9 ± 1 a	14 ± 2 b
18:4 *	27 ± 1 a	32 ± 0 d	30 ± 1 b	32 ± 0 e	23 ± 1 c	30 ± 1 f
20:5	82 ± 3 a	90 ± 3 c	85 ± 2 a	88 ± 1 c	79 ± 2 b	87 ± 2 d
22:6 *	93 ± 1 a	85 ± 1 c	95 ± 2 a	81 ± 0 c	84 ± 1 b	80 ± 5 d
Total *	225 ±10 a	231 ±8 c	235 ± 6 a	228 ± 6 c	207 ± 5 b	225 ± 11 d

The values do not add up to 1000 because minor fatty acids are not reported.

The heads byproducts have the highest level of total saturated fatty acids while the headless byproducts have the lowest (262 versus 242 g/kg of lipids). No significant difference was found between the byproducts and the respective oils produced.
The total amount of monounsaturated did not show any significant difference over the different types of byproducts, which was about 400 g/kg of lipids. However, with exception of the heads where an interaction effect for the model (tested as: $Y_{\text{Fatty Acids}}$ = type + product + type*product) was found between the type of raw material used and the product (byproducts or oil), the oil fractions present a significant higher value of monounsaturated fatty acids than the respective byproducts, showing the dependency of the fat extraction process applied (solvent versus temperature), as reported earlier (*59*). The level of monounsaturated was highly dependent on the content of docosenoic acid, the major compound in the group. Its content was the highest in the heads and the lowest in the headless byproducts (189 versus 175 g/kg of lipids).
The lowest level of unsaturation was found in the heads; it differed significantly from the mixed and headless oil. Obviously, this difference is related to the fact that heads presented the higher content of saturated fatty acids. Significant differences were found between the total content of PUFAs of the byproducts and the respective oils. A similar pattern concerning type and composition was found for the EPA and DHA. In all cases, the heads byproducts presented a significantly lower value as compared to mixed and headless byproducts.

After oil production, the level of DHA decreased significantly, the headless oil was the one with highest decrease and the heads had the lowest (around 15 and 5% of the byproducts, respectively). On the other hand, the EPA content in the oil was significantly higher as compared to the byproducts. In this case, the heads and mixed oil have an excess of about 10% while approximately only 3.5% of that excess was found in the headless oil. The facts lead us to the conclusion that during oil production, the headless oil was the one that lost the most of DHA, while on the other hand, EPA was mostly recovered from the heads. Although the total fat content did not differ significantly in the two fractions, there may be a difference on the content of neutral lipids and phospholipids present, although not further studied here, it represents an interesting topic for further research.

Initial oxidation status

The initial oxidation status of the three different produced herring oils is shown in Table 3.

Table 3- Oxidation status of the different produced herring oils. Mean values of three independent measurements and standard deviations are given. Values in the same column followed by different letters are significantly different ($P < 0.05$). – Not determined due to lack of sample.

	PV (meq per./kg)	AV	FC (area units/pg)	A_235 (area units/μg)	A_270 (area units/μg)	FFA (%)	α-Tocop. (mg/100g oil)
Mixed oil	6.4 ± 0.1 a	9.1 ± 0.1 a	1.2 ± 0.1 a	193.5 ± 0.5 a	112.5 ± 0.5 a	2.5 ± 0.0 a	5.97 ± 0.68 a
Headless oil	6.3 ± 0.1 a	6.8 ± 0.0 b	0.7 ± 0.0 b	194.5 ± 0.5 a	118.5 ± 0.5 b	2.3 ± 0.0 b	7.36 ± 0.02 b
Heads oil	13.1 ± 0.1 b	21.2 ± 0.6 c	-	-	-	2.4 ± 0.0 a	2.16 ± 0.05 c

The PV of the heads' oil was significantly higher as compared to the mixed and headless oil (13.1 versus 6.4 meq peroxides/kg lipids). The oil produced from heads presented also a significantly higher AV than those produced from mixed and headless byproducts. In fact, the headless oil shows the lowest found value for the AV (6.8). Obviously, it is related to the initial oxidation status of the raw material used; heads byproducts showed the higher level of oxidation products, and subsequently, the oil produced from it had also high oxidation values. It has been reported that compared to muscle, skin is more sensitive to high temperatures, since this is found to be the only type of tissue that was almost completely inactivated by heat treatment (*107, 174, 175*). This suggests a purer enzymatic nature of the pro-oxidative activity in skin rather than in muscle, possibly because transition metal catalysis is more probable there. No differences could be found with respect to conjugated dienes, contrary to the conjugated trienes and FC where significant differences were found between the mixed and headless oil (112.5 versus 118.5 area units/μg oil and 1.2 versus 0.7 area units/pg oil, respectively). Because of a lack of sample, no data are available for these measurements concerning the heads oil. The FFA was significantly lower to the headless oil as compared to the mixed and heads oils (2.3 versus 2.5%). The α-tocopherol content of the oils varied from 2.16 to 7.36 mg/100g of lipid. The headless oil had the significant highest content of α-tocopherol while the oil from the heads presented the lowest content, since most of it has been used while oxidation progressed. Note that although undetectable levels of α-tocopherol were found in the heads' byproducts, since it is a lipossoluble compound, small values could be measured in the respective oil. Apparently, the differences found in the α-tocopherol contents between the oils obtained from solvent method and by steam extraction from the same byproducts might be related to an extraction phenomena, originating a higher yield

for the last procedure. Summarizing the results, it can be concluded that the heads oil was of a lower quality since it is the one with higher oxidation values and lowest α-tocopherol content. However, mixed and headless oils are of similar quality, probably because only a small part of the mixed oil consists of heads oil.

Oxidative stability

Table 4 shows the outcome of the data analysis from the GLMs that were fitted to all of the data obtained from the chemical analyses. The data are related to the change in oxidation products and antioxidants during storage at two different temperatures.

Development of oxidation products

The first step in the statistical analysis is to check whether the data are normally distributed using probability plots followed by one sample Kolmogorov-Smirnov test. Only the PV data required a transformation in order to obtain normally distributed data.

To evaluate whether mixed or headless herring oil, stored at 20 and 50 °C, had the greatest effect on the oxidative changes, all of the data were subjected to a Least Squares Estimates (LSE) test which, for each measurement, gave rise to the following parameters in the model:

$$Y_{(\text{measurement})} = \beta_0 + \beta_1 t + \beta_{2,T} + \beta_{3,\tau} + \beta_{4,\, T*\tau}$$

In this equation, t (covariate) is the storage time in days, T (class variable) is the effect of the storage temperature, and τ (class variable) is the type of oil studied. $T*\tau$ represents the interaction term of the two class variables on the measurements studied. The estimation of the constant β_0 is given with the value determined for the intercept.

The fitted models (Table 4) showed that, with exception of the A_235 nm measurement, all were highly significant ($P << 0.05$). Concerning the primary oxidation products (PV), a significant influence was detected only to the temperature. For CT (A_270 nm), FC, and α-tocopherol, the effect of storage time and temperature showed to be highly significant. Related to AV, not only were time and temperature significant but also the type of oil was significant. For the FFA, all four effects (time, temperature, type, and interaction effect between temperature and time) were significant. No studied parameter was found to be significant ($P > 0.05$) in to the measurements of conjugated dienes (CD, A_235 nm); consequently, the described model was also not significant ($P = 0.247$).

In the next step of the statistical analysis, non-significant effects were omitted step-wise, with this, new models were derived (Table 5). This technique provided the possibility to compare the overall rates at which the different oxidation products are produced.

Table 4- Anova table from the GLM of the different measures on fish oil data comparing the effect of storage days, temperature, oil's type and interaction effects. MS= mean squares, df = degrees of freedom, P = probability level. $Y_{(measurement)} = \beta_0 + \beta_1 t + \beta_{2,T} + \beta_{3,\tau} + \beta_{4,T*\tau}$

Measure	df	MS	F	P	Measure	df	MS	F	P
Log PV					**AV**				
Model	4	0.947	24.84	0.000	Model	4	26.141	31.0	0.000
Intercept	1	7.153	195.09	0.000	Intercept	1	1780.2	2111.2	0.000
Error	57	0.037			Error	59	0.843		
Effects					Effects				
Days	1	0.0067	0.18	0.670	Days	1	4.83	5.73	0.020
Temp	1	2.97	81.15	0.000	Temp	1	62.23	73.79	0.000
Type	1	0.000	0.001	0.979	Type	1	37.45	44.42	0.000
Temp*Type	1	0.000	0.000	0.999	Temp*Type	1	0.54	0.636	0.428
A_235 nm					**A_270 nm**				
Model	4	7.505	1.39	0.247	Model	4	1.112	27.3	0.000
Intercept	1	38729.05	7200.2	0.000	Intercept	1	144.62	3544.2	0.000
Error	59	5.38			Error	59	0.0408		
Effects					Effects				
Days	1	6.07	1.129	0.292	Days	1	0.922	22.6	0.000
Temp	1	0.0126	0.002	0.962	Temp	1	4.299	105.4	0.000
Type	1	18.547	3.448	0.068	Type	1	0.0704	1.726	0.194
Temp*Type	1	6.025	1.120	0.294	Temp*Type	1	0.0826	2.025	0.160
FC					**FFA**				
Model	4	2706.26	25.98	0.000	Model	4	0.086	8.298	0.000
Intercept	1	17598.08	168.97	0.000	Intercept	1	161.56	15581	0.000
Error	60	140.15			Error	60	0.010		
Effects					Effects				
Days	1	1825.78	17.53	0.000	Days	1	0.0569	5.492	0.022
Temp	1	10354.8	99.42	0.000	Temp	1	0.216	20.80	0.000
Type	1	10.52	0.101	0.752	Type	1	0.067	6.488	0.013
Temp*Type	1	282.92	2.716	0.105	Temp*Type	1	0.048	4.618	0.036
α-tocopherol									
Model	4	3.981	10.992	0.000					
Intercept	1	1225.67	3383.9	0.000					
Error	54	0.362							
Effects									
Days	1	2.386	6.586	0.013					
Temp	1	5.404	14.918	0.000					
Type	1	0.318	0.879	0.353					
Temp*Type	1	0.105	0.289	0.593					

Where t (covariate) is the storage time in days, T (class variable) is the effect of the storage temperature and τ (class variable) is the type of oil studied. T*τ represents the interaction term of the two class variables on the measurements studied. The estimation of the constant β_0 is given with the value of the intercept.

As can be seen in Table 5, in all cases, an initial value was estimated indicating that the initial intercept values differed from zero and were always positive. Because different units are used for the different measurements, no comparison of the values can be made.

Table 5 – Statistics describing the outcome of the parameter estimates.
$Y_{(measurement)} = \beta_0 + \beta_1 t + \beta_{2,T} + \beta_{3,\tau} + \beta_{4,T*\tau}$ and the confidence intervals by least squares estimates for the fish oil data. Log PV is expressed as log meq. perox./kg of lipid; A_270 nm as area units/ng of lipids; FC as area units/10 pg of lipids; FFA in % as oleic acid; and α-tocopherol as mg/100g lipids. Refer to text for more information.

	β_0	β_1 **t**	$\beta_{2,T}$		$\beta_{3,\tau}$		$\beta_{4,T*\tau}$
			20 °C	50 °C [a]	SM	SH [a]	20 °C*SM
Log PV	0.278 ± 0.07	-	0.495 ± 0.096	0	-	-	-
A_270 nm	2.699 ± 0.084	0.003 ± 0.002	-0.607 ± 0.12	0	-	-	-
AV	9.026 ± 0.50	0.00808 ± 0.06	-2.482 ± 0.704	0	1.355 ± 0.682	0	-
FC	41.22 ± 4.14	0.148 ± 0.072	-29.63 ± 5.96	0	-	-	-
FFA	2.588 ± 0.056	0.00083 ± 0.000	-0.189 ± 0.078	0	0.0101 ±0.076	0	0.109 ± 0.102
α-Tocop.	7.397 ± 0.246	-0.0054 ± 0.004	-0.710 ± 0.362	0	-	-	-

[a] this parameter is set to zero because it is redundant to the model. – No significant effect found.

Concerning the storage time in days, it was shown that this did not have a significant effect to the log PV and had a negative influence on the α-tocopherol content (β_1 = -0.0054), which was expected. Because of the reduction in protection against oxidation, that takes place with the decrease of α-tocopherol content, oxidation was formed. This is shown by an increase of secondary and tertiary oxidation products measured as conjugated trienes, AV, and FC. The largest influence of time was found for the tertiary oxidation products, measured as FC (β_1 = 0.148) and the smallest for the formation of conjugated trienes (β_1 = 0.003). Furthermore, FFA was somewhat dependent on the storage time (β_1 = 0.00083). This implies that during storage, some hydrolysis of the oil occurred. The time courses of the different signals during autoxidation for the mixed herring oil stored at 20 and 50 °C and their models are shown in Figures 1 and 2, respectively. The log PV values are not shown since they were found to be independent of the storage time.

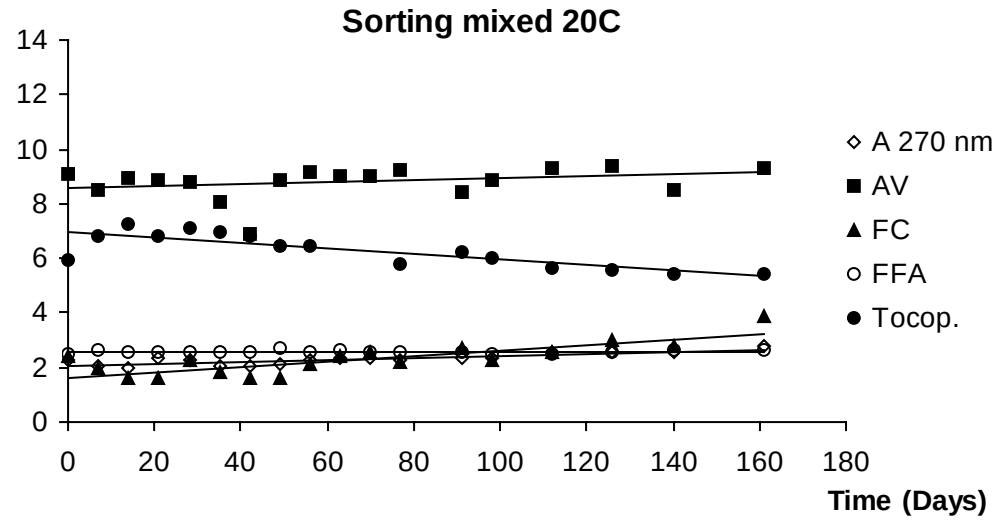

Figure 1– Trend lines and values determined for crude oil produced from mixed herring byproducts, stored under nitrogen and dark condition (20 °C). α-tocopherol is expressed as mg/100g lipids; A_270 nm as area units/ng of lipids; FC as area units/10 pg of lipids; and FFA in % as oleic acid.

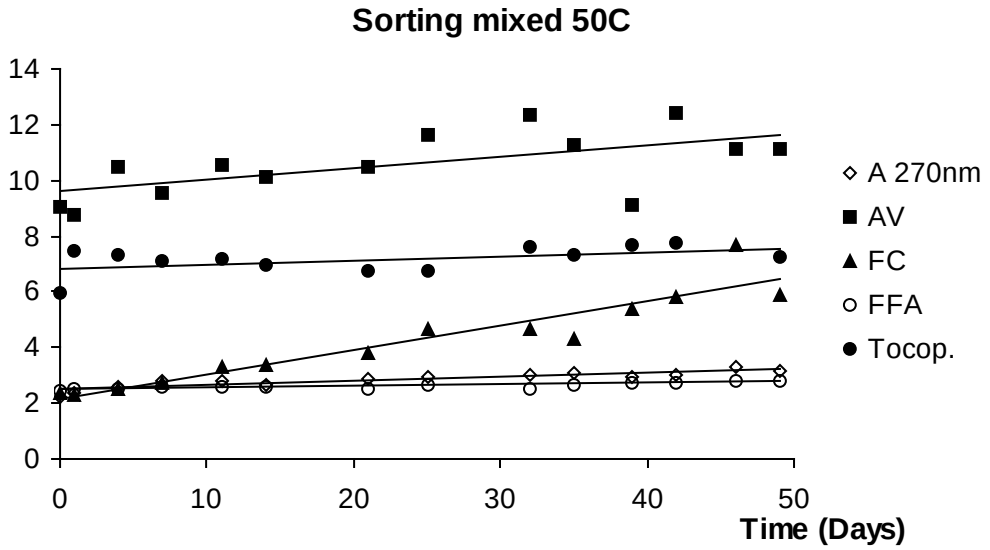

Figure 2- Trend lines and values determined for crude oil produced from mixed herring byproducts, stored under nitrogen and dark condition (50 °C). Same units as described in legend of Figure 1.

The storage temperature (Table 5) was found to be significant to all of the measurements. It can be observed that when the oil was kept at 20 °C, the log PV signal showed positive estimates in time while for the other signals negative estimates were found. The positive value of the log PV (β_2 = 0.495) means that higher values of hydroperoxides were

determined at 20 °C rather than at 50 °C. The results illustrate the instability of the hydroperoxides concerning the effect of temperature, showing that these compounds are rapidly decomposed to further oxidation products at higher temperatures. The results are in good agreement with reported literature (*176*) and our previous findings (*60*). As the lipid oxidation process progresses, the development of CT, AV, and FC was in all cases significant and occurred in a lower rate at 20 °C rather than at 50 °C, since negative values were found. Once again, the FC has revealed to be the measurement that progresses more rapidly at 20 °C (β_2 = - 29.63), as reported earlier (*59*) supported also by the findings of Aubourg (*93*). The increase in FFA level is significant, and it is more pronounced at a higher temperature of storage. The α-tocopherol content was found to be lower at 20 °C (β_2 = - 0.710). The fact means that a larger loss of this antioxidant seems to have occurred at 20 °C rather than at 50 °C. The reason of such phenomena might be related to a mechanistic reaction as reported previously (*60*) for oil produced from fresh herring byproducts.

The effect of origin of the oil from mixed or only headless byproducts, showed to have a significant difference for the AV and FFA (β_3 = 1.355 and β_3 = 0.0101, respectively). In both cases, the oil produced from mixed byproducts presented a higher value than the headless oil. A positive interaction effect between temperature (20 °C) and type of oil (SM) was only found for the development of FFA (β_4 = 0.109). For the other measurements, no significant difference was found.

Concluding Remarks

In conclusion, it can be stated that heads byproducts oxidized much faster than the mixed and headless byproducts. With the exception of the CT, the headless oil has significantly lower initial values for the oxidation parameters and the highest content of α-tocopherol. In contrast, the oil produced from the heads had the highest oxidation values and presented the lowest α-tocopherol content. Heads contained the lowest PUFAs content and the highest amount of saturated fatty acids. No significant differences were found between the fatty acid composition of the mixed and the headless either in the byproducts or in the oil. Some differences were detected between the byproducts and the produced oil for the EPA, DHA, and total content of PUFAs. When stored over time, the headless oil only presented significantly lower values for the AV and FFA measurements. Considering the small differences found in the different types of produced oils, it seems that sorting of herring byproducts does not result in strongly different oil qualities. A relatively simple

valorization procedure therefore seems to be suitable for the production of high quality and rather oxidation insensitive oil.

Acknowledgements

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3.3 Influence of Production Process Parameters on Fish Oil Quality in a Pilot Plant

ABSTRACT

A pilot plant used for upgrading herring byproducts into fish oil was analyzed on its operational efficiency and product quality. The temperature of the heat exchanger (T), the speed of the pump (MP) and the speed of the three-phase decanter (D) were varied according to a 2^3 fractional factorial design. The initial amount of oxidation products present in the crude oil, its storage stability and the yields from the different obtained products were determined. Multivariate data analysis of the advanced screening stage showed that T had neither influence on the quality nor on the yield in contrast to D and MP ($P < 0.05$). Process optimization results showed that the quality of the oil was influenced by an interaction effect between the speed of the MP and the D. The results showed that the system needed ~ 2 hours to stabilize in order to obtain the lowest oxidation levels for the oils. The lowest amount of oxidation products was obtained by processing at high pump speed and at intermediate decanter speed (47 Hz). Oils processed with the highest MP speed were the most stable during storage.

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INTRODUCTION

The use of ω-3 polyunsaturated fatty acids (PUFAs) has been suggested for the prevention and treatment of diseases such as cardiovascular and visual function in infants (*24, 25, 177, 178*). Therefore the food industry shows interest in adding fish oil, which is rich in PUFAs, into food products (*179-183*). Herring (*Clupea harengus*) byproducts, resulting from the filleting operation, have the potential to be utilized as raw material for production of fish oil since they are relatively rich in PUFAs throughout the year (*41, 59, 61, 62*). However, ω-3 PUFAs are susceptible to oxidation, which does not only lead to a reduced health effect, but also to the generation of highly unwanted off-flavors (*90*). It is therefore important to optimize the fish oil process such that the degree of oxidation in the extracted oil is as low as possible at profitable production process conditions.

Fish oil can be extracted by several methods, including fractionation (*32*), low temperature solvent extraction (*33*) and supercritical fluid extraction (*34*). However, wet and steam rendering are the most commonly used methods. They involve cooking the raw material by steam under pressure with or without the presence of water, respectively. Following this stage, the cooked material is pressed (either by hydraulic batch–wise or by continuous screw presses) and centrifuged and/or filtered to recover the oil from the micella (*35-39*). The possibility of using a three-phase decanter offers an opportunity to produce and separate fish oil in only one step after the heat exchanger (*6, 59, 61*). Enzymatic treatments have also been developed in order to increase the concentration of ω-3 PUFAs in fish oil (*184-187*).

In this work, a thermal rendering process with a three-phase decanter was studied. In our previous work (*59, 61*), it was demonstrated that crude oil from herring byproducts with relatively low oxidation products could be extracted efficiently. In this work, the evaluation of the performance of the fish oil pilot plant was performed using an experimental design consisting of three consecutive stages (*188*): first screening, advanced screening (AS) and an optimization (PO). The first screening stage consisted of identifying the most important process parameters and to search for their limits. An advanced screening experiment was subsequently performed to estimate the importance and interaction effects of the selected variables. In the final stage, the effects were studied in more detail with the purpose of optimization. The application of experimental design and multivariate techniques are effective and yield more information compared to the traditional way of evaluation by changing one variable at a time (*189*).

It is known that, while extracting fish oil, yield is reduced at low temperature while oxidation is enhanced at high temperature. The total throughput has a similar effect: high

throughput leads to a short residence time in the heat exchanger, and thus a low oil extraction yield; low throughput causes a long residence time and consequently unnecessary high oxidation levels in the oil. Low rotation speeds of the decanter may lead to incomplete separation of the oil; too high speeds may lead to oxidative stress in the oil. Previously obtained settings (*41, 59, 61, 62*) were used as first screening. Using the found settings, the advanced screening experiment was carried out. A two-level factorial design was used with three variables, namely, the speed of the mono-pump (MP) representing the total throughput, the temperature of the heat exchanger (T) and the speed of the three-phase decanter (D). Evaluation criteria were the quality and the yield of the oil. The data obtained at this stage was used in the setup of the PO stage. In both stages, the yield and residence times were also determined.

The main goal of this work was to optimize the process parameters from the fish oil pilot plant in terms of oil quality and yield. Since the most important aspect of the oil is the degree and stage of oxidation, this was investigated more specifically. The oxidative status and its development over time, in terms of type and concentration of lipid oxidation products, was followed as a function of the processing conditions.

MATERIALS AND METHODS

Fish oil

The oil recovery process used is presented elsewhere (*59*). For each experiment, an amount of ~ 2000 kg of fresh unsalted herring byproducts (heads, frames, skin, viscera, etc.) were minced (SAB, 49-033.2). Immediately after the mincing step, the byproducts were pumped (mono-pump, SW 032) towards an insulated scraped-surface heat exchanger that was indirectly heated by steam (Contherm®, type 6x9). The heated suspension was separated in a three-phase decanter (Alfa Laval, NX 409S-11G) into a solid phase (called the protein phase), a water phase (stickwater) and lipid phase (oil). Apart from the mincer, all equipment parts exposed to the products were made of stainless steel.

Two experiments denominated advanced screening (AS) and process optimization (PO) were performed. For the AS experiment the major fatty acids present in the produced oil were C14:0 (14.4%), C16:0 (17.1%), C16:1 (6.3%), C18:1 (8.5%), C20:1(12.2%), C22:1 (17.6%), C18:4 (3.9%), C20:5 (EPA, 6.5%), and C22:6 (DHA, 6.0%). For the PO experiment the major fatty acids were C14:0 (8.6%), C16:0 (13.1%), C16:1 (5.2%), C18:1 (10.0%), C20:1(15.7%), C22:1 (28.0%), C18:4 (2.1%), C20:5 (EPA, 5.5%), and C22:6 (DHA, 6.3%). The fatty acids composition was determined by capillary gas chromatography (GC) with flame ionization detection of the methyl esters prepared by transesterification using BF_3-methanol (*66*) with the applied conditions described earlier (*59*).

Sample set-up

Production. For each run all the tests were performed in triplicate. For quantification purposes, the flows for each run were determined in two ways: by weighing the products of the three different phases after five minutes of production, and by measuring the residential time. For the

last procedure, a color marker (Rayner's Green Food Color, Rayner and Co. Ltd., London, England) was used. The marker was added to the process just before the mono-pump and the time was recorded until it appeared in the stickwater phase.

Storage trial of the oil. A storage trial of the different oils was performed over 20 days in order to evaluate its oxidative stability. Part of the recovered oil was placed in an oven at 35 °C in open 250 mL dark glass bottles (opening ∅ 45 mm). Three oil samples (~ 10 mL) were taken, at regular intervals, from each produced oil. To avoid further oxidation, all samples were kept in a freezer (–80 °C) until analyzed. Prior to analysis, the oil samples were thawed at room temperature for 30 min. The samples were then analyzed for the level of oxidation products and FFA contents.

Analyses of lipid oxidation products

Peroxide Value. The peroxide value (PV) of the samples was determined according to the Official AOCS (*66*) Method Cd-8b-90 (n = 3, a = 1). The content is expressed in terms of meq of peroxides per kg of lipid.

Anisidine Value. The anisidine value (AV) of the oil was carried out according to AOCS (*66*) Official Method Cd 18-90 (n = 3, a = 1).

Totox Value. The Totox value (TV) was calculated according to the formula TV = 2xPV + AV. This value is often referred as total oxidation and has been suggested for the assessment of oxidation in oils (*190*).

Absorbance at 235nm (A_{235}, CD), 270 nm (A_{270}, CT), and 300 nm (A_{300}). A_{235}, A_{270}, and A_{300} nm of the oil samples were measured using flow injection analysis (FIA) as described by Undeland et al. (*98*) (n = 3, a = 1). Results were expressed as peak area units per nanogram of lipid. The repeatability were 2.0, 4.3, and 7.0 (n = 1, a = 6) for A_{235}, A_{270}, A_{300} respectively.

Fluorescence Compounds (FC). Total lipid soluble fluorescent oxidation products with an excitation (ex) maximum at 367 nm and an emission (em) maximum at 420 nm were measured in the oil samples using FIA as described by Undeland et al. (*98*) (n = 3, a = 1). Results were expressed as peak area units per nanogram of lipid. The repeatability was 7.0% (n = 1, a = 6).

Free Fatty Acids. The amount of FFA of the oil samples was determined by titration according to AOCS (*66*) Official Method Ca 5a-40. In each occasion, three oil samples (n = 3) were analyzed once (a = 1). The percentage of FFA was calculated as oleic acid.

Compositional analyses

Lipid content. The total lipid content in the samples was determined gravimetrically after extraction according to the Bligh and Dyer (*65*) procedure (n = 3, a = 1). Results were expressed as grams of lipid per kilogram of samples.

Moisture. Moisture was determined as described earlier (*59*) (n = 3, a = 1). Results were expressed as percentage of wet weight.

Protein. Total nitrogen in the homogenized samples was determined using the Kjeldahl digestion method as described previously (*59*). Results were expressed as percentage of wet weight.

Salt. Chloride content in all the samples was titrated according to Volhard's method as described by Kolthoff and Sandell (*67*) (n = 3, a = 1). Results were expressed as percentage of wet weight.

Apart from the fatty acids compositional analysis, internal reference materials were analyzed together with the samples.

Statistical Analysis

Multivariate Experimental Design. A 2^3 fractional factorial design (resolution IV) was used for the AS study. This means that the three variables, or factors, as given in Table 1, were investigated at two levels, +1 and –1. The center point in the design was repeated once to

calculate the reproducibility of the method. The results were then analyzed statistically using software Unscrambler™ (version 7.5, CAMO ASA, Norway). Residuals were tested for normality using probability plots. The data analysis resulted in a mathematical model that correlated with the three variables studied (MP, T, D) to the various responses: PV, AV, TV, FFA, FC, CD, CT, A_300 nm.

Table 1- Sampling sequence and conditions used for the advanced screening stage, using a 2^3 fractional factorial design.

Experiment	Speed Mono-pump (Hz) [a]	Temperature (°C) [b]	Speed Decanter (Hz) [c]
Center point 1	0	0	0
2	+1	-1	-1
3	+1	+1	+1
4	-1	-1	+1
5	-1	+1	-1
Center point 2	0	0	0

[a] +1 = 80, -1 = 40, [b] +1 = 100, -1 = 90, [c] +1 =50, -1 = 48. In all cases 0 = middle value.

For the PO a manual data set was created, in order to incorporate the knowledge acquired from the AS. In both experiments, the results were examined from response surface overviews. For each of the response variables, model summaries and lack of fit tests were analyzed for linear and quadratic models. From this information, the most accurate model was chosen, which was a linear and quadratic model for AS and PO, respectively. The optimal settings for the design variables were read from the response surface plots, choosing for the quality the lowest oxidation values and for the quantity the highest values. Significant levels were read from ANOVA tables. In order to check whether different processing conditions of fish oil contribute to different storage stability over time, General Linear Models (GLM) were tested. For that, the obtained data from the storage trial of the oil, at 35 °C, was checked. Observed power was used to evaluate the strength of the drawn inferences related to the failure to reject the H_0 (*68*). SPSS software (version 10.0) was used. The level of significance for all tests was set at 95%.

Table 2- Sampling sequence and conditions used for the process optimization stage. Note that reference points have the same settings as the center points used for the advanced screening stage. Temperature was set at 95 °C.

Run	Speed pump (Hz)	Speed decanter (Hz)
Reference point 1	60	49
2	80	48
3	80	50
Reference point 2	60	49
5	40	48
6	40	50
Reference point 3	60	49
Reference point 4	60	49
9	80	47
10	80	46
Reference point 5	60	49

RESULTS AND DISCUSSION

In the first part of this section the results from the advanced screening (AS) stage and the storage stability of the oil is discussed. The process optimization (PO) stage is evaluated in the second part.

Previously obtained settings (*41, 59, 61, 62*) were used for first screening. These results suggested that the temperature during the heat treatment should be varied between 90 ºC to 100 ºC, the total throughput should be between 200 kg/h and 400 kg/h (mono-pump speed from 40 to 80 Hz), while the decanter should be operated at 5693 to 5930 rpm (48 to 50 Hz, respectively).

Part I: Advanced Screening Stage

This stage was performed in order to estimate the main effects and interactions for the important variables. Linear models were fitted to the quality and quantity responses; results from analyses of variance (ANOVA) from the data analysis procedure are shown in Tables 3 and 4.

Quality parameters

The significant levels found between the design variables and the quality responses are shown in Table 3. As can be observed from the results, the temperature range tested did not influence any of the quality responses significantly. Heating coagulates the fish proteins, so that liquids and solids can be mechanically separated. At these temperatures, cell rupture is more or less complete. Therefore, a variation of the temperature did not result in a significant effect, even though the average residence time varied between ~ 5 to 12 min (MP speed of 80 and 40 Hz, respectively). Consequently, for the PO experiment, the variable T was set at the center point (95 °C).

Table 3- Summary of the ANOVA tables with p-values for the quality parameters from the advanced screening experiment. Bold values represent significant influences ($p < 0.05$).

Design variables	PV	AV	FFA	Totox	CD	CT	A_300	FC
T	0.91	0.18	0.82	0.37	0.77	0.14	0.11	0.11
MP	**0.02**	**0.01**	0.23	**0.00**	**0.02**	0.96	0.78	0.84
D	0.39	**0.02**	0.94	**0.02**	0.49	0.91	0.70	0.74

From the MP, significant effects were found on the PV, AV, Totox and CD values ($p = 0.02$, $p = 0.01$, $p = 0.0009$, $p = 0.02$, respectively). This implies that the concentration of primary oxidation products (measured by PV and conjugated dienes), secondary oxidation

product (AV) and hence the Totox value were influenced by the speed of the MP. Further data analysis showed that the response surface plots presented a minimum level of primary (PV) and secondary oxidation products at the highest speed of the MP, 80 Hz. The PV increased when samples were processed with longer residence times (lower MP speed). The speed of the decanter showed a significant effect only for the AV and consequently for the Totox value ($p = 0.017$ and $p = 0.018$, respectively). Hence, the results suggested that the decanter speed influenced only the amount of secondary oxidation products. Response surface plots exhibited a minimum AV at the lowest decanter speed, 48 Hz (5692 rpm). For the PO stage the value of this variable was therefore set at the lowest speed where good oil separation (evaluated visually) and production could be achieved (which was found to be at ~ 46 Hz).

It was further observed that the two center points, which were separated in time, presented different oxidation values for the PV, AV and consequently to the Totox. This led us to the conclusion that a time effect was present in the process. Therefore, for the process optimization stage, several reference points over time were incorporated in the design and the processing time was recorded also.

Oxidative Oil Stability at 35 °C

In order to evaluate the oxidative stability of the produced oil, the different extracted oils were subjected to a storage trial. PUFAs in oxidized lipids can produce conjugated chromophores that can be detected by the absorbance of ultraviolet (*91*). Therefore, the concentration of conjugated dienes (CD), conjugated trienes (CT), tetraenes and fluorescent compounds (FC) were followed over time. For each of the responses, General Linear Models were tested as follows:

$$Y_{(response)} = \beta_0 + \beta_1.\text{days} + \beta_2.\text{MP} + \beta_3.\text{D} + \beta_4.\text{T} + \beta_5.\text{MP*days} + \beta_6.\text{D*days} + \beta_7.\text{T*days}$$

The development of conjugated tetraenes was not significant ($p > 0.05$). The coefficients of independent variables as storage time (days), mono-pump settings (MP), temperature set point (T), and decanter speed (D) determined for GLM for level of total CD, CT and FC of the processed and stored crude herring oil were:

$$Y_{(CD)} = 64.832 - 0.449 \text{ days}$$

$$Y_{(CT)} = 28.420 - 11.738 \text{ days} - 0.136 \text{ MP} + 0.268 \text{ days*D}$$

$$Y_{(FC)} = 1.081 \text{ days} - 0.095 \text{ MP} + 0.363 \text{ T}$$

The size of the β-coefficient shows how large the influence from a specific variable is for each response. It should be emphasized that positive values on the coefficients imply that the variable had an accelerating effect, whereas negative values mean a retarding effect on the responses. It can be seen that on the CD and CT responses a high value on the

intercept (β_0) was obtained, indicating the occurrence of oxidation in the freshly extracted oil. The results showed that the level of FC increased over time in contrast to the CD and CT levels, where their development slowed down. Possibly, over time, the rate of degradation of such compounds is high, producing further substrates for maintaining the oxidation process and to promote the development of tertiary oxidation products found in a later oxidation stage. At the same time, it would also explain the absence of such latter compounds from the original processed oil.

The influence of the different processing conditions was significant in the development of CT and FC compounds. In both cases, the development of oxidative compounds was negatively correlated with the MP speed, e.g. oils produced with higher MP speed presented lower levels of CT and FC compounds. This suggests that, for oil quality, the residence time in the heat exchanger should be as low as possible. A significant interaction effect between days and D was detected only for the formation of CT. The oil extracted with higher D speed favored the development of CT over time, possibly because high D speeds provoked high shears force which results in an increase of temperature in the system and may lead to the development of CT. For the development of tertiary oxidation products quantified by FC, the processing temperature presented a significant effect. Overcooking results in a breakdown in the coagulated muscle tissue to an extent that promotes the development of fluorescent compounds. Clearly, a longer residence time in the system, at the highest processing T promoted the development of fluorescent compounds over time.

Quantity parameters

The herring byproducts contained a high moisture content (73%), whereas the protein and fat content presented values of 15 and 10%, respectively. The yields of the three obtained phases were established from the first center point, based on 100 kg of herring byproducts. The protein phase from the separation process was the major fraction (55.7%) and consisted mainly of protein, water and fat, 24, 68, and 4%, respectively. The stickwater phase, representing 34.4%, contained more than 90% of water and only ~ 7 and 2% of protein and fat content, respectively. The oil fraction accounted for 9.9%.

To evaluate the effect of the studied variables with the quantity responses, the flows and ratios for each phase were assessed (Table 4). The results showed that values of D and T of the heat exchanger did not have a significant effect on the yield ($p > 0.05$). In contrast, the speed of the MP had a strong effect on the flows of the three fractions ($p = 0.04$, $p = 0.01$, $p = 0.03$ for protein, stickwater and oil, respectively). The surface plots showed, as

expected, that the maximum flows were obtained by producing at the highest MP speed, 80 Hz. Figure 1 shows the yields obtained for individual fractions as well as for the total throughput as a function of MP. Higher water yield results from the loss of protein.

Table 4- Summary of the ANOVA tables with p-values for the quantity parameters (flows and ratios) from the advanced screening experiment. Bold values represent significant influences.

Design variables	Protein	Water	Oil	Protein ratio	Water ratio	Oil ratio
T	0.26	0.08	0.52	0.72	0.46	0.17
MP	**0.04**	**0.01**	**0.03**	0.47	0.34	0.38
D	0.98	0.98	0.62	0.82	0.60	0.27

Apparently, high MP speeds and using the same decanter conditions results in a better protein/water separation. It can also be concluded that the flows can be easily described using linear models, since the responses were only dependent on the MP. Within the range of conditions used, no significant effect was found for the different ratios of the obtained products (Table 4). Chantachum et al. (*39*) found that for precooked tuna heads a lower oil yield was obtained when heating at 95 °C for 10 and 20 min than at 75 and 85 °C. This was explained by the observation that protein denaturation generates a tightly packed protein structure, leading to the retention of oil in the protein matrix. However, in this study, possibly due to the short residence times in the heating treatment (between ~5 and 12 min), differences between the oil ratios were not observed.

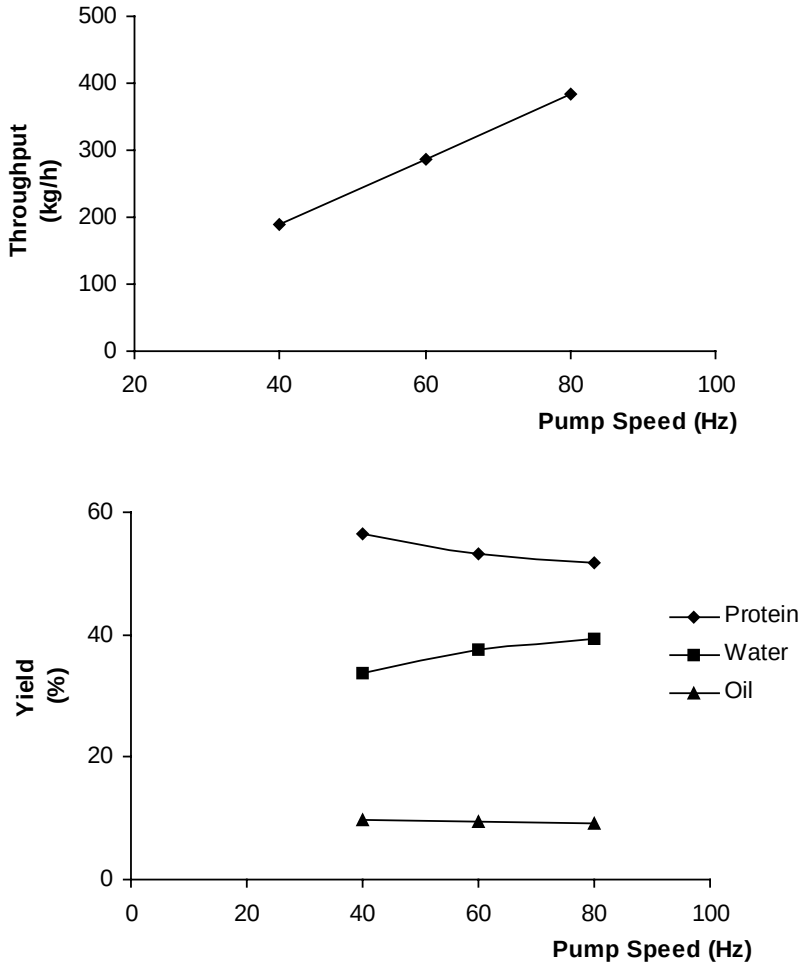


Figure 1– Variation of the total throughput and yield between mono pump speeds and quantity responses.

Conclusions from the Advanced Screening Stage

Several conclusions could be drawn: the temperature did not influence the quality of the oil. The oil from the two center points, produced at separate time, differed in quality. High MP and low D speed presented the best combination for good quality oil. Quantity has shown to be only dependent on the MP of the system. Higher MP speeds produce higher individual and total flow, maintaining similar ratios between the fractions.

Part II: Process Optimization Stage

Since MP and D speeds were the major variables in the process, it was necessary to study their main and interaction effects in more detail. With this knowledge, process optimization was performed, as described in Table 2. T was kept constant, the decanter speed was lowered and the same conditions as applied before were used for the MP. The

obtained results were fitted in quadratic models that were used to generate response surfaces of the oxidation products formed during processing. A quadratic model is more likely to approximate the true response surface correctly and to give more accurate predictions in the neighborhood of the optimum (*188*). Optimization designs can be particularly useful in minimizing a single response, finding a stable region, or a compromise between several responses in order to determine which combinations of design variables lead to the best compromise between several responses (*188*).

Quality parameters

From the reference points data, it became clear that AV and FFA decreased over time (Figure 2) while for the other quality parameters relatively constant values were measured (data not shown). Apparently the system needed ~ 2 hours to stabilize. As these values decreased over time, it was assumed that all values obtained from the process optimization need to be corrected. Therefore the ultimate stable measurements were used as baseline values to correct all the results by subtraction. For the other quality responses no corrections were made since no effect over time was detected.

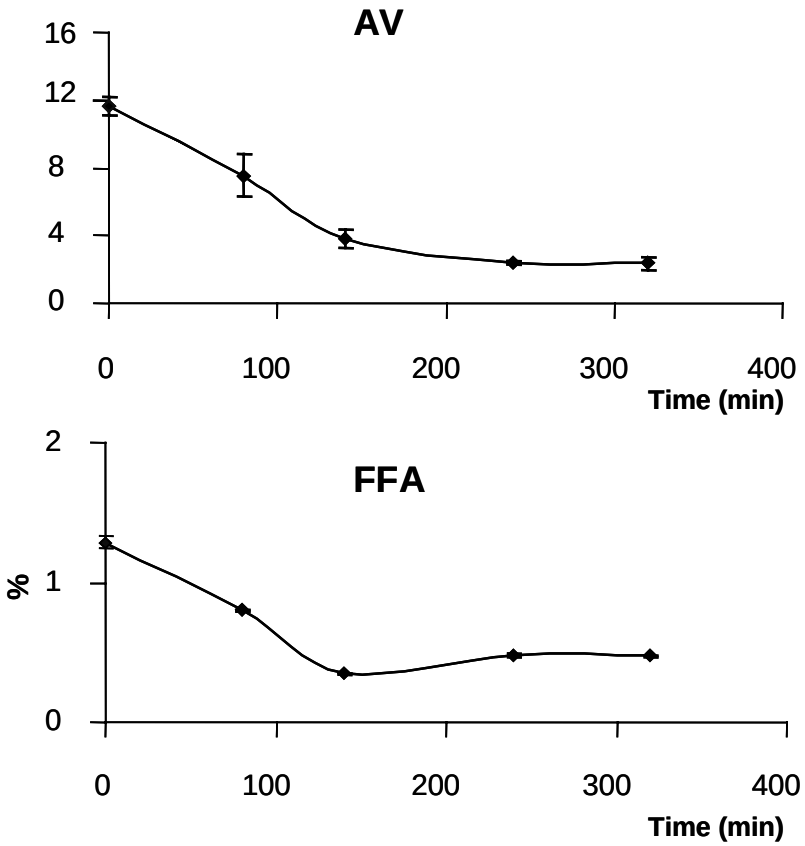


Figure 2- AV and FFA values of the crude herring oil for the reference points produced over time.

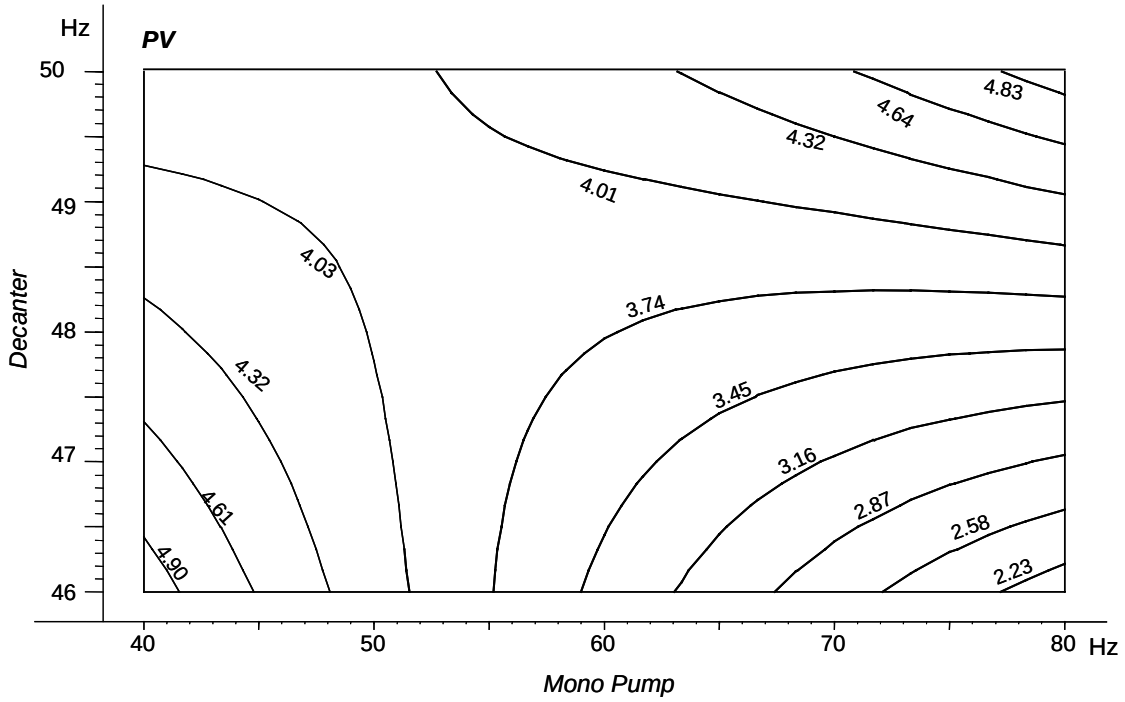
The statistical outcome from the experiments is shown in Table 5. For the AV and FFA responses a significant influence from all the design variables and possible interaction effects was found ($p < 0.05$). A significant effect of the D and an interaction effect between the MP and D was found on PV ($p = 0.01$ and $p = 0.006$, respectively). Apart from the effect of the D speed, main effect of MP and all the other interaction effects were found significant for the corrected Totox value (in all cases $p < 0.05$). The CD response showed no significant influence of any of the variables; therefore these results were not used for further analyses.

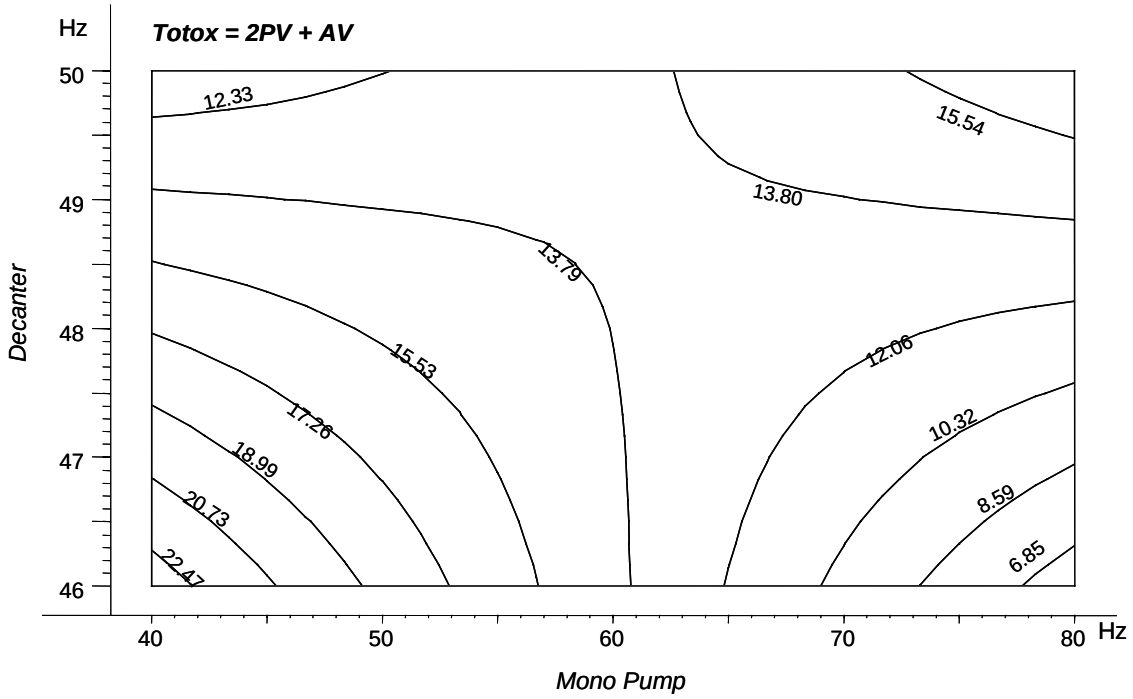
Table 5- Summary of the ANOVA tables with the p-values for quality parameters from the process optimization experiment. Bold values represent significant influences.

Design variables	PV	Corrected AV	Corrected FFA	Corrected Totox	CD
MP	0.75	**0.00**	**0.00**	**0.00**	0.44
D	**0.02**	**0.00**	**0.00**	0.18	0.15
MP*D	**0.01**	**0.00**	**0.00**	**0.00**	0.97
MP*MP	0.43	**0.00**	**0.00**	**0.01**	0.46
D*D	0.91	**0.00**	**0.00**	**0.01**	0.74

Surface plots results showed that for all the significant responses (PV, AV, FFA and Totox) the minimum amount of oxidation products were a result of the highest MP speed, 80 Hz. However, for the D more complex conclusions needed to be drawn: the lowest levels of the different responses were obtained at different D speeds. Figure 3 shows contour plots for the effects of MP and D speed on the PV, Totox and FFA responses. The PV level was lowered to less than 3 meq perox./kg oil at a MP speed of 68-80 Hz and a D speed of 46.5-46 Hz. Secondary oxidation products, measured as AV, were present in lower concentrations when the oil was processed with a MP speed of 70-80 Hz and D at 47-46 Hz. The results showed that in the region where higher hydroperoxides concentrations were measured (highest D and MP speed), favored their decomposition and consequently an increase of secondary oxidation products was found. Similarly, the Totox values showed a minimum with a MP speed of 70-80 Hz and D at 47.5-46 Hz. Changes in the Totox value provided information regarding the progressing of the formation of primary and secondary oxidation products. The total oxidation index (Totox value) is often considered to have the advantage of combining evidence about the past history of the oil (as reflected in the AV, for the processing) with its present state (as

evidenced in the PV), being therefore used extensively to estimate oxidative deterioration of lipids (*191*).





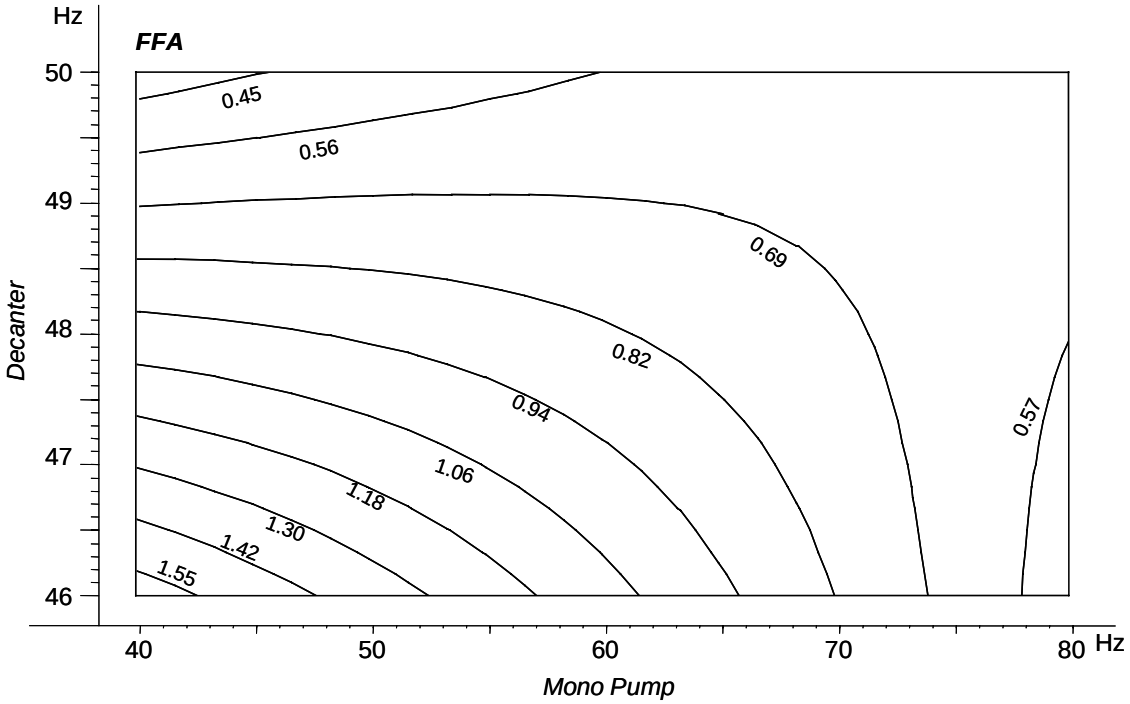


Figure 3- Contour plots for the effect of mono-pump and decanter speed on PV, Totox and FFA content of the extracted crude oil from herring byproducts during the process optimization experiment. The plots illustrate where in the experimental domain the lowest values could be obtained.

According to these results, the formation of both primary and secondary lipid oxidation products was highest at the combination of the lowest MP and the lowest D speed. In contrast, the lowest oxidation values were found with the combination of highest MP and lowest D speed. Apparently, the processing residence time is a very important factor in the extraction of fish oil. From the contour plots, the MP speed of 75-80 Hz and D of 46-50 Hz were suggested to be good experimental conditions ranges for processing herring byproducts into oils with low levels of FFA. It is known that oil can undergo hydrolysis in the presence of moisture and heat (*192*). Our results indicate that the degree of hydrolysis of triglyceride ester bonds is less at higher MP speed, possibly because these conditions represent short heating times in the system. Therefore, these results justify the assumption that oxidation values in the oil are controlled by the MP and D speed in the process of extracting fish oil. Graphical representation of the data on contour plots (Figure 3) showed that these minima were located outside the experimental region. However, since neither MP could be higher nor the D lowered due to technical constraints, the obtained results are acceptable. It can be concluded that inside of the experimental region the lowest oxidation values were obtained by combining maximum MP speed (80 Hz) with an intermediate/lowest D speed (47-46 Hz).

Concluding Remarks

The results of this work show that the fish oil production method used is a stable process. The process seems to be sound within the chosen limits for the production parameters. The main variables that have influence on the product quality in the process are the separation speed of the decanter and the mono-pump. The advanced screening stage showed that the range of temperature selected did not influence either the quality or the quantity of the oil. By choosing the process settings it is possible to produce crude herring oil with the desired product quality (e.g. lowest oxidation values) and yield. The most important responses in the processed oil were PV, AV, Totox and FFA, which were found to positively correlate with the processing lowest mono-pump speed. Therefore, the best quality oil was obtained while producing oil with the maximum mono-pump speed, 80 Hz. The AV and FFA contents were negatively correlated with time until a period of two hours was reached, even though the average total residence time was ~5 min. The yields of the three fractions (water, protein and oil) were not dependent on the settings. During storage of the oil, the one that was produced with the highest mono-pump speed presented lower levels of conjugated trienes and fluorescent compounds. The analysis of the results, and the assessment of all the variables involved, allow us to assert that a good quality crude fish oil can be obtained from herring byproducts with a thermal processing method.

Acknowledgements

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Chapter 4

Product Quality

4.1 Stability of Crude Herring Oil Produced from Fresh Byproducts: Influence of Temperature during Storage

ABSTRACT

Crude herring oil, extracted from fresh byproducts, was stored at 0, 20 and 50 °C in order to study the effect of temperature on lipid oxidation. The obtained oil had an initial peroxide value (PV), anisidine value (AV), and free fatty acids of only 0.7 meq perox./kg of lipid, 0.4, and 0.6%, respectively. During storage, the oil reached the secondary oxidation stage for all three temperatures. The formation of fluorescent compounds was inhibited at 0 °C. Significant decrease of the α-tocopherol content was found after storage at 0 and 20 °C, but no consumption occurred at 50 °C. The development of oxidation products over time exhibited a temperature-dependency with a very good correlation. All measures could be described with linear regression models with the exception of PV where a second order polynomial function was required. AV, conjugated trienes and α-tocopherol loss were the best quality parameters to follow the oxidation progress.

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INTRODUCTION

In recent years, utilization of marine resources for human consumption has increased rapidly worldwide. This is induced by the health benefits of polyunsaturated fatty acids (PUFAs), in particular those of the ω-3 family (*24, 25*), which are present in high proportion in fish species with a high fat content (*11, 142*).

Atlantic herring (*Clupea harengus)*, a fatty fish, is an important raw material for the Dutch fish processing industry. After the filleting operation a substantial amount of fish and fish leftover is not used. In a time where depletion of the marine resources has become all too real, there is a clear need for more sustainable fisheries. A 100% utilization of fish processed for human consumption can be achieved by processing heads, fins, viscera, and so on into fish meal and fish oil. Converting herring byproducts into fish oil is an opportunity of adding value to byproducts.

PUFAs, such as ω-3 fatty acids, are interesting from a nutritional point of view, but at the same time can adversely affect sensory product quality due to oxidation (*118*). In general, the rate of oxidation increases rapidly with the increase in unsaturation content and, therefore, oxidation is a major concern in fish oils. Autoxidation of oils causes rancidity and the development of unpleasant flavors and odors. After initiation, autoxidation proceeds, in general, through a mechanism of free radical-mediated chain reactions and, finally, termination steps (*90*).

Although it is commonly recognized that temperature influences fish oil deterioration, research has not focused in finding relevant indicators for measuring oxidation in crude herring oil according to the storage temperature. The aim of this study was to investigate the chemical quality and stability of crude herring oil produced from fresh herring byproducts and stored at three different temperatures as well as to establish the best chemical measurements to evaluate oxidative changes. In the first part of the study, characterization of the raw materials and the crude oil was made. In the second part stability of the oil was investigated. To measure the lipid oxidation process, several methods were used to unravel the oxidation kinetics. Attention was focused on the loss of antioxidants, change in the free fatty acids content, and development of primary, secondary, and tertiary oxidation products. Linear regression models were used as a tool to evaluate whether any changes occurred on a statistically significant level during storage and to compare the different autoxidation processes at the different temperatures.

MATERIALS AND METHODS

The sources used in this study were byproducts from fresh herring (*Clupea harengus)* caught in October 1999 off 51.20 N, 02.60 E, with an average weight of 138 ± 23 g, length 24 ± 1 cm and maturity stage V (n = 19).

Equipment

A production of about 1000 kg of fresh herring byproducts (such as heads, frames, skin, viscera) was minced. Immediately, the minced byproducts were pumped towards an insulated scraped-surface heat exchanger indirectly heated by steam (about 8 min at 95 °C). The heated suspension was then separated in a three-phase decanter into a solid phase (called protein phase), a water phase (stickwater), and a lipid phase (oil). The recovery process used is presented elsewhere (*59*). All equipment parts exposed to the products, with the exception of the mincer, were made of stainless steel.

Sampling set-up

Samples were collected from five consecutive stages of the production chain, i.e., the herring fillets, the minced byproducts, the oil, the stickwater, and the protein phase, and these were analyzed.

Part of the recovered herring oil was divided into several containers and exposed to three different temperatures: 0 °C, ambient temperature (~ 20 °C) and 50 °C, in order to study oxidative changes at different temperatures. 50 °C was chosen to accelerate the oxidative process and therefore sampling was stopped earlier than for the other two temperatures. In all cases, the oil was blanketed with an inert atmosphere (nitrogen) and kept in closed dark containers.

Two oil samples (~ 100 mL) were taken, at regular intervals, from each storage condition and analyzed for their level of oxidation products, FFA formation, and α-tocopherol content. Sampling was stopped when the oils developed a quite strong off-odor, and therefore different storage times were used for the three temperatures studied (155 days for 0 and 20 °C and 52 days for 50 °C). In order to avoid further oxidation, all the samples were kept at –80 °C until analyzed. Prior to the analysis, the oil samples were thawed at room temperature for 30 min. The averages of the two sample measurements have been used for the final results.

Methodology

Fatty acid composition. Lipids from herring fillets and byproducts were extracted according to the method of Bligh and Dyer (*65*). Fatty acid methyl esters (FAMEs) from the lipid extractions and the oil samples were prepared according to AOCS (*66*) Official Method Ce 1b-89 and analyzed with regard to the content of individual fatty acids. In each case, three samples (n = 3) were analyzed once (a = 1). The different FAMEs were separated from each other with gas chromatography (GC) and detected with a flame ionization detector (FID) with the applied conditions described earlier by Aidos et al. (*59*). Results are expressed as g/kg of lipid.

Extraction of lipids. Total lipids for determination of peroxide value, anisidine value, free fatty acids and fluorescent oxidation products were extracted from the herring byproducts according to the method of Burton et al. (*94*) as modified by Undeland et al. (*95*).

α-Tocopherol. α-Tocopherol was analyzed according to the slightly modified method of Lie et al. (*97*) as described earlier (*59*). On each occasion n = 2, a = 1. The repeatability of the method was 5.1% (n = 1, a = 6). Results are expressed as mg/100 g of lipids.

Free Fatty Acids. The amount of FFA in the oil samples was determined by titration according to AOCS (*66*) Official Method Ca 5a-40 (n = 2, a = 1). The percentage of FFA was calculated as oleic acid equivalents.

Peroxide Value: The PV of the herring oil samples was determined according to Official AOCS Method Cd-8b-90 (*66*) (n = 2, a = 1). The content is expressed in terms of meq of peroxides per kg of lipid.

Anisidine Value. The AV of the herring oil was carried out according to AOCS (*66*) Official Method Cd 18-90 (n = 2, a = 1).

Absorbance at 235nm (A_{235}) and 270 nm (A_{270}). A_{235} and A_{270} nm of the oil samples were measured using flow injection analysis (FIA) as described by Undeland et al. *(98)* (n = 2, a = 1). Results were expressed as peak area units per microgram of lipid. The repeatability were 2.0 and 4.3% (n = 1, a = 6) for A_{235} and A_{270}, respectively.

Fluorescence compounds (FC). Total lipid-soluble fluorescent lipid oxidation products with an excitation (ex) maximum at 367 nm and an emission (em) maximum at 420 nm were measured in the oil samples using FIA as described by Undeland et al. (*98*) (n = 2, a = 1). Results were expressed as peak area units per picogram of lipid. The repeatability was 7.0% (n = 1, a = 6).

Lipid content. The total lipid content in the samples was determined gravimetrically after extraction according to the Bligh and Dyer (*65*) procedure (n = 2, a = 2). Results were expressed as grams of lipid per kilogram of samples.

Moisture. Moisture content of the oil samples was determined according to the Karl Fischer method (n = 2, a = 2). In the other samples moisture was determined by weighing the samples, until a constant weight was achieved, after drying in an oven at 105 °C (n = 2, a = 2). Results were expressed as percentage of wet weight.

Protein. Total nitrogen in the homogenized samples was determined using the Kjeldahl digestion method. Protein content was calculated as N*6.25 (n = 2, a = 2). Results were expressed as percentage of wet weight.

Salt. Chloride content in all the samples was titrated according to Volhard's method as described by Kolthoff and Sandell (*67*) (n = 2, a = 2). Results were expressed as percentage of wet weight.

Analysis of copper (Cu). To make copper available for analysis wet sample digestion was used. The samples (about 0.5 g of oil and 1.0 g of the other samples) were digested with concentrated nitric acid in a microwave oven for 45 min in Teflon destruction vessels and the procedure described earlier (*59*) was employed. Results are expressed as mg/kg of wet sample (n = 2, a = 2). The limits of detection were 0.1 mg/kg. The repeatability of the method was 6.0%.

Analysis of iron (Fe). About 1.0 g of each sample was ashed at 500°C (*100*) and a similar procedure was employed as described previously (*59*). Results are expressed as mg/kg of wet sample (n = 2, a = 2). The limits of detection were 0.01 and 0.1 mg/kg and the repeatability of the methods was 10.0 and 5.0% for the oil and the other samples, respectively.

Apart from the fatty acids analyses, internal or certified reference materials were analyzed together with the samples.

Statistical Analysis

Data from the α-tocopherol and stability measurements were subjected to regression analysis in order to fit a model describing whether there was a significant increase or decrease over time. Linear models ($y = \beta t + \alpha$) were tested and r^2 calculated. This technique also provided the possibility to compare the overall rates at which the different oxidation products are produced. Analysis of variance (ANOVA) for testing the hypothesis H_0: $\beta = 0$ against H_A: $\beta \neq 0$ was used. Confidence intervals for the parameter β (regression coefficient) and α (intercepts) of different measurements were estimated. The p-value was set at 95%. The slopes found from the linear models of the oxidation products (apparent rate) were plotted against temperature.

RESULTS AND DISCUSSION

In the first part of this section, the composition of the raw materials and produced oil is presented. In the second part, the stability of the oil during storage is discussed.

Compositional data:

Crude composition

As can be observed in Table 1, a higher protein content was determined in the fillets compared to the byproducts (16.2 vs. 14%). It is known that moisture and fat content are inversely related in herring (*41, 70, 73, 74*). This has been proven also true in our case, as the total lipid content in the herring fillets and the byproducts differs (16.8 vs. 9.6%) as well for the moisture content (66.7 vs. 73.6%), respectively. After processing, the protein and stickwater fraction contained 5.2 and 3.9% of total lipid content, respectively. The produced herring oil showed a moisture content of approximately 0.1%. This value meets the quality assurance specification (Young 1982; Bimbo 1998).

Table 1– Compositional data of fillets, byproducts, oil, protein and stickwater phase extracted from fresh herring (*Clupea harengus*).

	Fillets	Byproducts	Herring oil	Protein	Stickwater phase
Crude composition					
Protein (% w/w)[a]	16.2 ± 0.3	14.0 ± 0.5	-	22.7 ± 0.4	4.6 ± 0.1
Moisture (% w/w)[a]	66.7 ± 0.4	73.6 ± 1.6	0.10 ± 0.01	68.3 ± 0.5	90.8 ± 0.4
Salt (% w/w)[a]	0.2 ± 0.0	0.2 ± 0.0	-	0.3 ± 0.1	0.5 ± 0.1
Total lipids (g/kg wet sample)[a]	168 ± 2	96 ± 2	-	52 ± 5	39 ± 3
α-Tocopherol (mg/100g lipid)[a]	0.2 ± 0.1	0.7 ± 0.2	8.1 ± 0.4	-	-
Trace elements					
Fe (mg/kg wet sample)[b]	7.4 ± 0.4	29.0 ± 2.0	0.030 ± 0.006	38.0 ± 1.5	5.1 ± 0.8
Cu (mg/kg wet sample)[b]	0.87 ± 0.11	1.05 ± 0.19	<0.1	0.96 ± 0.06	0.58 ± 0.01

[a]: n = 3, a = 1, results are given as: mean value ± sd.

[b]: n = 2, a= 2, results are given as: mean value ± (max-min)/2. For each of the two samples, a = 2. Mean values from these two analyses were used to establish sample variation. **-:** Not determined.

α-Tocopherol

The α-tocopherol content showed large distribution variations between the fractions analyzed. However, it should be pointed out that a large individual difference has previously been found (*107*). Lipids from herring byproducts, which are composed of a considerable amount of dark muscle, contained three times more α-tocopherol than that of the fillets. These results are in accordance with the results reported for lipids from mackerel dark muscle where it was reported that this fraction contains three times as much α-tocopherol as the light muscle lipids (*74, 82, 108*). The fact was explained by the

authors due to the abundance of mitochondria in dark muscle. The amount of α-tocopherol present in the oil, 8.1 mg/100g lipids, is lower than the values reported for herring fish oil (*106*, *109*) but higher than values stated earlier (2.8 mg/100g lipids) (*59*). The isolated herring oil contained 10 times more α-tocopherol than the byproducts. The fact might be related to an extraction phenomenon, achieving higher efficiency of α-tocopherol recovery by the steam isolation process compared to the solvent extraction, as suggested elsewhere (*61*).

Trace elements (Fe and Cu)

As shown in Table 1, the iron and copper contents varied among the different fractions analyzed. Herring byproducts contained an almost four times higher iron content compared to the fillets, and concentrations of copper were higher (approximately 21%) in the byproducts than in the fillets. The higher iron content reflects the high levels of various hemoproteins (*74*, *82*) and low molecular weight iron (*193*) present in byproducts. With the exception of the oil fraction, iron concentrations were much higher than copper concentrations. The iron was mostly accumulated in the protein fraction, while the copper was more evenly distributed over the other fractions. The oil fraction had the lowest iron and copper content with values of 30 μg/kg and less than 0.1 mg/kg of wet weight, respectively. The values found are in accordance with values reported in the literature for herring oil (*106*) and used for acceptance levels of copper and iron in fish oil (*88*, *89*). Notevarp and Chahine, (*106*) showed that for capelin oil the oxidative stability had some tendency to correlate negatively with the copper content. In addition, Young (*89*) pointed out the importance of metals content, in particular copper, as a catalyst for the oxidation of oils and fats. Therefore, it can be suggested that our low oxidation levels (initial and developed over time) might be partly due to the low content of copper present in the crude herring oil, as already suggested earlier (*59*).

Fatty acid composition

Table 2 shows the fatty acid composition of the lipids from the three different stages of the production chain: herring fillets, byproducts, and the obtained oil.

Table 2- Fatty acid profile (g/kg lipid)[a] in total lipids from fillets, byproducts and oil produced from fresh herring (*Clupea harengus*) byproducts.

Fatty acids	Fillets	Byproducts	Oil
Saturated			
14:0	93 ± 7	84 ± 4	91 ± 1
16:0	137 ± 7	145 ± 4	133 ± 2
18:0	15 ± 1	18 ± 1	17 ± 1
Total	245 ± 15	247 ± 9	241 ± 4
Monounsaturated			
16:1	46 ± 4	43 ± 1	46 ± 1
18:1	81 ± 2	76 ± 1	74 ± 1
20:1	120 ± 1	117 ± 3	120 ± 0
22:1	214 ± 3	210 ± 6	215 ± 2
Total	461 ± 1	445 ± 10	456 ± 5
Polyunsaturated			
18:2	19 ± 6	18 ± 5	13 ± 3
18:3	15 ± 1	9 ± 7	15 ± 1
18:4	28 ± 2	27 ± 1	32 ± 0
20:5 (EPA)	53 ± 1	57 ± 3	58 ± 1
22:6 (DHA)	75 ± 1	83 ± 5	65 ± 1
Total	189 ±10	194 ± 21	183 ± 5

[a] Mean value ± SD, (n = 3, a = 1)

The studied fractions showed the following ranking order for the amount of saturated, monounsaturated and polyunsaturated fatty acids of their fat; monounsaturates > saturates > polyunsaturates. For monounsaturated, the total amount is very similar in the three fractions analyzed; C22:1 being the one present in more abundance. It is known that the feed of the herring is composed mainly of copepods (natural marine zooplankton) from relatively high latitudes, which are rich in this fatty acid (*11, 102, 103*). The total contents of polyunsaturated fatty acids in the lipids from fillets, byproducts, and in the fish oil fractions were identical. However, the values determined are lower than those reported previously (*59*). A reason might be that the herring used was caught during a different period of the year and from another catching area, as discussed elsewhere (*41*). The main contributors in this class were, as expected, the C20:5 (5, 8, 11, 14, 17-eicosapentaenoic acid) and C22:6 (4, 7, 10, 13, 16, 19-docosahexaenoic acid) commonly known as EPA and DHA, respectively. It is interesting to note that in the three fractions the content of DHA was higher than EPA. A lower DHA value was found for the oil comparatively to the byproducts. This is in disparity with our previous findings (*59*) on frozen herring

byproducts. Apparently, the oil produced from fresh byproducts is more susceptible to the loss of this fatty acid than the oil from frozen herring byproducts.

Oxidative Stability

FFA are more susceptible to oxidation than esterified fatty acids (*45, 110*); therefore, their concentration is considered to be an important quality parameter. Quality specifications for crude fish oil state that the FFA content should vary usually between 2-5% (*88*), but maximum acceptance values of 4.0% have also been suggested (*35*). The initial values of FFA were 0.78 ± 0.01% for the byproducts and 0.60 ± 0.01 % for the oil. The similar FFA levels indicated comparable hydrolysis in the herring byproducts and in the oil. The results (Figure 1) show that the amount of FFA present in the fresh herring oil was low (0.6%) and remained almost constant during storage time for the three different storage conditions.

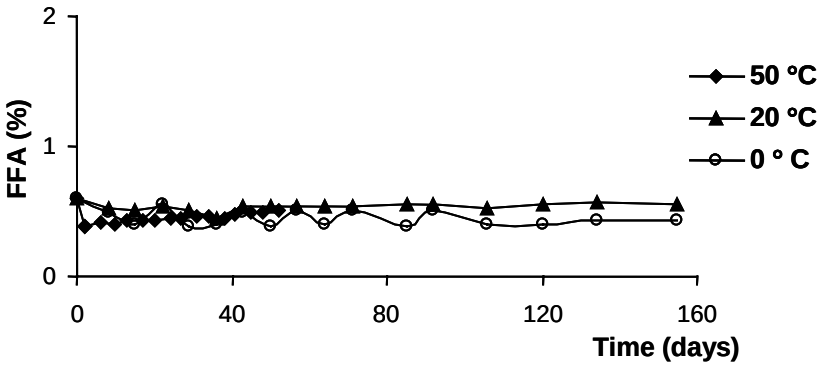


Figure 1- Free fatty acids of herring oil during storage.

In fact, no significant increase or decrease over time was revealed for the referred studied storage conditions (Table 3). This suggests that hydrolysis of the oil did not occur during the storage period, possibly because residual lipases and phospholipases were irreversibly denatured during the heating step at 95 °C. In crude capelin oil, Notevarp and Chahine (*106*) found a positive correlation between FFA and iron contents. Therefore, the low FFA content measured in the crude herring oil could also be due to the low iron content.

Table 3- Statistics describing the outcome of the linear regression analysis of the data obtained for herring oil stored at different temperatures (see also Figures). For all cases average values, obtained from duplicated analysis, were used. α and β represent the intercepts and slopes, respectively, with the correspondent confidence intervals; r^2 is the correlation and P the significance level found. Values given in bold represent significant differences.

Measure		0 °C (n = 17)	20 °C (n = 17)	50 °C (n = 16)
FFA	α	0.49 ±0.06	0.52 ± 0.03	0.44 ± 0.05
(Fig. 1)	β	-0.00057 ± 0.00075	0.00018 ± 0.00037	0.0008 ± 0.0017
	P	0.128	0.316	0.304
	r^2	0.148	0.067	0.075
PV	α	2.61 ± 0.77	1.59 ± 0.40	1.83 ± 0.56
(Fig. 2)	β	**0.0128 ± 0.0099**	**0.0474 ± 0.0052**	-0.0131 ± 0.018
	P	**0.0153**	**4.77exp-12**	0.141
	r^2	0.333	0.962	0.159
A 235 nm	α	33.41 ± 1.65	34.67 ± 0.82	33.45 ± 2.17
(CD)	β	0.0168 ± 0.0219	**0.0235 ± 0.0105**	0.0361 ± 0.0718
	P	0.122	**0.00025**	0.298
	r^2	0.174	0.602	0.077
A 270 nm	α	1.54 ± 0.11	1.44 ± 0.07	1.44 ± 0.12
(CT)	β	-0.0011 ± 0.0014	**0.0024 ± 0.0008**	**0.0099 ± 0.0039**
	P	0.132	**2.686exp.-5**	**0.000113**
	r^2	0.145	0.702	0.695
AV	α	0.53 ± 0.10	0.66 ± 0.26	0.98 ± 0.40
(Fig. 3)	β	**0.0025 ± 0.0013**	**0.0139 ± 0.0033**	**0.0305± 0.0129**
	P	**0.0011**	**2.209exp-7**	**0.0002**
	r^2	0.520	0.841	0.668
FC	α	26.56 ± 2.07	24.52 ± 1.714	25.40 ±2.59
	β	**-0.0299 ± 0.0265**	0.014 ± 0.022	0.0554 ± 0.0858
	P	**0.0294**	0.195	0.187
	r^2	0.278	0.109	0.1206
α-Tocop.	α	8.81 ± 0.75	8.84 ± 0.63	8.32 ± 0.75
	β	**-0.0099 ±0.0097**	**-0.0085 ± 0.0080**	0.0106 ±0.0255
	P	**0.0459**	**0.0388**	0.381
	r^2	0.240	0.254	0.065

As initial values for the oxidation parameters, the herring byproducts and crude oil presented PV of 0.90 ± 0.11 and 0.65 ± 0.17 meq. perox./kg lipids and AV of 2.6 ± 0.3 and 0.36 ± 0.06, respectively. The difference regarding AV found in the byproducts and in the oil may come from some oxidation during the lipid isolation process.

The time course of PV and AV during autoxidation of the herring oil stored at three different conditions is shown in Figures 2 and 3, respectively.

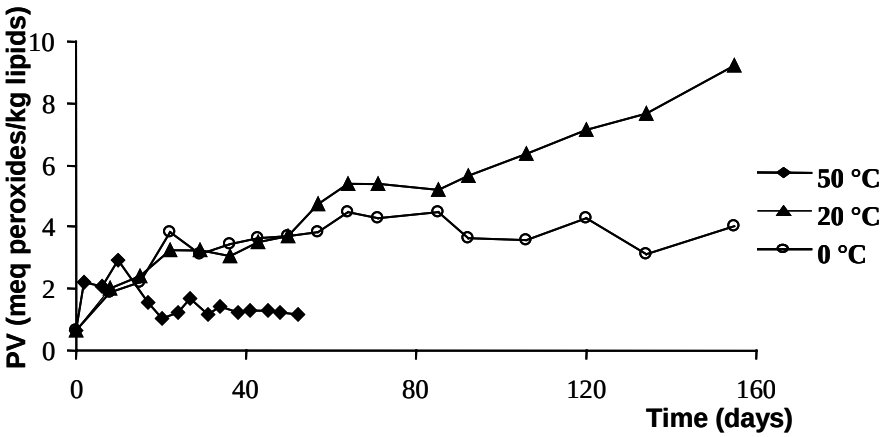


Figure 2- Peroxide value of herring oil during storage.

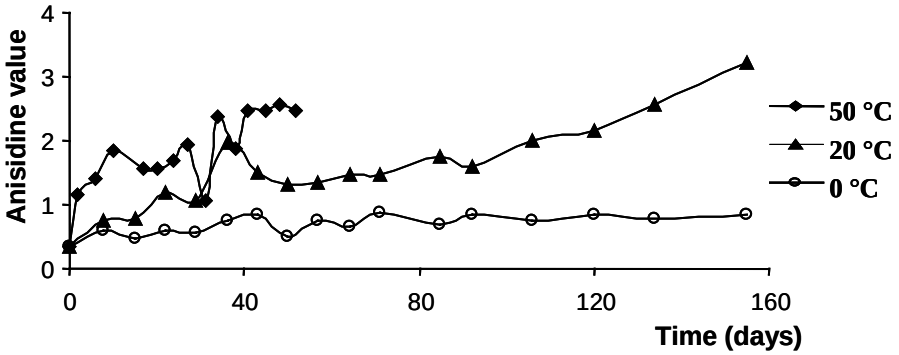


Figure 3- Anisidine value of herring oil during storage.

Influence of temperature on oxidative stability

Condition 0 °C

With increasing time of storage at 0 °C there was a significant increase in formation of primary oxidation products determined by the formation of hydroperoxides measured by the PV ($\beta = 0.0128$; $P = 0.015$). However, apparently, the higher hydroperoxide levels co-exist with the levels of conjugated dienes and trienes, since no significant changes were detected in the absorbance at 235 and 270 nm, respectively (Table 3).

Expectedly, the hydroperoxides formed in the first stage of oxidation were decomposed into secondary oxidation products, measured by AV. As a result, AV formation rate showed a positive slope developed over time ($\beta = 0.0025$). Although the correlation was not very high ($r^2 = 0.520$), the change was significant ($P = 0.0011$).

At 0 °C, the formation of secondary oxidation products was faster than the degradation; therefore, no significant amounts of tertiary oxidation products were formed. This is

confirmed with a significant, negative slope determined for the fluorescent compounds (FC) formed over time (β = - 0.0299; P = 0.0294).
During storage of the oil, at 0 °C, the α-tocopherol content decreased significantly, as shown in Table 3 (β = -0.0099; P = 0.0459), revealing that this antioxidant must have been consumed during the autoxidation process.

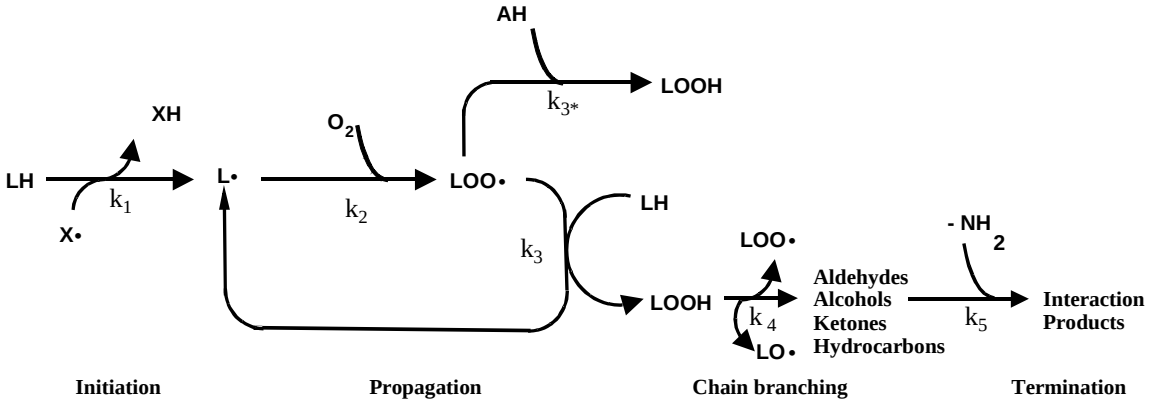
Condition ambient temperature (~ 20 °C)

In the oil kept at ambient temperature, hydroperoxides accumulated during storage (Figure 2). The increase was significant (β = 0.0474; P <<0.05) and with a high correlation (r^2 = 0.962), as it can be observed in Table 3. In contrast to 0 °C, a significant development of conjugated dienes and trienes was verified (β = 0.0235; P = 0.00025 and β = 0.00236; P << 0.05; respectively). The primary oxidation products, like at 0 °C, were decomposed to secondary oxidation products quantified by the AV (Figure 3). The development of these secondary products was significant and increased over time (β = 0.0139; P << 0.05). These secondary oxidation products did not continue further to the next step of the oxidation process since a non-significant development over time was found for the fluorescent compounds. Similarly to 0 °C, α-tocopherol was consumed significantly (β = - 0.0085; P = 0.0388).

Condition 50 °C

At this storage condition, it appeared that the rate of degradation of hydroperoxides in the oil was much higher than the formation; consequently, a negative, but non-significant, slope over time was determined. Apparently, the oxidative process also did not raise conjugated dienes (Table 3). These results suggested that the conjugated dienes, similarly to development of hydroperoxides, were immediately decomposed into secondary oxidation products. The formation of conjugated dienes during the oxidation of PUFAs (mainly EPA and DHA) can be attributed to the further oxidation of conjugated monohydroperoxides to yield conjugated triene products (*123*) which is confirmed by a significant increase of these compounds (β = 0.0099; P < 0.05). Besides, it has been reported (*126*, *194*) that at 270 nm, not only conjugated trienes, but also various bifunctional oxidation products, such as ethylenic diketones and oxodienes, are detected. As the lipid oxidation process progresses, the development of secondary oxidation products, measured as AV, was significant (β = 0.0305; P = < 0.05). The reaction from secondary to tertiary oxidation products clearly does not occur. Possibly, a termination reaction is favored above an interaction reaction, resulting in a non-significant increase of tertiary oxidation products.

The fact that the hydroperoxides are rapidly decomposed to secondary oxidation products could explain why α-tocopherol was not consumed (Table 3). It is known that α-tocopherol competes with lipid substrates (PUFA) in donating a hydrogen atom to the lipid peroxyl radical, thereby breaking the chain of reactions involved in lipid autoxidation (*91, 124*). This is shown in the following scheme:



The type of initiation reaction is the attack by any oxidizing species ($X^{\bullet}$) having sufficient energy to abstract a hydrogen atom from the fatty acids (LH) yielding an alkyl radical, $L^{\bullet}$. After an induction period, varying in length, the propagation step involving several possible reactions will drastically increase the reaction rate (*195*). The two propagation reactions (with rate constants k_2 and k_3) form the basis of the chain reaction process. The first part is the oxygenation reaction, namely the reaction between an alkyl radical and ground state oxygen, yielding a peroxyl radical, $LOO^{\bullet}$. At normal oxygen pressure, this reaction occurs very fast and its activation energy is almost zero and, consequently, the concentration of $L^{\bullet}$ is much smaller than that of $LOO^{\bullet}$. The rate-limiting step is the subsequent reaction between the peroxyl radical and a new fatty acid or antioxidant (AH) under formation of a hydroperoxide (LOOH) and an additional alkyl radical (*195*). The overall rates of reactions k_2 and k_3 are made equal by the adjustment of $[L^{\bullet}]$ and $[LOO^{\bullet}]$. It is in this way that the requirement for two independent reactions to have equal rates that the ratio of the concentrations of the radicals $L^{\bullet}$ and $LOO^{\bullet}$ are controlled (*195*). Another significance is that the rate of production of $LOO^{\bullet}$ is the same as the rate of formation of the product LOOH (*195*).

In the presence of an antioxidant, the rate of the formation of hydroperoxides is related to the ratio of lipid concentrations (LH) to that of the antioxidant (AH) concentrations (*91, 195*). This is observed at 0 and 20°C. At elevated temperatures, α-tocopherol loses its efficiency because of decomposition of hydroperoxides and by the production of a chain-carrying peroxyl radical (*91*). High temperature selectively lowers the activation energy

for the k_3 reaction more than the activation energy for the k_{3*} reaction. Furthermore, at elevated temperature, the oxygen concentration is limiting, and the rate of hydroperoxide formation is then related to the ratio of oxygen concentration to that of the antioxidant concentration (*91*, *195*, *196*). The relative lack of oxygen, since at higher temperatures the oxygen solubility is usually lower, would explain the non-consumption of α-tocopherol at 50 °C, favoring further steps on the oxidation process. The theory is corroborated with low PV and much higher values of AV and FC formed through k_4 and k_5, respectively (Table 3), than for the previous storage conditions.

This results from the temperature-dependence of the various autoxidation rates. Figure 4 shows the apparent accumulation rate for all the autoxidation measurements. All apparent rates increased upon increasing temperature, except for the PV accumulation rate. As lipid peroxidation proceeded as a function of temperature, more primary decomposition products were formed which is in line with the decrease of PV. Similar patterns were reported (*90*, *117*) concerning peanut butter and mackerel muscle, respectively. At 50 °C, there is extensive formation of PV, but the hydroperoxides do not accumulate due to their instability at high temperatures. Therefore a fast degradation into secondary oxidation products occurs. This is stressed by the increase of the apparent oxidation rates at 50 °C resulting in a rapid increase of FC.

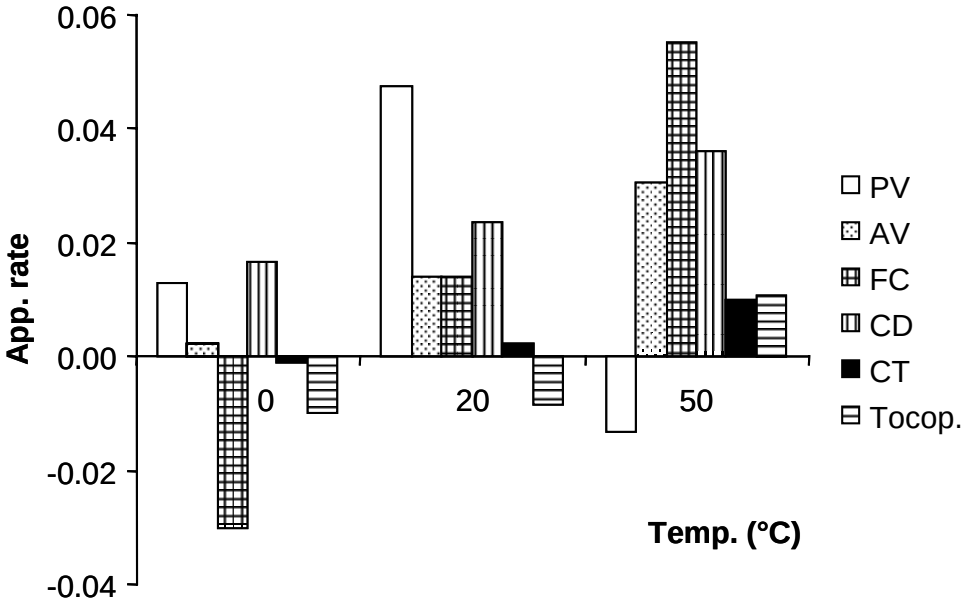


Figure 4- Apparent autoxidation rates of different oxidation measurements for crude herring oil as a function of temperature.

In conclusion, the PV alone is not necessarily a good indicator of the actual extent of lipid oxidation. At higher temperatures, in our case 50 °C, the hydroperoxides are broken down into different oxidation products and a considerable amount of secondary products was formed at an early stage of oxidation. Although it is known that oxidized fatty acids that contain a conjugated dienes system absorb ultraviolet light strongly at 235 nm, this was only observed at 20 °C. The presence of conjugated trienes was detected at 20 and 50 °C, their formation was to approximately four times faster at 50 °C than at 20 °C. The time course of AV during autoxidation of herring oil revealed that the oils were at the secondary oxidation stage. Contrary to reported results (*59, 93*) in this case, the FC was not a useful measure, since no significant changes were detected during storage. The determination of secondary oxidation products is suggested, and due to the simplicity and the useful information given, the measurement of conjugated trienes is also advisable. Loss of α-tocopherol can also be followed as a measure for oxidation progress at 0 and 20°C.

CONCLUSIONS

The present study shows that oxidation proceeds much faster at 50 than at 0 °C. At 20 °C the lipid hydroperoxides (k_2 and k_3) are formed 3.7 times faster than at 0 °C. The consumption of α-tocopherol is related to the development of peroxides during the autoxidation process, it is slightly consumed more rapidly at 0 than at 20 °C, however it is not consumed at all at 50 °C.

At 50 °C the formation of secondary oxidation products progressed 2.2 times faster than at room temperature and 12.4 faster than at 0 °C ($\beta = 0.0305$ vs. $\beta = 0.0139$ vs. $\beta = 0.00246$, respectively). FC was inhibited at 0 °C; however, it increased over time as a function of temperature, but the change was not significant. It seems obvious that the best temperature to keep the oil, as expected, is at 0 °C. However, cold storage will involve costs and also solidification of the oil. In spite of the conclusion that temperature has a strong effect on the preservation of the studied oil, it is also shown that the oil presents low levels of oxidation products over time with the applied conditions.

Acknowledgements

We thank Eelco Scherpenisse and Gerda Booy for their valuable help with part of the analytical work.

4.2 Volatile Oxidation Products Formed in Crude Herring Oil under Accelerated Oxidative Conditions

ABSTRACT

Crude fish oil produced from herring byproducts was evaluated on its oxidative stability by a number of complementary methods. The oil was placed in a closed vessel, exposed to pure oxygen, and kept at 50 °C for up to 53 hours. Six volatile compounds contributing to off-flavor in the oil were followed over time. Simultaneously, conventional oxidative chemical measurements were carried out. The results showed that, apart from 4-(*Z*)-heptenal, all the other components increased with time. The rate of formation of fluorescent compounds, being only time dependent, showed to be the simplest model to describe the progress of oxidation, in opposition to the development of hexanal. Development of 1-penten-3-one correlated well with peroxide and anisidine values ($r = 0.938$ and $r = 0.931$, respectively). Correlations between the volatiles were also found. Within the applied conditions 1-penten-3-one and 2, 4-(*E, E*)-heptadienal could be useful as volatiles indicators for the oxidative status of the oil.

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INTRODUCTION

Fish oil contains long chain n-3 polyunsaturated fatty acids (PUFAs) that are considered to have a positive effect on several human diseases (*21, 24, 26, 115, 197*). The perspective of using n-3-PUFAs as beneficial ingredients in industrially prepared foods presents an opportunity to the fish oil industry and the food industry in general. Moreover, upgrading herring byproducts into oil would increase the revenue in the fish processing industry and at the same time reduce their waste problems. Previous results have shown that it is indeed possible to produce a stable crude oil from the herring byproducts (*41, 59-61*).

It is generally accepted that fish oil, rich in n-3-PUFAs, is prone to oxidation. The oxidative deterioration of lipids involves the formation of peroxides from unsaturated fatty acids, and this process of autoxidation in turn leads to the development of various unpleasant flavors and odors generally classified as rancid (*90, 198*). Primary oxidation products, although tasteless and odorless, will act as precursors of flavor compounds. The decomposition products, from the primary oxidation products, are directly related to the flavor compounds affecting quality (*199*). The degree of oxidation should be monitored by more than one method and by measuring different types of products, including primary and decomposition products of lipid oxidation (*199*). Concentration of primary oxidation products can be estimated by determining the peroxide value or by the level of conjugated dienes. Volatile decomposition products of oxidation can be measured by gas chromatography. In fish oil a number of potent odorants have been suggested to have a strong effect on fishy flavors. The precursors of most of these are derived from the n-3 fatty acid family (*200, 201*). The formation of secondary oxidation products such as volatile aldehydes and ketones is responsible for other sensory changes. Volatile components have been characterized in fish oil (*201-203*), in fish itself (*204, 205*) and in other food systems as fish oil enriched mayonnaise (*183, 206, 207*). Some headspace volatiles as hexanal and pentanal (*201, 208*) have been commonly used to measure the extent of lipid oxidation since these off-odor and off-flavor components have very low threshold values. 1-Penten-3-one (pungent, green odor), 4-(*Z*)–heptenal (fishy odor), 2, 4-(*E, E*)-heptadienal and 2, 6-(*E, Z*)-nonadienal (cucumber odor) are volatiles that have been characterized as very potent odorants, contributing to the unpleasant rancid and fishy off-flavor in bulk fish oil, fish meat and fish oil-enriched mayonnaise (*207, 209-211*). Hexanal (with a pungent, green and grassy odor) and nonanal (green plant and compost-like), are oxidation products from the n-6 PUFAs family mostly present in vegetable oils. These volatiles have been included in the present study since they are considered

important contributors of metallic, green, and rancid off-flavors in fish oil and in mayonnaise based on fish oil only (*207, 212*).

The overall purpose of this exploratory work was to identify suitable volatile oxidation indicators in crude oil produced from herring byproducts. Our previous findings (*59-61*) have shown that the crude herring oil, when kept at room temperature, was quite stable over time, presenting low values of the traditional chemical measurements like peroxide and anisidine value. These unexpected results led us to investigate whether volatiles might be a more sensitive measure of oxidation. Therefore, our aim with this study was to correlate both chemical and volatile changes in fish oil oxidation. The techniques employed included determination of concentration of lipid hydroperoxides (by peroxide value, PV, and conjugated dienes, CD), measurements of concentration of secondary oxidation products (by anisidine value, AV, conjugated trienes and tetraenes and volatiles by dynamic headspace GC-MS) as well as concentration of tertiary oxidation products following the development of fluorescent compounds (FC). The formation of six volatiles (1-penten-3-one, hexanal, 4-(*Z*)-heptenal, 2,4-(*E, E*)-heptadienal, nonanal, and 2,6-(*E, Z*)-nonadienal), responsible for off-odors, was followed using oxidative isothermal conditions. The correlations between the measurements were tested.

MATERIALS AND METHODS

Fish oil

Crude herring oil was produced from fresh herring byproducts, processed in our pilot plant, using the same system and conditions as described earlier (*59*). The major fatty acids were C14:0 (9.4%), C16:0 (10.9%), C16:1 (4.4%), C18:1 (6.5%), C20:1(13.1%), C22:1 (21.7%), C18:4 (4.1%), C20:5 (EPA, 7.2%), and C22:6 (DHA, 6.3%), as determined by capillary gas chromatography (GC) with flame ionization detection of the methyl esters prepared by transesterification using BF_3-methanol according to AOCS (*66*) Official Method Cd-8b-90 and using the conditions described earlier (*59*). Other analytical data were as follows: iron, 0.4 mg/kg; copper, 0.071 mg/kg; α-tocopherol, 3.8 mg/100g.

Rapeseed oil (low-erucic acid variety), used for dilution of the oxidized fish oil in headspace sampling procedure, was supplied from Aarhus oil, Aarhus, Denmark with a PV < 0.3 meq perox/kg, FFA < 0.1% and being practically free of volatiles.

Standards

The standards used for quantification of volatiles were: 1-penten-3-one (97%), 2,4-(*E, E*)-heptadienal (90%), nonanal (95%), and 2,6-(*E, Z*)-nonadienal (95%) from Aldrich-Chemie (Steinheim, Germany), hexanal (98%) from Riedel-de Haen AG (Germany) and 4-(*Z*)-heptenal was from TCI Tokyo in Japan.

Oxidation

40 g of fish oil were placed in each of six individual (100 ml) reaction vessels, covered with Teflon® sealed plastic lids, connected by tubing to the pressure-transducers of the Oxidograph®

(Mikrolab, Aarhus, Denmark). The reaction vessels were flushed with 2.5 liters/min of O_2 for 30 sec, placed in a thermostat-controlled aluminum block at 50°C, and tightened after 15 min. The oil was continuously stirred during the reaction period. Samples for determination of volatiles and of traditional oxidation parameters were collected through a 53 hours period. Analyses of the initial, unoxidized oil were used as time = 0-hour data. Two series of measurements were carried out: 1st series: Analyses based on 20 g samples taken from the reaction vessels (sampling after 1, 3, 7, 13, 30, and 45 hours). 2nd series: After removal of the samples for the first series, the reaction vessels now containing 20 g of oil were again covered with the lid, sealed, flushed with 2.5 liters/min of O_2 for 30 sec, tightened after 15 min and kept at 50 °C until 2nd sampling was performed (after 2, 5, 10, 21, 37 and 53 hours). It may be assumed that the headspace of the vessels consisted of pure oxygen. The volume ratio oxygen/oil was calculated to be 1.3 and 3.5 for 1st and 2nd sampling, respectively. The two-series set-up was chosen in order to double the number of data points obtainable in one Oxidograph-run, for assessment of corresponding chemical and volatile oxidation parameters.

Dynamic Headspace Sampling (DHS)- 200 mg of sample was weighed into a pear-shaped glass flask equipped with a purge head and mixed with 3.8 g of rapeseed oil, which previously had been found practically free of volatiles. The dilution was required, as the use of undiluted raw fish oil would result in an overload of volatiles. The volatiles from the oil were collected using a DHS system as described elsewhere (*205*). Briefly, volatiles were stripped from the oil by a stream of nitrogen carrying the volatiles to an adsorbent trap. The outlet from the bottle head was connected by a stainless steel fitting and Teflon ferrule to the adsorbent Tenax-GR trap. Three parallel samples were purged with nitrogen (99.995%, 150 ml/min, Hydrogas, Fredericia, Denmark) for 30 min at 75 °C for trapping of the volatiles. The traps contained 225 mg of Tenax-GR (Chrompack, Bergen op Zoom, The Netherlands), placed in ¼ -inch steel tubes (Perkin-Elmer, Buckinghamshire, U.K.). Blank runs were periodically carried out during the study.

Thermal Desorption

A Perkin-Elmer (Norwalk, CN) ATD-400 automatic thermal desorber with a Tenax TA-packed cold trap was used for thermally desorbing the collected volatiles. The gas flow from the trap to the transfer line to the capillary column in the gas chromatograph (GC) was split in the ratio of 5.0 ml min^{-1}/ 1.3 ml min^{-1}.

GC-MS- The transfer line of the ATD was connected to a Hewlett-Packard (Palo Alto, CA) 5890 IIA gas chromatograph equipped with a HP 5972 A mass-selective detector. A DB 1701 column (30 m x 0.25 mm x 1.0μm, J&W Scientific, Folsom, CA) with a flow of 1.3 ml of helium/min and the following temperature program was used: 35 °C for 3 min, 35 to 140 °C at 3°C/min, 140 to 170 °C at 5 °C/min, 170 to 240 °C at 10 °C/min and finally hold at 240 °C for 8 min. The GC-MS transfer line temperature was kept at 280°C. The ionization energy of the mass spectrometer was set at 70 eV in EI mode and the detector operated with a mass range of 30-250 amu (atomic mass units) with a repetition rate at 2.2 scans/s and a threshold of 50.

Quantification

Headspace sampling of standards. For quantification purposes five aldehydes and one ketone (C5-C9) were accurately weighed and dissolved in a stock solution of 10 g of rapeseed oil. Five sets of concentrations were prepared with a range of values from 125 to 2000 ng/g. Quantification of the amounts collected on the trap was done using calibration curves for known amounts of each volatile compound. Results from the collections of standards were used to prepare a calibration curve for each standard compound, using the HP ChemStation software (version G1701AA). Compounds were verified on the basis of two or three qualifier ions and the

chromatographic retention time and identified by MS library (NIST) or by RI of literature and molecular external reference compounds. All collections were made in triplicate.

Limit of detection. Detection limits were determined as 3x noise (*213*). The noise was determined from six GC-MS analyses on blank tube and the magnitude of baseline fluctuation was determined over a period of 1 min. The "distance" (expressed in abundance units) between minimum and maximum in this period was defined as baseline noise. The response calculated as 3x noise was converted to concentration by use of the calibration curve prepared for each standard compound. The values found were 0.06; 0.12; 0.18; 0.22; 0.58 and 11.1 ng/g for 1-penten-3-one; 2,4-(*E, E*)-heptadienal; hexanal; 4-(*Z*)-heptenal; 2,6-(*E, Z*)-nonadienal and nonanal, respectively.

Peroxide Value. The peroxide value (PV) of the samples was determined according to the official AOCS (*66*) method Cd-8b-90 (n = 3, a = 1). The content is expressed in terms of meq of peroxides per kg of lipid.

Anisidine Value. The anisidine value (AV) of the oil was carried out according to AOCS (*66*) Official Method Cd 18-90 (n = 3, a = 1).

Absorbance at 235nm (A_{235}), 270 nm (A_{270}), and 300 nm (A_{300}). A_{235}, A_{270}, and A_{300} nm of the oil samples were measured using flow injection analysis (FIA) as described by Undeland et al. (*98*) (n = 3, a = 1). Results were expressed as peak area units per nanogram of lipid. The RSD% were 2.0, 4.3, and 7.0 (n = 1, a = 6) for A_{235}, A_{270}, A_{300} respectively.

Fluorescence Compounds (FC). Total lipid soluble fluorescent lipid oxidation products with an excitation (ex) maximum at 367 nm and an emission (em) maximum at 420 nm were measured in the oil samples using FIA as described by Undeland et al. (*98*) (n = 3, a = 1). Results were expressed as peak area units per nanogram of lipid. The repeatability was 7.0% (n = 1, a = 6).

Statistical analysis

The data was subjected to the ANOVA univariate method ($P < 0.05$) using SPSS software (version 10.0). The relation of oxygen injected (sampling: 1^{st}, 2^{nd}) and the sequence of the triplicates (sample: a, b, c) in the oils was also subjected to an ANOVA test. For comparison, the reaction time was placed as a covariate factor, while sampling and analysis' order were considered as fixed factors. General Linear Models (GLM) were tested for the stored oil data in order to describe whether there was a significant effect of the reaction time, sampling (influence of oxygen present) or sample's order. The residuals were tested for normality using probability plots followed by one sample Kolmogorov-Smirnov test. Pearson's correlation coefficient (r) was calculated in order to study the correlation between various measures. The data were also analyzed by ANOVA Partial Least Squares (APLS) regression using the Unscrambler version 7.6 software program (CAMO, Oslo, Norway). The design variables time (T0, T1,...T53) and samplings (sampling 1 and sampling 2) were used as X-data and the measured variables (FC, A235, A270, A300, PV, AV and the volatiles as well as the time in hours) were used as Y-data. All variables were standardized by 1/standard deviation and full cross validation was used. By using the jack knifing facility in the Unscrambler program it was possible to calculate whether regression coefficients between the design variables and the measured variables were significant (*214*), and obtain a correlation loadings plot from the APLS analysis. In this plot the inner and outer ellipses indicate 50 and 100 % explained variance, respectively. Variables located close to each other and near the outer ellipse are thus positively correlated with a correlation coefficient of ~1.0. The level of significance for all tests was set at $p = 0.05$.

RESULTS AND DISCUSSION

In the first part of this section, the oxidative stability of the crude herring oil is presented. In the second part, the development of oxidation products in the oil and the correlation between the various oxidation products are discussed. The data are related to the change in oxidation products and the selected volatiles while the oil was submitted to accelerated oxidation conditions.

Oxidative stability of the oil

Figure 1 shows the TIC chromatograms from the volatile profile of the initial herring oil, after 45 and after 53 hours under oxygen at 50 °C. The total volatile content as well as the number of volatiles developed over time showed the progressive deterioration in the herring oil. These changes and the volatile compounds responsible for them were evaluated during an accelerated oxidation process. The oil initially had a considerable amount of volatile compounds that are known to be responsible for the original flavor. Comparison of Figure 1A and B showed that new volatiles (breakdown products of lipid hydroperoxides) had been formed in addition to those that were already present in the initial sample. The increase in total volatiles and in the concentration of some specific volatile compounds such as pentanal and hexanal also indicated that the oxidation process in the oil had progressed further. The development of pentanal (retention time of 11.28 min) was conspicuous. Although this volatile was not selected for quantification in this study, its abundance was estimated to increase almost five times compared with the original value. This supports previous findings that for fish oil, which is rich in n-3 fatty acids, pentanal is a useful indicator of oxidation (*201*).

The time courses of the chemical oxidation parameters and the analyzed volatile compounds, during autoxidation of the herring oil, are shown in Figure 2. Increasing concentrations of all compounds during reaction time were found, apart from 4-(*Z*)–heptenal. In our study, 4-(*Z*)-heptenal was below the detection limit of the GC-MS analysis and therefore is not shown. This compound, derived from n-3 PUFAs, possesses a fishy and sweet odor (*207*) and it is known that with a very low odor threshold level (0.010 mg/kg of oil) it can be responsible for sensory changes (*200*).

It was detected in fish oil (*203, 209*), fish oil enriched mayonnaise (*207*) but was found to be absent after frozen storage of salmon (*204*). The absence of 4-(*Z*)-heptenal during the oxidation process suggested that retro-aldol degradation from the conversion of 2, 4-(E, Z)-nonadienal to yield this volatile, as proposed elsewhere (*215*), did not occur. On the basis of PV, AV and 1-penten-3-one determinations (Figure 2), it can also be easily

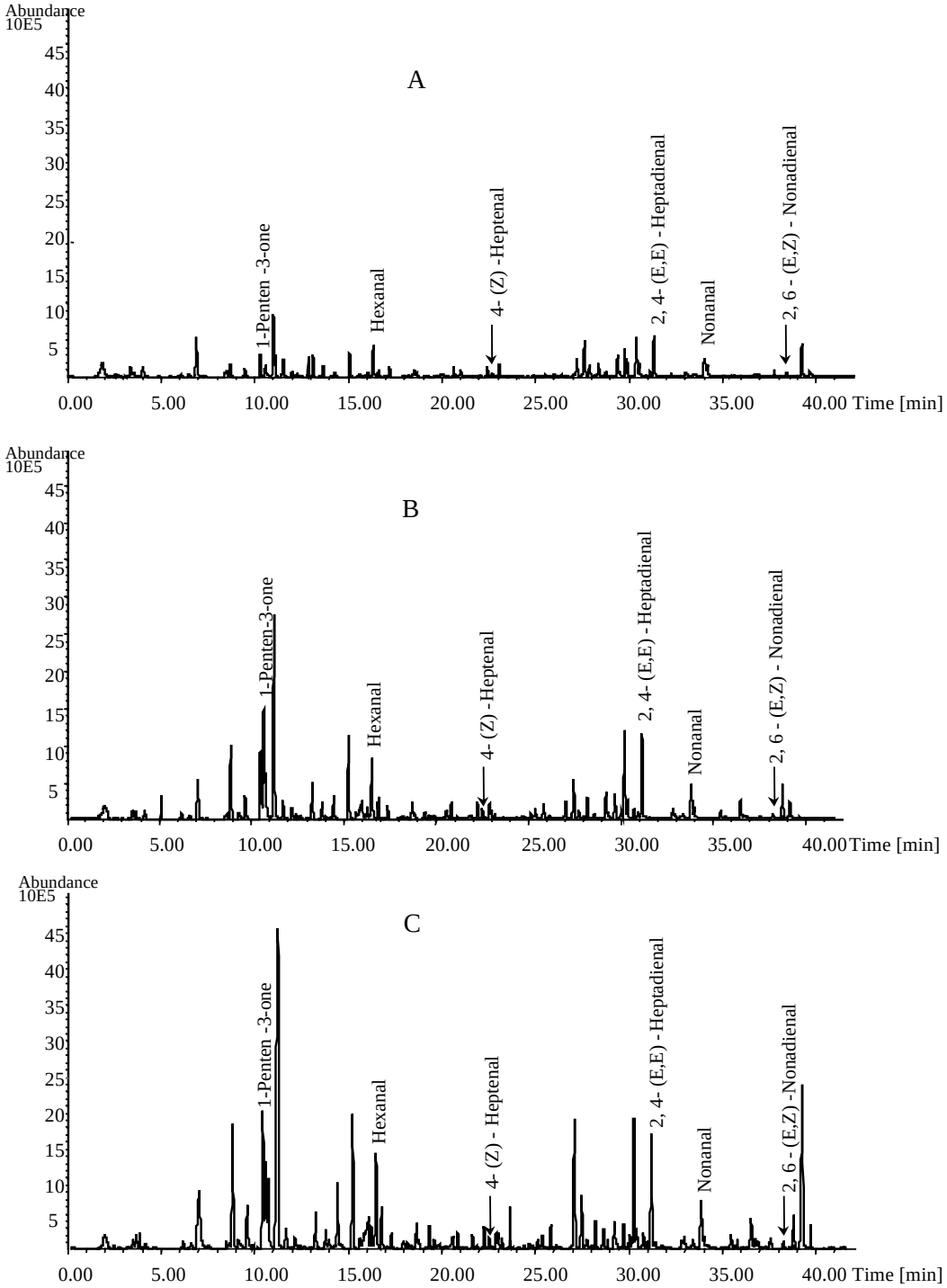

Figure 1- TIC (total ion current) chromatograms (0 – 42 min) for volatiles of crude fish oil produced from herring byproducts: (A) initial oil; (B) oil after 45 hours of accelerated oxidation at 50 °C in oxygen atmosphere (closed vessel with a volume oxygen/oil ratio of 1.3); (C) oil after 53 hours of oxidation at 50 °C after renewed oxygen supply (closed vessel with a volume oxygen/oil ratio of 3.5).

perceived that herring oil oxidized more rapidly with a higher ratio of oxygen to fish oil (volume ratio 3.5 vs. the initial ratio of 1.3, respectively 2nd and 1st sampling).
The results for conjugated dienes, trienes and tetraenes will be discussed in the following section.

Development of oxidation products

General linear models were fitted to all the obtained data under the assumption that no induction period was present. The first step in the statistical analysis was to check whether the data are normally distributed using probability plots followed by one sample Kolmogorov-Smirnov test. Only the 1-penten-3-one data required a square root transformation in order to obtain normally distributed data.
To evaluate whether time, first or second sampling of herring oil (oxygen effect), and analysis (samples in triplicate) had an effect on the oxidative changes all the data was subjected to a Least Squares Estimates (LSE) test. For each measurement the following parameters in the model were estimated:

$$Y_{(measurement)} = \beta_0 + \beta_1 t + \beta_{2,S} + \beta_{3,\tau} + \beta_{4,t*\tau} + \beta_{5,t*S}$$

In this equation, t (covariate) is the reaction time in hours, S (class variable) is the effect of the sampling time, and τ analysis (class variable) is the sequence order that samples were collected and analyzed. t*S and t*τ represents the interaction term of the two class variables on the measurements studied. The estimation of the constant β_0 is given as the value determined for the intercept.
The fitted models were all highly significant ($P << 0.05$) with exception of the model for the 4-(*Z*)-heptenal measurement. No significant intercepts were found for the FC and for hexanal indicating the absence of such compounds from the initial oil.
In the next step of the statistical analysis, non-significant effects were omitted step-wise. With this, new models were derived (Table 1). This technique provided the possibility to compare the overall rates at which the different oxidation products were produced. As can be seen in this table, initial values were estimated indicating that the initial intercept values differed from zero and were always positive, except for FC and hexanal. Hence, tertiary oxidation products (measured as FC) were apparently not present in the initial oil. Hexanal is formed mainly from the n-6 fatty acids family, present primarily in vegetable oils. This volatile, almost absent from the initial fish oil, is formed by degradation of 2,4-decadienal (*202, 208, 216*) apparently after an induction period of approximately 10 hours (Figure 2).
Among the studied volatiles, nonanal presented the highest initial concentration (~ 21 ng).

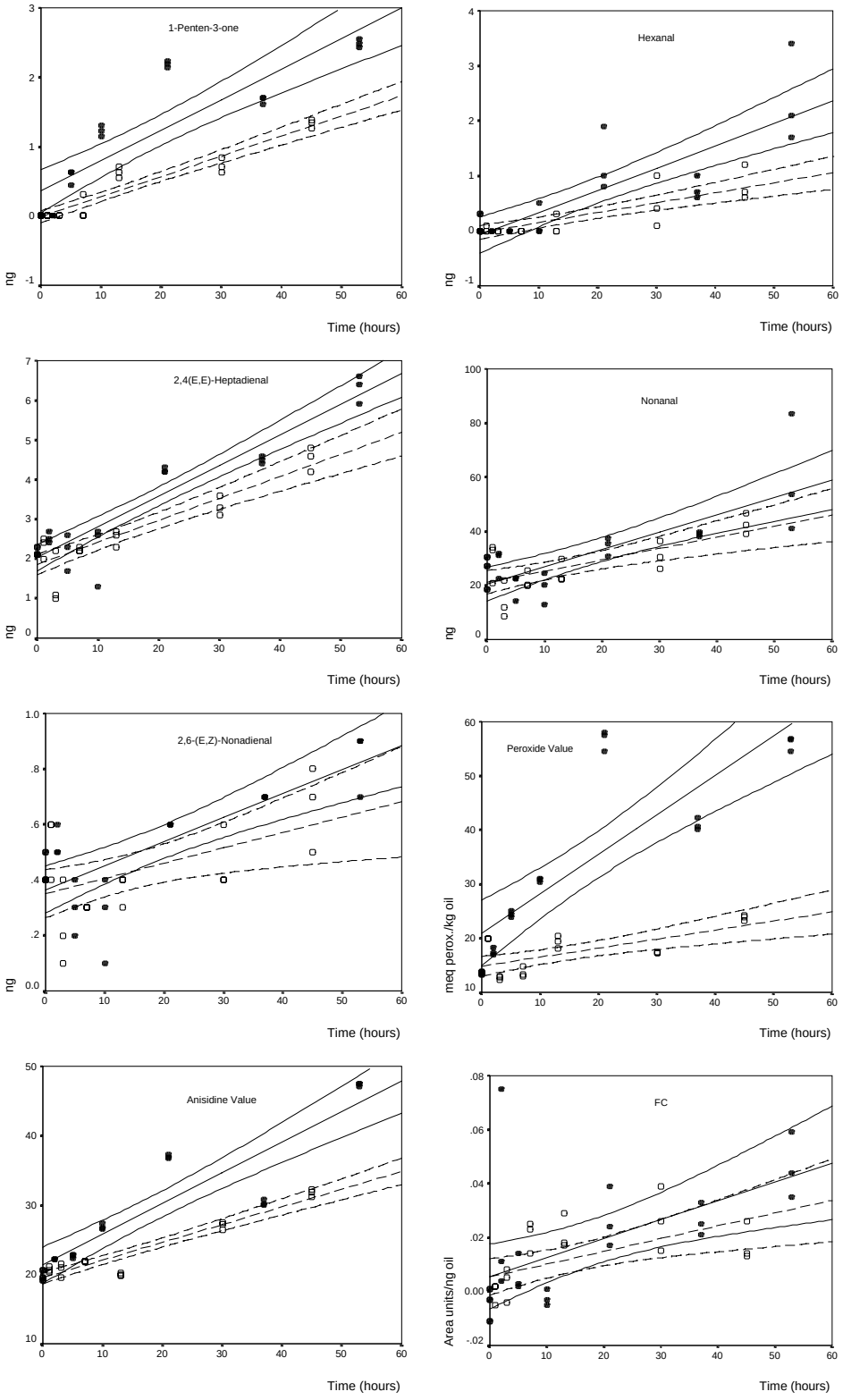

Figure 2- Linear regression lines, of the different lipid oxidation measurements, illustrating the change in (--- and ▫) = oil sampled after first oxygen exposure, and (— and ▪) = oil after a second oxygen exposure. Models describing these lines are shown in Table 1. The confidence bands are based on p = 0.05.

The progress of oxidation during the oxidative treatment is seen by the increase of all measured parameters of primary, secondary and tertiary oxidation products. The largest influence of time was found for the PV, followed by nonanal and AV measurements (β_1 = 0.728, β_1 = 0.554, and β_1 = 0.443, respectively) and the smallest for the formation of FC (β_1 = 0.00062).

The sampling order (Table 1) was found to be significant for PV and 1-penten-3-one measurements. The oil with only one oxygen injection presented negative estimates signals in time for these two parameters (β_2 = -6.142, and β_2 = -0.372, respectively). These negative values showed that the samples taken after exposure of oil only to the first amount of oxygen (1st sampling) presented lower hydroperoxides and volatile values than the samples taken after additional exposure to a new supply of oxygen (2nd sampling). It is known that the oxygen concentration can influence the oxidation pathways, and can lead to different oxidation products (*200*).

The obtained results illustrate the instability of the hydroperoxides concerning the effect of oxygen, showing that these compounds are rapidly decomposed to further oxidation products in the presence of oxygen, and in this way leading to the formation of different compounds such as ketones and aldehydes. The lack of sampling effect for the other analyzed volatiles indicates that the breakdown of peroxides led to formation of other secondary oxidation products in addition to those pursued in this study.

The interaction effect between sampling (1, 2) and reaction time in the oil was shown to have a significant effect for the PV, AV and 1-penten-3-one, hexanal and 2, 4-(*E, E*)-heptadienal (β_4 = -0.561, β_4 = -0.187, β_4 = -0.0149, β_4 = -0.0213, β_4 = -0.0269, respectively). In all cases, the oil from the second sampling, i.e. after exposure to a new amount of oxygen and with a high oxygen/oil ratio, presented a higher value than the first sampling oil. A negative interaction effect between analysis (sample's order) and time (hours) was found only for the development of hexanal (β_4 = -0.079), showing that the last sample analyzed presented significantly higher values than the two previous ones. For the other measurements no significant differences were found.

Table 1– Statistics describing the outcome of the parameter estimates:
$Y_{(measurement)} = \beta_0 + \beta_1 t + \beta_{2,S} + \beta_{3,T*t} + \beta_{4,S*t}$ and the confidence intervals by least squares estimates for the fish oil data. PV is expressed as meq. perox./kg of lipid; A_235, A_270, A_300 nm and FC as area units/ng of lipids; all volatiles are expressed in ng. Abbreviations used are as follows: Heptadienal to 2, 4-(*E, E*)-heptadienal and Nonadienal to 2, 6-(*E, Z*)-nonadienal.

	β_0	β_1 **t**	$\beta_{2,}$ **mpling**		$\beta_{3,}$ **sample* hours**			$\beta_{4,}$ **Sampling*hours**	
		hours	1	2 [a]	a	b	c [a]	ampling 1* hours	Sampling 2* hours [a]
PV	20.98 ± 2.15	0.728 ± 0.082	-6.142 ± 2.97	0	-	-	-	-0.561 ± 0.127	0
A_235 nm	36.46 ± 0.816	0.093 ± 0.034	-	-	-	-	-	-	-
A_270 nm	4.015 ± 0.324	0.0406 ± 0.014	-	-	-	-	-	-	-
A_300 nm	0.419 ± 0.019	0.0035 ± 0.001	-	-	-	-	-	-	-
AV	21.33 ± 0.938	0.443 ± 0.036	-	-	-	-	-	-0.187 ± 0.055	0
FC	-	0.00062 ± 0.0002	-	-	-	-	-	-	-
1-Penten-3-one	0.359 ± 0.110	0.044 ± 0.004	-0.372 ± 0.152	0	-	-	-	-0.0149 ± 0.006	0
Hexanal	-	0.0523 ± 0.005	-	-	-0.079 ± 0.162	-0.127 ± 0.162	0	-0.0213 ± 0.004	0
Heptadienal	1.946 ± 0.101	0.0797 ± 0.005	-	-	-	-	-	-0.0269 ± 0.006	0
Nonanal	20.66 ± 1.82	0.554 ± 0.077	-	-	-	-	-	-	-
Nonadienal	0.354 ± 0.030	0.00756 ± 0.001	-	-	-	-	-	-	-

[a] This parameter is set to zero because it is redundant to the model.
– No significant effect found.
[b] Data square root transformed prior to analysis.

Correlation

Table 2 describes the correlation coefficient between the different analytical parameters followed during the oxidation of crude herring oil. As can be seen, in all cases positive and significant correlations were found. Within the chemical tests used to monitor lipid oxidation, PV correlated with AV (r = 0.892). This is expected since, as the lipid oxidation process progresses, the hydroperoxides are broken-down into secondary oxidation products that can be measured with AV. These two measurements also correlated quite well with 1-penten-3-one (r = 0.938 and r = 0.931, respectively). The low correlation values between the other selected volatiles data and PV is not surprising since lipid peroxides are not themselves responsible for off-flavor formation as previously shown (*202*, *217*). It is known that determination of PV by titration provides an empirical measure of lipid oxidation that is less sensitive and precise than sensory and headspace methods for measuring volatiles (*199*). AV was shown to correlate also with hexanal and

Table 2- Correlation as described by Pearsons correlation coefficient (r) between the different measures used to follow quality changes in herring oil during oxidograph experiment. N = 42. Abbreviations used are as follows: Pent to 1-penten-3-one; Hepta to 2, 4-(*E, E*)-heptadienal and Nonadi to 2, 6-(*E, Z*)-nonadienal.

Measure	A_{235}	A_{270}	A_{300}	FC	PV	AV	Pent [a]	Hexanal	Hepta	Nonanal	Nonadi
A_{235}		0.485	0.769	0.341	0.542	0.492	0.537	0.481	0.461	0.416	0.377
A_{270}			0.520	0.764	0.518	0.587	0.506	0.517	0.514	0.400	0.364
A_{300}				0.529	0.392	0.426	0.507	0.414	0.523	0.442	0.402
FC					0.473	0.575	0.502	0.469	0.601	0.459	0.455
PV						0.892	0.938	0.786	0.783	0.601	0.598
AV							0.931	0.898	0.912	0.748	0.692
Pent [a]								0.822	0.862	0.657	0.610
Hexanal									0.868	0.873	0.719
Hepta										0.870	0.881
Nonanal											0.858
Nonadi											

Correlation is significant at the 0.05 level (two-tailed).
[a] Data square root transformed prior to analysis.

2, 4-(*E, E*)-heptadienal (r = 0.898 and r = 0.912, respectively). The findings that AV did not correlate well with all the selected volatiles may indicate that the sensitivity and

specificity of the AV measurement is too low to correlate with the changes in concentration of volatiles responsible for the off-flavor determination, as previously suggested (*217*). Low correlation values were found between the conjugated dienes, trienes, tetraenes and FC content. The amounts of conjugated dienes formed in the oxidation of PUFAs (mainly from EPA and DHA) can be attributed to the further oxidation of conjugated monohydroperoxides to yield conjugated trienes and tetraenes products (*123*). This is confirmed by an increase in the concentrations of these compounds. However, none of these compounds correlated well with the studied volatiles. This might be due to a low specificity of the measurements as well as to interference of different oxidation compounds (*126, 194*).

Correlations were also found between the volatiles. After a square root transformation, 1-penten-3-one correlated best with hexanal and 2, 4-(*E, E*)-heptadienal (r = 0.822 and r = 0.862, respectively). 1-Penten-3-one has been identified as a product of lipid oxidation in fish oil (*202, 203, 209*) and it has been suggested to contribute to unpleasant off-flavors described as sharp-fishy in fish oil (*200*) or pungent, rancid green and glue in fish oil-enriched mayonnaise (*207*). Hexanal also showed a relatively good correlation with 2, 4-(*E, E*)-heptadienal and nonanal (r = 0.868 and r = 0.873, respectively). In fish oil enriched mayonnaise, the increased fishy and rancid off-flavors developments seemed to correlate to high concentrations of nonanal and 2,4-(*E, E*)-heptadienal (*218, 219*). On the other hand, the development of 2, 4-(*E, E*)-heptadienal showed also a correlation with the development of nonanal and 2, 6-(*E, Z*)-nonadienal (r = 0.870 and r = 0.881, respectively). The compounds 2, 4-(*E, E*)-heptadienal and 2,4–(*E, E*)-decadienal have been identified as off-flavors from pure fish oil (*220*), and apparently they play a significant role in the development of fishy and rancid off-flavors in fish-oil-enriched mayonnaise (*218*). The development of 2, 6-(*E, Z*)-nonadienal correlated better with the development of nonanal (r = 0.858) than with the other volatiles. The n-3 PUFAs derived potent odorants, as 4-(*Z*)-heptenal, and 2, 6-(*E, Z*)-nonadienal have been associated with fishy off-flavors in oxidized fish oil (*209*) and in related fish oil products (*207*).

In order to facilitate clear comparisons and relationships between the data obtained, APLS analysis was also performed (Figure 3). Two principal components (PC) were validated, explaining 25% and 65% of the variation in the X- and Y-data, respectively. The scores plot of the data is not shown since the location of the various objects corresponds to the location of the various design variables for time (T0-T53).

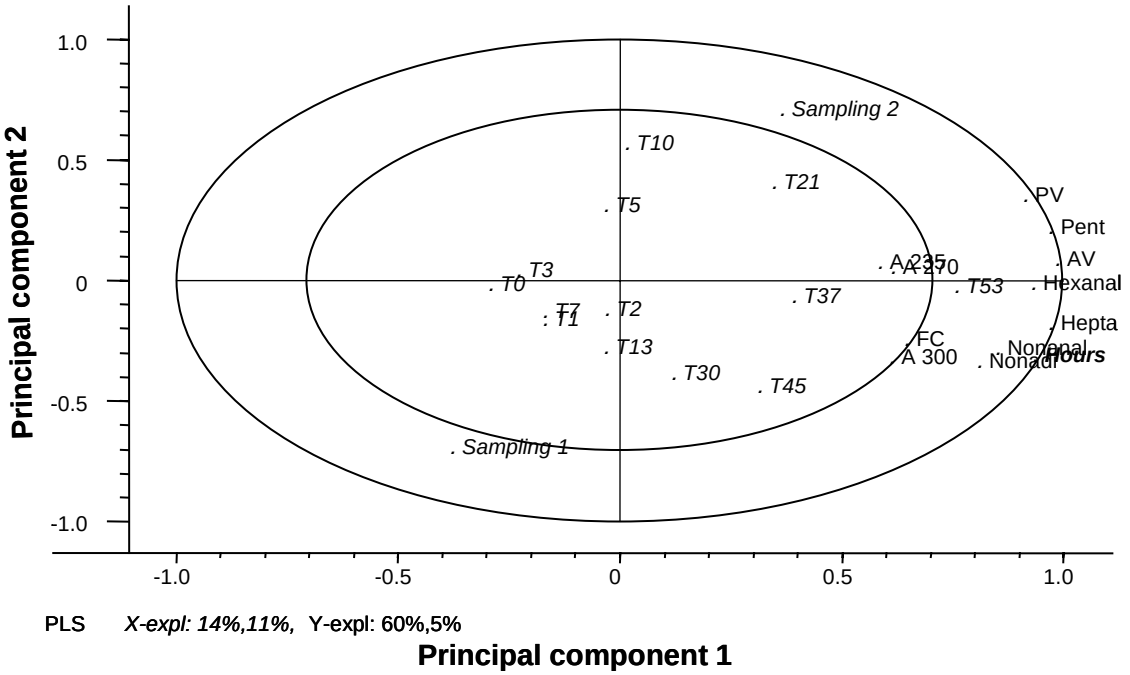

Figure 3- Correlation loading plot from the APLS analysis on the studied volatiles and chemical measurements of the oxidized crude oil. The designed variables time (T0-T53) and sampling (sampling 1 and sampling 2) were used as X-data and all the measured variables were used as Y-data. The inner and outer ellipses indicate 50 and 100 % explained variance, respectively. Volatiles' abbreviations used were the same as in Table 2.

In the correlation loadings plot (Figure 3), the design variables indicating oxidation time (T0-T53) moved from left to right with increasing oxidation time. The design variable for sampling 1 was located in the lower left corner, while the design variable for sampling 2 was located diagonally in the upper right corner. Hence, the time effect was mainly explained by PC1 while the sampling effect was explained by both PC1 and PC2. All the measured variables were located to the right. FC, A235, A270, and A300 were located within the inner ellipse, which indicates that these variables were not well explained by the model. PV, AV, hours and the volatiles were all located far to the right, indicating that these variables were well explained by the model. PV, AV and 1-penten-3-one were located close to each other. Hence, these variables seemed to correlate with each other to a high degree, which is also in accordance with the Pearson's correlation coefficients (Table 2). Furthermore, especially PV and 1-penten-3-one were located near the design variable sampling 2. This indicates a particular strong effect of the sampling on these two parameters, which is also in accordance with the data in Table 1. However, also AV and hexanal were located relatively close to the sampling 2 design variable. This means that the sampling also influenced these parameters. The design variable T53 and the measured variable "hours" were both located far to the right. Hence, all the measured variables

correlated strongly with oxidation time. This was also confirmed by the significantly positive regression coefficients ($p < 0.05$) that were found between T53 and all the measured variables.

CONCLUSIONS

As lipids oxidize, they form hydroperoxides, which are susceptible to further oxidation and to further decomposition into products such as aldehydes, ketones, etc., compounds that adversely affect flavor, taste, nutritional value and overall quality (*221*). Therefore the control, quantification, and prediction of oxidation are important issues from both scientific and economic points of view. This work provides an extension of the volatile data available for fish oil and also confirmed that pentanal was developed substantially over time. In contrast, 4-(*Z*)–heptenal was not detected. The model that described the rate of formation of FC, was the simplest model to describe the progress of oxidation as it was only time dependent. However, since FC are tertiary oxidation products developed after the break-down of secondary oxidation products, which are responsible for changes in odor and taste, and also because low correlation values were found for the secondary oxidation products, other measurements should be followed in the earlier stage of the oxidation process. Our results suggested that, within the studied oxidative system, 1-penten-3-one could be a useful volatile to be followed, due to its good correlation with PV and AV, thus giving a good indication of the hydroperoxides formed and decomposed. Furthermore, 1-penten-3-one concentrations correlated well to hexanal concentrations. Also 2, 4-(*E, E*)-heptadienal could be targeted because of the low threshold value and its association to fishy and rancid off-flavors. Moreover, this volatile also had shown a good correlation to nonanal, 2, 6-(*E, Z*)-nonadienal and hexanal. Thus, together, 1-penten-3-one and 2, 4-(*E, E*)-heptadienal are good indicators for the oxidative status in fish oil. The combined odor-contributing volatiles may be used as a degradation index in crude fish oil obtained from herring byproducts and may provide a basis for future research.

Chapter 5

General Discussion

Abstract

A compilation of the major conclusions of the study concerning the upgrading of herring byproducts into fish oil is given. In general, it can be stated that herring byproducts resulted in oil that it is rich in PUFAs and with initial low levels of oxidation products. The crude oil retained low oxidation levels when stored. It was concluded that the decrease of α-tocopherol content as well as the level of FFA present were good parameters to monitor the progress of lipid oxidation in crude herring oil.

Relatively low levels of chemical contaminants such as PCBs and dioxins were found in the oil. The oils extracted from herring caught in the English Channel and in the North Sea presented the highest level of contaminants. Crude and refined oil processed from herring byproducts behave similarly to the commercially available menhaden oil, extracted from the whole fish.

On the basis of the results, suggestions for future research were given. The results of this study may help to produce good quality fish oil using herring byproducts, contributing to a better sustainability of the marine resources.

Introduction

To unravel the possibilities of using herring byproducts as raw material for fish oil production, certain pertinent questions needed to be answered. For example, it was necessary to characterize and to perform storage stability trials of the oil extracted from herring byproducts, and to study whether it was possible to reach a constant composition of the oils when it is known that the lipid content and composition of herring change seasonally. Other issues were to compare the composition and storage stability from the oil obtained from different sorted herring byproducts to the oil extracted from mixed byproducts as well as to find out the relationship between the freshness of the byproducts and the quality of the extracted oil. Another factor of interest was to check whether oil could also be extracted from salted herring byproducts. In this study, high quality crude oil was defined as oil that is rich in polyunsaturated fatty acids (PUFAs) and with low levels of oxidation products. Ideally, the level of oxidation should remain low, in order to have a good storage stability.

The work presented in this thesis aimed at finding out the answers to those questions. As explained in the introduction of this thesis, crude fish oil was extracted from herring byproducts with a thermal processing method and separated from the fish tissues by mechanical means. During the investigation other questions rose, such as what is the best parameter to monitor lipid oxidation and which technique to choose for data interpretation. It also became necessary to answer questions about how to influence the PUFAs content, about the level of pollutants in the oil and about the feasibility of an industrial process. In this chapter attention is given to these questions.

A further objective of this study was to relate the quality of the extracted crude oil to the quality of the starting raw material, herring byproducts. This was done by assessing the influence of the starting raw material quality, by processing the raw material into fish oil and by evaluating the product quality.

This chapter integrates results of the previous chapters. First, conclusions are drawn that summarize the results of the chapters 2-4 and on the use of statistical data analysis methods. Second, the overall conclusions concerning the lipid analysis methodology are described. Next, recommendations concerning the maximization of the ω-3 PUFAs content and toxic residues in fish oil are assessed. Thereafter, the scope for commercialization is discussed. Finally, recommendations for future research are given.

Compilation of conclusions

The obtained results described in chapters 2-4 can be summarized as follows:
In **Chapter 2** the influence of the raw material quality is described. The main conclusions drawn were:

- The fatty acid composition in the byproducts is relatively independent of the catch season. However, during summer they are found to be somewhat richer in PUFAs (with a level of ~ 23%). Thus the herring byproducts from the entire year appear to be an adequate raw material for fish oil production.
- Salted byproducts of the maatjes herring industry were found to be a suitable raw material source for the production of fish oil. However, the oil obtained from maatjes byproducts, presented a much higher content of iron and copper ions than the other types of studied oils (from unsalted frozen and fresh byproducts). It is known that metals, by catalyzing the decomposition of hydroperoxides, may produce misleading low peroxide value (PV), even though the rancidity increases and the sensory quality decreases (*222*). This was indeed observed for the maatjes oil. This oil, with very low PV, presented the fastest oxidative odor development. It was therefore concluded that maatjes oil was less stable than the other oils, which could be attributed to the presence of these metals.
- Byproducts generated from different herring processing yielded oils with differing quality. Oil extracted from fresh and unsalted herring byproducts has the lowest levels of oxidation products and consequently possesses less unpleasant sensorial attributes. It was also shown that an increase of free fatty acids (FFA) levels was directly related to a deterioration of the odor, as evident in odor notes as fishy, train, marine and acidic in the oil.

The influence of handling of the byproducts on oil quality is given in **Chapter 3**. In this chapter the major conclusions were:

- A crude oil richer in EPA (eicosapentaenoic acid), DHA (docosahexaenoic acid) and the total PUFAs (polyunsaturated fatty acids) can be produced from byproducts that are stored longer than 8 hours compared to oil from fresh byproducts. In the oil from stored byproducts, low levels of oxidation products were found; only the FFA level increased significantly over time. In the byproducts material, it was found significant increases of the levels of total volatile bases (TVB), histamine, putrescine, cadaverine, and tyramine. The microbial count was high but constant, indicating that a maximum level had already been reached even without extra storage time. Furthermore, by determining the putrescine content of the herring

byproducts it is possible to predict the value of FFA present in the produced crude oil, which turn is related to the total oil quality. However, since the FFA determination is easier and simpler than any biogenic amine determination it may be more convenient to measure the level of FFA directly in the byproducts.

- The oil extracted from herring heads is of a lower quality. It yielded the higher oxidation values, lower PUFAs and lower α-tocopherol levels compared to the oil extracted from headless or mixed byproducts. However, oils recovered from mixed and headless byproducts are of similar initial quality and storage stability. Therefore, it seems that sorting of herring byproducts does not result in oils of strongly different qualities and it is therefore not necessary.
- The main variables that influence the oil quality in the extraction process of fish oil from herring byproducts are the rotational speed of the decanter and the throughput of the pump. The best quality oil was obtained while producing oil with the shortest residence time, i.e. at maximum pump speed (80 Hz), and at intermediate decanter speed (47 Hz). The anisidine value (AV) and FFA contents were negatively correlated with processing time until a processing time of two hours was reached. The yields of the three fractions (water, protein and oil) did not depend on the settings.

The product quality was evaluated in **Chapter 4**. It was concluded that:

- Storage of the produce oil can lead to loss in quality, due to adverse chemical changes. Crude herring oil, extracted from fresh byproducts, was stored at 0, 20 and 50 °C in order to study the effect of temperature on lipid oxidation. The level of oxidation products over time is clearly dependent on temperature. Increasing the storage temperature accelerates not only the propagation reactions, but also the peroxide decomposition. This results in a negative influence on the apparent autoxidation rate. As the concentration of peroxides increases over time, a steady state concentration of hydroperoxides is reached. The results showed that, during storage, the oil reached the secondary oxidation stage at all three temperatures. It was shown that the best temperature to keep the oil is 0 °C. However, cold storage will involve economical implications and solidification of the oil. Since the oil still presents low levels of oxidation products over time when stored at 20 °C, this storage temperature is therefore deemed acceptable as well.
- In crude herring oil, pentanal, 1-penten-3-one and 2, 4-(*E, E*)-heptadienal were volatiles that developed substantially over time. The presence of 1-penten-3-one correlated well with the traditional PV and AV measurements. Therefore, pentanal,

1-penten-3-one and 2, 4-(*E*, *E*)-heptadienal together are good indicators for the oxidative status in fish oil and could be used as a degradation index.

As with many processes involving agri-feedstocks, the quality parameters used in this study shown extensive variation. Therefore, the collection of experimental data that is statistically significant is not trivial. The usefulness of an appropriate experimental design to plan and conduct experiments in order to extract the maximum amount of information from a limited number of experimental runs is of great and indisputable value (*188, 223*). This type of multivariate design is more effective than designs in which one variable at the time is changed. It also offers the possibility to make of use previous data when performing a new experiment. Experimental design was used successfully for the optimization of the oil extraction process employed, as described in chapter 3.3.
The application of multivariate data analysis to multivariable processes, where several variables interact, allows the investigation of many variables simultaneously and at the same time reveals their relationship (*224*). An example of multivariate data analysis is the principal component analysis (PCA) that can be applied to large quantities of data providing overviews with useful visualization and summarization of the data, as used in chapters 2.3 and 3.1. Clusters and trends in data are more easily recognized as well as outliers detected (*225*). A useful interpolation method is the partial least squares method (PLS), which allows prediction of responses from the independent variables (*226, 227*), as was used in chapters 2.3 and 4.2. Several other statistical methods were used in this thesis allowing to draw conclusions, to optimize systems and even to find correlation among variables or between variables and responses.

The overall practical conclusion of this research is that it is possible to upgrade byproducts from herring processing industry into crude fish oil, which fulfils the requirements of stability after recovery and during storage at room temperature and in an inert atmosphere.

Mechanism and experimental indicators of lipid oxidation in crude herring oil

Lipid oxidation is the degradation process responsible for the development of objectionable odors and flavors and therefore has great impact on the oil quality (*90, 200, 228*). The autoxidation mechanisms are traditionally categorized into the initiation, propagation and termination reactions (*229-231*), as shown in Figure 1. Oxidation can occur not only from oxygen present in the headspace above the oil but also from air that is dissolved in the oil itself.

Initiation

$RH + O_2 \longrightarrow R\cdot + RO_2\cdot + OH\cdot + H_2O$

Propagation reactions

$R\cdot + O_2 \longrightarrow RO_2\cdot$

$RO_2\cdot + RH \longrightarrow ROOH + R\cdot$

$ROOH \longrightarrow RO\cdot + OH\cdot$ (Homolytic β-scission)

$2\ ROOH \longrightarrow ROO\cdot + RO\cdot + H_2O$

$RO\cdot + RH \longrightarrow ROH + R\cdot$

$\cdot OH + RH \longrightarrow H_2O + R\cdot$

Termination reactions

$2R\cdot \longrightarrow RR$

$R\cdot + RO_2 \longrightarrow ROOR$

$RO_2\cdot + RO_2\cdot \longrightarrow ROOR + O_2$

$RO_2 + R\cdot \longrightarrow ROR$

Figure 1- Classical free radical autoxidation (*230*), where RH = fatty acid, R· = fatty acid radical, $RO_2\cdot$ = peroxy radical, ROOH = fatty acid hydroperoxide, RO· = alkoxy radical.

Several methods to assess the extent of oxidation have been reviewed extensively (*91, 199*). To monitor oxidative changes, all methods rely upon the determination of the concentration of specific oxidation products in the oil or food containing lipids (*91, 222, 232, 233*). However, which measurement is most suited, depends on the matrix involved. For a reliable prediction of the shelf life of oils containing PUFAs it is essential to use more than one method to determine lipid oxidation (*222*). At 20 °C, α-tocopherol loss, the PV, and the levels of conjugated dienes (CD), conjugated trienes (CT), and AV were found to be good quality parameters to follow the oxidation progress in crude oil extracted from herring byproducts. Most of these measurements are related to the detection of hydroperoxides. It is known that hydroperoxides do not themselves contribute significantly to the flavors and odors of oxidized fish oil. In most cases, the organoleptically detectable components appear to have low molecular weights; they are formed by the decomposition of the peroxides. A large number of saturated and unsaturated aldehydes, ketones, acids, and other products has been isolated from oxidized fish oil, and have been shown to contribute to the development of undesirable flavors and odors (*201-203*). The highly unsaturated fatty acids of fish oil are not only more susceptible to oxidation, but they also develop highly objectionable odors and flavors at even low levels of oxidation (*200*). Determination of the level of fluorescent compounds (FC) can be used as a method to evaluate the progress of lipid oxidation in oil originated from low graded byproducts, e.g. salted byproducts or byproducts originated from frozen

herring, as described in chapter 2.2 and 2.3. The development of CT is also a suitable measurement for oil extracted from unsalted byproducts originated from frozen herring. The FC measurement from the organic phase, as performed in this study, was not a good measurement to evaluate oxidative stability of the stored byproducts, as shown in chapter 3.1. Apparently, as lipid and protein damage increases with storage, FC become progressively more soluble in the water phase resulting in a decreasing value from the lipid phase. FC seems to be more suitable as a quality measurement of the oil when it is produced from fresh (non-stored) byproducts.

In chapters 2.2, 2.3 and 4.1, it was shown that the crude herring oil kept at room temperature was relatively stable over time, resulting in low values of the traditional chemical measurements (PV, AV, CD, CT, and FC). Other research showed that analysis of volatiles by GC is, as expected, closely related to flavor evaluation and is therefore the most suitable method for comparison with the results of sensory panel (*222*). Since, ultimately, the volatiles are generated by the oxidation process, became of interest to correlate both chemical and volatile changes in fish oil oxidation. The results showed that 1-penten-3-one could be a useful volatile to be followed, due to its good correlation with PV and AV, thus giving a good indication of the amount of hydroperoxides formed and decomposed.

In this thesis, a relationship has been established between the sensory perception of off-odors during oil deterioration and the levels of FFA in the oils (Figure 4, chapter 2.3). A good correlation was also found between FFA measurements and lipid oxidation products (measured as AV) as shown in Figure 1, chapter 2.3. This is in accordance with the suggested influence of lipid hydrolysis on the lipid oxidation process (*123*). It is known that FFA arise from the hydrolysis of triacylglycerols in the presence of water and heat (*192*). The amount of FFA in oil samples is used for evaluation purposes (*234*); the higher the FFA content, the lower the commercial value is, due to the acceleration of oxidative deterioration and the interference with processing (e.g. in the hydrogenation step).

The oils that were extracted during this study were crude oils, i.e., they still need to be refined. All oxidation compounds may be removed during refining. Procedures employed to refine the oils differ among manufactures but are essentially designed to remove, besides the oxidation products and FFA, pigments, odors, and flavors from the crude oil in order to enhance its usage and stability. However, during the refining, particularly at the bleaching and deodorization steps, further oxidative stress on the oil might be induced and the levels of natural antioxidants such as α-tocopherol may decrease considerably (*234-236*).

Tocopherols perform the important function of protecting the oil from oxidation. For this reason, they may be classified as highly desirable constituents of most oil and fat products. According to Kinsella (*109*), α-tocopherol is the major tocopherol present in marine oils. Kulas and Ackman (*237*) studied the influence of α-tocopherol on the rate of formation of hydroperoxides in menhaden oil. Crude oil contains significant levels of α-tocopherol whereas refined oil contains considerably less. In the purified oil, formation of hydroperoxides was very rapid with no apparent induction period (*237, 238*), but the non-purified menhaden oil exhibited an induction period of about four days (*237*). As in menhaden oil, crude herring oil contains significant levels of α-tocopherol. Also in this type of oil, the antioxidant suppresses the levels of peroxides, as shown in chapter 3.2 and 4.1, but it does not improve the odor stability, as evident on chapter 2.3, Figure 4. When the crude oil is kept at room temperature, under an inert atmosphere, oxidation of the oil progresses slowly, partly due to the protective role of the α-tocopherol. To make optimal use of this effect, the refining step should be as late as possible on the production chain, just prior to incorporation in a final product, as discussed in chapter 3.2 and 4.1. If necessary, some antioxidant may be added to protect the oil further (*237, 238*).

Optimization of the ω-3 PUFAs yield

As the importance of PUFAs in human nutrition and disease prevention is becoming clear, production of ω-3 fatty acid is also becoming a topic of interest (*187*). Methods for the isolation of ω-3 PUFAs from e.g. fish oils are numerous including adsorption chromatography (*239*), fractional or molecular distillation, enzymatic splitting (*189*), low-temperature crystallization, supercritical fluid extraction and urea complexation (*186*). Each technique has its own advantages and drawbacks (*187*).

Due to its relatively high content of EPA and DHA, herring oil may be a good raw material for production of ω-3 fatty acid concentrates. Marine oils could be used as such or as modified triacylglycerols and as FFA or as their alkyl esters (*240*). Industrially it is attractive to start with a richer raw material, e.g. fish oil richer in EPA and DHA content. It was shown in chapter 2.1 that herring oil richer in PUFAs could be obtained when extracted from herring caught during the summer months.

A further option, as emerged from the results related to the storage of the byproducts (chapter 3.1), is to allow fermentation to take place. After tissue degradation, obtained in byproducts stored for a period longer than eight hours, significantly higher values of EPA, DHA and consequently a larger yield of PUFAs were obtained in the extracted oil. Possibly, the higher degree of tissue disruption promotes recovery of higher levels of

DHA and EPA. Moreover, although one might expect an effect of the fermentation on the quality of the oil, the produced oils still showed low oxidation values, although the FFA levels increased significantly over time. Even though the sensory quality attributes may be lower, the herring oil richer in PUFAs content is produced from byproducts stored for longer periods at 15 °C.

Levels of chemical contaminants in crude herring oil

Due to increased ocean pollution, fish oils may contain highly undesirable and toxic contaminants such as organochlorine pesticides, polychlorinated biphenyls, dioxins, toxaphene and other persistent organic pollutants. Fish accumulate these contaminants, especially in the liver. Therefore, levels are likely to be higher in fish oil compared to vegetable oils and attention should be drawn to this problem. Important examples of undesirable constituents in fish oil are dioxins and PCBs.

Dioxins are polychlorinated, planar aromatic compounds with similar structures, chemical and physical properties formed as residues of chemical processes. The term dioxins covers a group of 75 polychlorinated difenzo-p-dioxin (PCDD) and 135 polychlorinated difenzofuran (PCDF) congeners, 17 of which are of toxicological concern, 2, 3, 7,8-tetrachlordibenzo-p-dioxin (TCDD) is the most toxic congener and is classified as human carcinogenic (*241*, *242*). Dioxins originate from natural events such as volcano eruptions and forest fires but mostly from industry, in the production of chemicals, pesticides, steel, paints, paper bleaching, through exhaust emissions and during incineration. Dioxins are strongly lipophilic compounds that can be bound to sediment and organic matter in the environment and are absorbed in animal and human fatty tissues. In addition, dioxins are not biodegradable and consequently once released to the environment, via air or water, they are persistent and tend to accumulate in the food chain.

Polychlorinated biphenyls (PCBs) are chlorinated aromatic hydrocarbons synthesized by direct chlorination of biphenyls. PCBs are a group of 209 different congeners, which can be divided into two groups according to toxicological properties. Only 12 congeners exhibit toxicological properties similar to dioxins and therefore are often termed “dioxin-like PCBs” (*241*, *242*).

According to the Scientific Committee on Animal Nutrition (SCAN), which deals with dioxin and PCB contamination of animal feed, the European fish oil and fish meal are the most heavily dioxin contaminated feed materials. Therefore their use in feed for farmed fish, or other food-producing animals, let alone in human consumption, raises concern.

Following SCAN’s recommendation, comparable and reliable data on dioxins and PCBs contamination in our fish oils was collected. For the general human population, the major

pathway of exposure to dioxins and PCBs is food. Therefore, specific maximum legislative limits for dioxins (dioxins and furans, not yet for dioxin-like PCBs) of 2 pg TEQ/g fat have been defined for fish oil intended for direct human consumption (*242*). In animal nutrition, legal maximum contents of 6 and 2,25 pg TEQ/g fat have been defined for fish oil and feed for fish/pet foods, respectively (*241*). Table 1 shows the results of PCBs and dioxins in three crude herring oil samples taken at random from our studies. A more detailed list is given in Appendix 1. The results show that crude oil samples extracted from herring byproducts were above the limit for human consumption. However, it should be remarked that normally crude oil is not used for human consumption and the level should become lower by the refining process. For application as fish oil for fish/pet foods the June and July results (5,0 and 4,7 pg TEQ, respectively) were below the limit of 6 pg TEQ/g fat. Due to the migratory nature of herring it is hard to conclude whether these higher values detected in October compared to June and July are caused by herring being caught in a more polluted area (different geographic location), or by varying levels of fat tissue in the fish. It is known that the Channel and North Sea regions are heavily polluted areas. Previous studies (*243*) have shown that herring caught in these regions contained levels up to 73 pg of PCDD/Fs, while herring from the Atlantic Ocean presented much lower levels (1.1 pg of PCDD/Fs). The PCBs levels followed the same tendency. Hence, most likely the geographical origin is an important determinant for concentrations of dioxins and PCBs in the oils.

Table 1: Results of polychlorinated contaminant analysis present in fish oil samples. Values are given in pg TEQ/g oil. TEQ is expressed as TCDD **t**oxicity **eq**uivalent concentration for the upper-bound concentration. Please, refer to chapter 2.2, Figure 1 for exact location of the catching area.

Sample	Catching area	PCDD/Fs	PCBs	Total-TEQ
June	60.50N 02.50W	4.7 [1]	4.4	9.1
July	60.40N 02.75W	5.0 [1]	3.7	8.7
October	51.20N 02.60E	9.7 [1,2]	7.9	17.6

[1] Above the allowable levels for human consumption (2 pg TEQ/g fat).
[2] Above the limit for application as fish oil for fish/pet foods (6 pg TEQ/g fat).

A reduction of the contaminant levels can be achieved by using less contaminated raw material, or by removal of the contamination. Research has shown that the levels of organochlorine contaminants and PCBs decreased only during the deodorization step, which follows the neutralization and bleaching of the refining process (*244*). In order to remove these contaminants from the oil, steam distillation, at 180 °C for two hours was

used during the deodorization step by Hilbert et al. (*244*). It is likely that such severe treatment may negatively influence the oil quality. Hence it is advisable to find alternatives. Apparently, these undesirable components can also be removed during the refining process by adsorption on specific lypophilic adsorbents, e.g. active carbon (*245*). For future utilization of the herring oil, pre-treatment with active carbon or other future developments on the refinery methods is recommended. Such treatment could lower the dioxins and PCBs concentrations to acceptable levels, fulfilling the legislative requirements without affecting the general quality characteristics of the oil.

Preliminary comparison between herring oil and commercially available oil [1]

Our studies show that valuable oil can be extracted from the byproducts from herring production. To evaluate the commercial attractiveness of this type of byproducts valorization, a study comparing the initial composition and stability of the oil extracted from herring byproducts relative to fish oil commercially available (menhaden oil) was performed. As can be observed in Table 2, menhaden oil is richer in PUFAs content than herring oil. This is due to a direct influence of the diet, since the former fish species is mainly caught at the American and Canadian West coast, areas richer in ω-3 plankton than the North Sea, the natural habitat of the herring used in this study.

These two crude oils have also been refined. Figure 2 summarizes the major refining steps of fish oil processing and the type of impurities removed during each step. Each processing step has a specific function and may affect the quality of the resultant oil by removing certain major and minor compounds such as proteins, FFA, pro-oxidants and/or antioxidants. The fish oil refining process comprised phospholipids removal during the degumming stage and FFA removal during the neutralization stage. The elimination of other material, that may have an effect on the stability of the refined oil, was achieved during the soda silicate boil stage. Residual soaps and pigments were removed during the bleaching stage and finally all the off-flavors and odors were excluded during the deodorization stage. All the steps were carried out under a nitrogen atmosphere. More details concerning the refining stage are given in Appendix 2.

[1] This part of the study was performed in collaboration with Unilever Research, Vlaardingen, The Netherlands. I would like to acknowledge Dr. Ron Potman, Otto Rosier and Dr. Frans-Jos Jansen.

Table 2- Fatty acid composition (w/w %) of commercial crude menhaden oil and herring oil extracted from herring (*Clupea harengus*) byproducts.

Fatty acids	Menhaden	Herring
Saturated		
14:0	8.6	9.4
16:0	22.4	10.9
18:0	4.3	1.0
Total	35.3	21.3
Monounsaturated		
16:1	9.7	4.4
18:1	13.3	7.5
20:1	0.7	13.1
22:1	0.2	21.7
Total	23.9	46.7
Polyunsaturated		
18:2	1.6	1.4
18:3	1.4	2.5
18:4	1.6	4.0
20:4	2.2	0.4
20:5(EPA)	12.9	7.2
22:5	2.3	0.7
22:6 (DHA)	7.2	6.3
Total	29.2	22.5

The values do not add up to 100 because minor fatty acids are not reported.

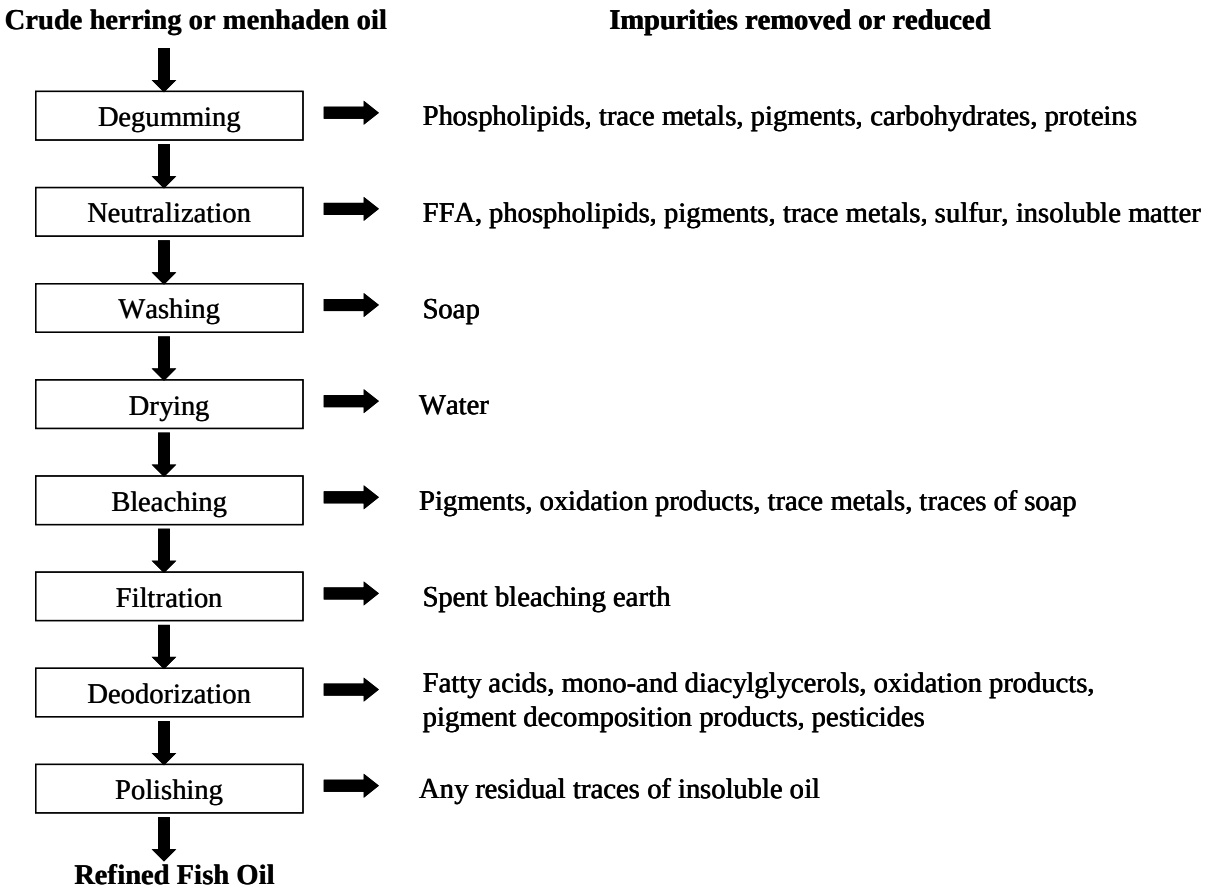

Figure 2- Scheme for the refining stages of fish oils and the major impurities removed.

Table 3 shows initial crude and refined characterization of the oils.

Table 3- Characterization of crude and refined menhaden and herring oil. Nt stands for not tested.

	Crude		**Refined**	
	Menhaden	Herring	Menhaden	Herring
PV (mmol O_2/2kg)	4.7	2.6	0.2	0.2
AV	17.8	6.9	Nt	Nt
Cu (mg/kg)	0.27	0.07	< 0.001	0.002
Fe (mg/kg)	4.7	0.4	< 0.01	0.02
FFA (%)	2.9	5.6	0.2	0.1
Taste	Nt	Nt	Bland	Bland

Apart from the FFA content, crude menhaden oil presented higher oxidation values (as reflected in PV and AV measurements) and a much higher metal content (copper and iron) than the crude herring oil. However, after the refining step, the two oils showed similar levels.

Both refined oils were evaluated on the oxidative stability in emulsions while kept at 60 °C. Since the content of EPA and DHA in the two oils differed, emulsions were prepared in such a way that the overall contents of PUFAs were similar. The oxidative stability of the emulsions, measured by the intake of oxygen, was identical for both oils (Figure 3). During storage time the herring oil emulsion presented a slightly slower off-taste development than the menhaden oil emulsion. Nevertheless, after a 50h period, both emulsions were scored by the panelists with score 9, which implies a high rejection level. It can be concluded that fish oil extracted from herring byproducts behaves, at least equivalent to the well-known commercially available menhaden oil.

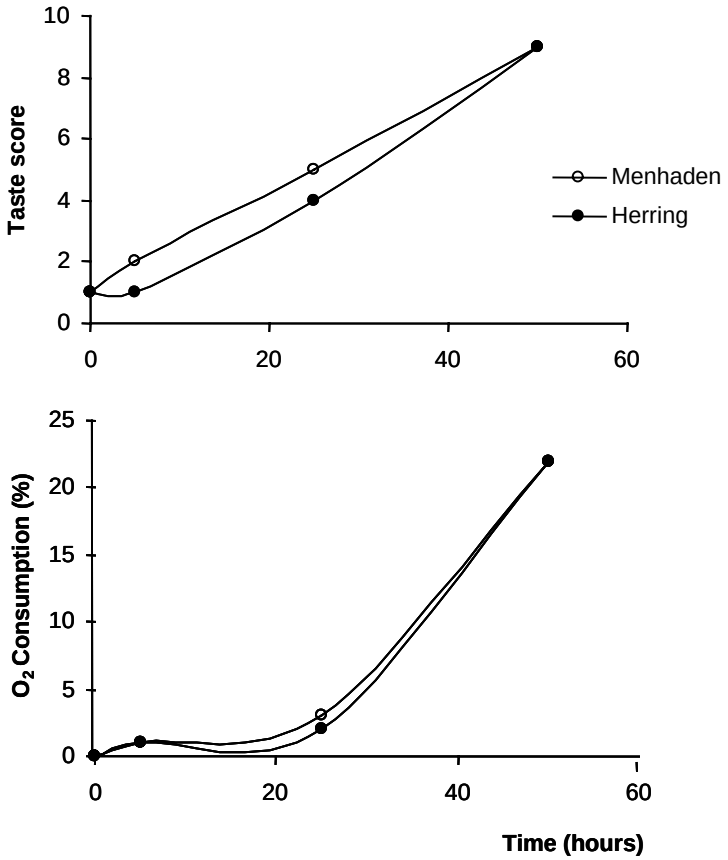

Figure 3- Comparison of O_2 consumption and off-taste development in emulsions with equal amounts of EPA + DHA content {menhaden (35%) vs. herring (53%)}, while kept at 60 °C.

Further, the development of volatiles in the crude and refined oils was compared. The oxidation was followed using static headspace GC for four volatiles: hexanal, 1-penten-3-one, 2-butenal and acetaldehyde. The results are shown in Figure 4. Different effects were observed for the different volatiles. In general, the refined oils were more stable than the crude oils. This may be due to the removal of metal ions, FFA, oxidation products and other contaminants during the refining process as well as to the addition of citric acid

during the deodorization step. Apart from the hexanal development, the herring oil was slightly more stable than the menhaden oil. The difference can be attributed to the fatty acids composition, since herring oil has a lower degree of unsaturation.

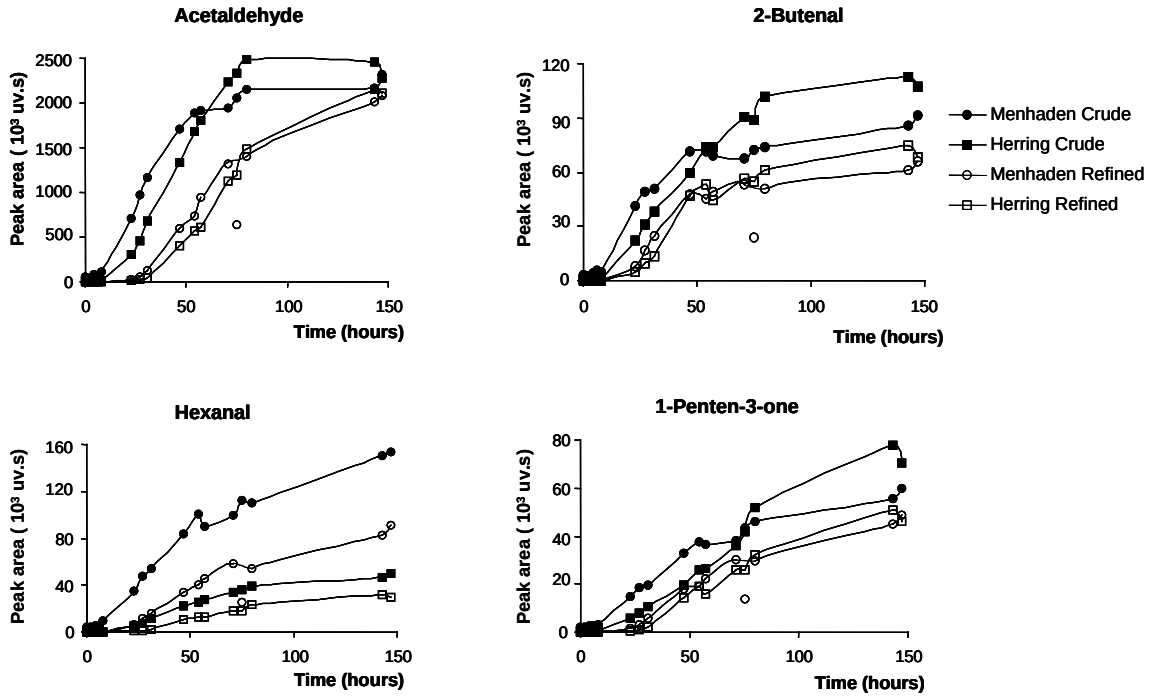

Figure 4- Oxidative stability of crude and refined herring and menhaden oil, when stored at 60 °C.

In conclusion, crude and refined oil extracted from herring byproducts do not behave differently from the commercial oil extracted from menhaden. However, the herring oil contains a lower level of ω-3 fatty acids. Consequently larger amounts of this oil are needed to achieve the same content of PUFAs. Since the raw material used is a waste product with little value, (€ 0.39 per kg for herring byproducts compared to at least € 0.64 per kg for pelagic fish species), its use may well be commercially attractive (see chapter 1 for a preliminary economic analysis).

Besides human consumption, there is also a market for fish oil for feed at animal farming, and for a number of non-food applications, including manufacture of cosmetics, detergents and paints. For these applications, the quality requirements may be less severe. Given the low costs of the raw material, it can be expected that also for these applications, its use may be commercially attractive.

Suggestions for future research

Although the feasibility of extracting a good-quality fish oil from herring byproducts has been established with this study, more research is still needed on the subject to strengthen the results and hypotheses. Some suggestions are:

- The study of the influence of refining procedure and the comparison between herring oil and commercial oil was only a preliminary one. It is therefore recommended that further work should be performed on the influence of the refining techniques on the stability of the herring oil before this is a valid and reliable model for investigating the addition of fish oil into different products.

- It would be of interest to investigate in more detail whether spontaneous fermentation of the byproducts changes the ratio of FFA, neutral lipids and phospholipids in the stored byproducts and the corresponding extracted oils.

- Future studies should be conducted to test the hypothesis that fluorescent compounds become more soluble in the aqueous phase when byproducts are stored for some time. It may be of importance to clarify whether measurement of FC in the aqueous phase is more suitable for the oxidative assessment of herring byproducts.

- The measurement of FFA is recommended as a quality index of the byproducts. FFA have been related to sensorial changes but was not related to the development of volatiles. It is not yet clear how the FFA levels are coupled to the development of volatiles such as pentanal, 1-penten-3-one and 2, 4-(E, E)-heptadienal. Elucidation of the mechanisms involved would raise our understanding of the stability of these oils. In addition, sensory evaluation should also be performed.

- New approaches may be needed to establish the relationship between volatiles (and pertinent non-volatile) lipid derivative components and specific oxidized odors and flavors more precisely.

Concluding Remarks

In this work, the feasibility of producing high-quality fish oil from herring byproducts was evaluated in various ways. With this, a contribution has been made to a more efficient usage of natural resources while yielding a high-quality product. Crude oil extracted from herring byproducts is relatively rich in essential ω-3 PUFAs such as EPA and DHA, and is relatively stable during storage. In most cases, the level of FFA increased over time. However, since it is crude oil, the off-odors, flavors and oxidation products are eliminated again during the refining process. This refining procedure should be a compromise between removing “undesirable” compounds and keeping beneficial components, such as α-tocopherol, that might improve the oil quality. The currently obtained knowledge of fish oil extracted from herring byproducts is a first step towards the valorization of byproducts from different fatty fish species into production of fish oil, thereby contributing to a better utilization of the marine resources that are available.

Appendix 1- Result of dioxin, non-ortho- and mono-ortho-CB analysis in fish oil. Results are given in pg or ng/g oil. TEQ expressed as TCDD toxic equivalent concentration for the upper-bound concentration. Please refer to chapter 2.2, Figure 1 for exact location of the catching area.

	October	July	June
Dioxins (pg/g oil)			
2,3,7,8-TCDF	14	4.6	4.5
1,2,3,7,8-PeCDF	2.7	1.6	2.0
2,3,4,7,8-PeCDF	10	5.3	5.5
1,2,3,4,7,8-HxCDF	1.5	1.2	1.5
1,2,3,6,7,8-HxCDF	0.95	0.62	0.75
2,3,4,6,7,8-HxCDF	1.3	0.67	0.58
1,2,3,7,8,9-HxCDF	0.42	0.51	0.67
1,2,3,4,6,7,8-HpCDF	*	0.66	*
1,2,3,4,7,8,9-HpCDF	0.32	*	0.53
OCDF	0.53	1.1	*
2,3,7,8-TCDD	0.58	<0,50	<0,50
1,2,3,7,8-PeCDD	1.9	0.92	*
1,2,3,4,7,8-HxCDD	*	<0,50	*
1,2,3,6,7,8-HxCDD	1.2	*	0.44
1,2,3,7,8,9-HxCDD	*	<0,25	<0,25
1,2,3,4,6,7,8-HpCDD	1.0	*	0.48
OCDD	3.4	7.6	2.8
Total content in pg TEQ/g fish oil	9.7	5.0	4.7
non-ortho-CB's (pg/g oil)			
PCB 81	2.7	*	2.1
PCB 77	176	53	61
PCB 126	54	20	24
PCB 169	16	11	11
Total content in pg TEQ/g fish oil	5.6	2.2	2.6
mono-ortho-CB's (ng/g oil)			
PCB 123	*	*	*
PCB 118	11	7.4	8.6
PCB 114	0.16	0.14	0.15
PCB 105	3.4	2.3	2.8
PCB 167	0.62	0.35	0.34
PCB 156	1.1	0.71	0.83
PCB 157	0.33	0.22	0.26
PCB 189	0.13	0.08	0.09
Total content in pg TEQ/g fish oil	2.3	1.5	1.8
Sum pg TEQ/g oil	17.6	8.7	9.1

* Not quantifiable due to interference.

Appendix 2- Description of the refining process performed in the oils.

1. **Degumming**

   Phosphoric acid (0.25% of 50% solution) is added slowly to the stirred oil at 85°C. After 5 min fast stirring the oil is settled and water washed.

2. **Neutralization**

   The oil is heated to 90 °C and neutralized with 4N NaOH (50% excess on FFA) with slow stirring. After settling and washing the still oil with water the soapstock is run off. After a further water wash and 0.1 NaOH wash on the stirred oil, the remainder of the soap is washed out with hot water.

3. **Drying**

   The oil is heated to 95 °C. 6N sodium carbonate (4% vol/wt on oil) and 30% sodium silicate solution (4% vol/wt on oil) are added to the oil with fast stirring. The temperature is then raised to 100 °C by sparging with live steam and sparging continued for 20-30 min. The oil is cooled to 95 °C and settled for 30 minutes. The still oil is washed with water and allowed to settle. The aqueous layer is run off. The stirred oil is then washed until neutral and dried under vacuum.

4. **Bleaching**

   The oil is bleached under vacuum for 30 min at 90 °C with 4% activated bleaching earth Tonsil Standard FF. The oil is cooled to 50 °C and filtered and collected under N_2 and transferred to a deodorizer.

5. **Deodorization**

   The oil is deodorized for 5 hours at 180-185 °C and 3-5 mbar. During heating and cooling 50 mg/kg citric acid is added to the oil as 50% solution. Steam sparging is discontinued at ~ 110 °C and replaced with N_2 sparging while cooling the oil to < 40 °C under high vacuum. The oil leaves the deodorizer via a polishing filter and is collected in polypropylene containers.

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SUMMARY

In recent years, the utilization of marine resources for human consumption has increased rapidly worldwide because of perceived nutritional benefits of polyunsaturated fatty acids (PUFAs), in particular those of the ω-3 family. The consumption of ω-3 PUFAs has been suggested for, among others, the prevention and treatment of cardiovascular and neurological diseases. It is known that fish species with a high fat content contain high concentrations of ω-3 PUFAs. Therefore the food industry shows interest in adding fish oil, which is rich in PUFAs, into food products. As the awareness of ω-3 PUFAs nutritional importance has risen, attention for their supply has increased. Presently, most of the fish oil available on the market is fish body oil, i.e., produced from the whole fish. However, fish oil can also be produced from byproducts from the processing industry, contributing in this way to a better utilization of the available natural resources.

However, ω-3 PUFAs are susceptible to oxidation, which does not only lead to reduced or perhaps even adverse health effects, but also to the generation of highly undesirable off-flavors and off-odors. In general, the rate of oxidation increases rapidly with the degree of unsaturation and, therefore, oxidation is a major concern in fish oils. Lipid oxidation takes place in fatty fish species during processing and storage. It is therefore important to optimize the fish oil process in order to ensure that the degree of oxidation in the extracted oil is and remains as low as possible.

Atlantic herring (*Clupea harengus)*, a fatty fish, is an important raw material for the Dutch fish processing industry. It is estimated that the three major Dutch fish processing companies produce approximately 27,000 tons/year of herring byproducts. A 100% utilization of fish processed for human consumption can be achieved by processing heads, fins, viscera, and so on into fish meal and fish oil. Moreover, converting herring byproducts into fish oil is an opportunity of adding value to the byproducts.

Upgrading of these herring byproducts into crude fish oil was investigated in the present study. The objectives of the study were to relate the quality of the extracted crude oil to the quality of the starting raw material, herring byproducts. The work aimed at creating knowledge in order to reliably develop routes to a better conservation and valorization of the bio-diversity. This thesis intends to give an overview of the current status and the contributions made.

An introduction is given in **Chapter 1** summarizing the state-of-the-art of herring processing and collection of the respective byproducts. A possible fish oil recovery process as well as a preliminary feasibility analysis for a complete fish meal plant are presented.

The quality of the starting raw material is discussed in **Chapter 2**. Firstly, the seasonal variation of the byproducts and the respective extracted oils was assessed. It was concluded that the herring byproducts from the entire year were an adequate raw material for fish oil production, though during the summer months higher PUFAs levels were found. Next, the use of salted byproducts was investigated. The results showed that good quality fish oil can be extracted from salted herring byproducts. Finally, oils obtained from different productions were studied from a chemical and sensory point of view. Byproducts from different herring processing generated oils with different qualities. Fresh and unsalted herring byproducts yielded oil with the lowest oxidation products and less unpleasant sensorial attributes.

The processing of raw material into high-quality crude fish oil was investigated in **Chapter 3**. The importance of the freshness as well as the necessity of sorting the byproducts *prior* to fish oil extraction was assessed. Sorting of the byproducts did not originate different oils quality showing that it is not required. However, it was concluded that stored byproducts generated oil with higher levels of PUFAs content. Next, process optimization of the pilot plant in order to yield a high quality and quantity fish oil was considered. The best quality oil was achieved while processing at the highest throughput of the pump and at intermediate decanter speed. The yield of the output fractions did not depend on the settings.

The oil product quality was evaluated in **Chapter** 4**.** Firstly, the storage temperature of the oil was investigated. As expected, the storage temperature has a strong effect on the stability of the crude herring oil. It has been proven that the best temperature to keep the oil is 0 °C. However due to economical implications and the fact that the oil may solidify, and since room temperature (~ 20 °C) still induces low concentrations of oxidation products, the latter temperature may also be used. Secondly, the development of undesirable volatiles and its correlation with chemical measurements was studied. The results showed that pentanal, 1-penten-3-one and 2, 4-(*E, E*)-heptadienal are good indicators for the oxidative status in fish oil and could be used as a degradation index.

In **Chapter 5**, a general discussion including the main conclusions of the study and final considerations is given. The content of pollutants present in the oil as well as the possibility to use fish oil extracted from herring byproducts at an industrial level are investigated and discussed. The extracted crude herring oil presented relatively low levels of contaminants, PCBs and dioxins. Finally, the crude herring oil was refined and compared with commercially available menhaden oil. Similar properties such as oxidative stability and off-taste development were found.

Curriculum Vitae

Isabel Aidos was born in Vouzela, Portugal, on the 23rd of July of 1972. She graduated in Food Chemistry from the University of Aveiro, Portugal, in September 1996 after a six months internship at the University of Bergen, Norway. From September 1996 to August 1997, she taught Physics and Chemistry at the High-School of Trancoso, in Portugal. From September 1997 to July 1998 she completed a postgraduate program for the Norwegian Research Council, at the Institute of Nutrition, Directorate of Fisheries, Bergen, Norway. In September 1998 she started her Ph.D. training at Food and Bioprocess Engineering Group, Wageningen University, with work placement at the Netherlands Institute for Fisheries Research (RIVO), in IJmuiden. The project aimed at studying the possibility of upgrading byproducts from the herring processing industry into high-quality fish oil.

Mar Salgado

Ó mar salgado, quanto do teu sal
São lágrimas de Portugal!
Por te cruzarmos, quantas mães choraram,
Quantos filhos em vão rezaram!

Quantas noivas ficaram por casar
Para que fosses nosso, ó mar!
Valeu a pena? Tudo vale a pena
Se a alma não é pequena.

Quem quer passar além do Bojador
Tem que passar além da dor.
Deus ao mar o perigo e o abismo deu,
Mas nele é que espelhou o céu.

Fernando Pessoa, 1932, in "*Mensagem*".

Portuguese Sea

Salt-laden sea, how much of all your salt
Is tears of Portugal!
For us to cross you, how many sons have kept
Vigil in vain, and mothers wept!
Lived as old maids how many brides-to-be
Till death, that you might be ours, sea!

Was it worth? It is worth while, all,
If the soul is not small.
Whoever means to sail beyond the Cape
Must double sorrow - no escape.
Peril and abyss has God to the sea given
And yet made it the mirror of heaven.

Translated into English by Jonathan Griffin, 1974.

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