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Quality analysis of marine zooplankton oils and identification of target molecules

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Contents

1. Introduction

In recent decades, the growing interest in seafood products has exerted pressure on wild resources, resulting in significant overexploitation (FAO, 2016). This increased pressure on resources is primarily due to global population growth, and the rising consumer awareness of the benefits of consuming fish-based products (FAO, 2016). With the increase in population, the demand for fish, for both human consumption and fish feed in aquaculture, has become highly competitive (FAO, 2009). The aquaculture industry has experienced extraordinary growth in the past three decades, now providing approximately half of the globally consumed fish (FAO, 2009). This growth has led to an increased demand for fish oil (Akoh, 2017). In order to meet the growing nutritional needs of fish, feed must provide essential elements such as polyunsaturated fatty acids, astaxanthin, proteins, and amino acids (Lall & Dumas, 2015). Furthermore, there is a growing interest in finding new alternatives for fish feed production and, especially, in fish oil and dietary supplements for human consumption. The use of secondary raw materials and byproducts from fish processing has become a solution to alleviate the pressure on wild resources and reduce waste (FAO, 2016). These byproducts are often rich in valuable ingredients, such as astaxanthin, chitin, collagen, and marine oils (FAO, 2016). Additionally, sustainable, and eco-friendly methods are being developed for the recovery of oils and other valuable components, avoiding the use of harsh chemicals (Akoh, 2017).

The increasing demand for fish has inevitably led to the overexploitation of fishery resources, with a dramatic decrease in the available natural resources and sustainability of fisheries (FAO, 2018). This has driven the search for alternatives, including the production of omega-3 fatty acids from marine resources such as zooplankton (Khozin-Goldberg et al., 2011; Miller et al., 2010). The use of zooplankton, however, remains a contentious topic due to environmental concerns

related to the potential impact of harvesting species that form the foundation of fish diets (Bachiller et al., 2018; Prokopchuk & Sentyabov, 2006). In Iceland, there is no direct capture of zooplankton; however, substantial amounts of zooplankton are indirectly collected as bycatch during atlantic mackerel (*Scomber scombrus*) catching during the summer.

Atlantic mackerel has become a species of great economic importance for the Icelandic fishing industry (Jansen et al., 2016). The mackerel primarily feeds on the zooplankton species *Calanus finmarchicus,* which is rich in polyunsaturated fatty acids (PUFA) (Óskarsson et al., 2016). The oil derived from *C. finmarchicus* is rich in omega-3 fatty acids and in the astaxanthin carotenoid; it is suitable for human consumption (Cook et al., 2016) and could also be used in aquaculture feeds, reducing dependence on wild catches (Oxley et al., 2009). Extraction of *Calanus* oil from the viscera of fished atlantic mackerel would be a good opportunity to obtain a high biological value product with low environmental impact. Currently this zooplankton enters the pelagic fishmeal and oil production, which it is starting to take part in the ichthyic sideproducts market thanks to its high concentration of polyunsatrated fatty acids (PUFA, especially Omega-3) and a decent quantity of the carotenoid astaxanthin (Daase et al., 2021). Concerning the quality, due to the origin and the composition of the raw materials, the *C. finmarchicus* contains very fast degrading digestive enzymes, which causes several problems during pelagic fish processing, including muscle degradation andgreater risk of nutrient loss, which requires greater precautions during processing (Pedersen, 2016). By removing the *Calanus* from the pelagic fishmeal and oil processing not only the pelagic fishmeal and oil products' quality can be improved, but this presents an opportunity for product development from the *Calanus* finmarchicus bycatch in itself (Eysteinsson et al., 2020).

Given these challenges and the pressure on global fishery resources, there is a growing need to explore innovative ways to fully harness the potential of fish byproducts during pelagic processing.

2. Aim of the thesis

The aim of this study was to extract oil from a zooplankton-rich side-stream obtained from pelagic processing, focusing on the visceral content of atlantic mackerel (*Scomber scombrus*), using two different extraction methods. The study also involved comparing the yield and composition of each extraction method and comparing it to five commercially available oils derived from different raw material origins. The chemical properties and compositions of the oils were also analyzed.

More specifically, the two extraction methods are the cold extraction and the Bligh and Dyer method which were applied to obtain *C. finmarchicus* oil from mackerel viscera. These types of oil drawing are supposed to affect the least as possible the lipids, from heat oxidation.

The oils were analyzed for their fatty acids' composition using a Flame Ionization Detection-Gas Chromatography (FID-GC), comparing them with other commercial marine oils (from *Calanus*, fish and krill), to evaluate the correlation among these oils and to provide that the extracted oil had similar properties, even after fish digestion.

Astaxanthin was also examined and compared among extracted oils and commercial ones, to see if the carotenoid was oxidated during feeding and storage.

Finally, this study also analyzed the amount of heavy metals and total oxidation compounds, to see if the extracted oils could be safe for human consumption and if the extraction method could have an effect on these compounds.

In summary, this study wanted to test if extracting oils from byproducts of the pelagic fishing industry was feasible, and if the oil had qualitative characteristics equal to, or better than, market competitors.

3. State of the art

3.1. Circular Economy in the Fish Productions

Over the past few decades, humans had to address the challenges of meeting the growing demands for food supply due to an ever-increasing global population. To achieve this goal, in addition to exploring alternative ways to produce larger quantities with reduced inputs, efforts have focused on minimizing food waste. By doing so, the environmental impact generated by waste can be reduced, and economic benefits can be derived from what, until half a century ago, had little or no value or occupied a marginal and unprofitable market (for example, converting animal and plant processing byproducts into feed or fertilizers) (Cooney et al., 2023).

As a result, numerous opportunities for the development of alternative products have emerged, contributing to the evolving of the concept known as the "circular economy". This concept involves preserving and increasing the value of raw materials within the production chain, minimizing waste, and optimizing the use of all its components, focusing on the replacement or reduction of raw materials use and minimizing waste (European Court of Auditors, 2023; Monsivais-Alonso et al., 2020). Indeed, as seen in **Figure 1**, the remanufacturing branch of the value chain is the focus to considerer in the food industry. Considering only the food waste generated from the processing and transformation of fishery products resulting from fishing activities, it is estimated that under- or unutilized raw materials from the fisheries sector accounts for between 30% and 70% (depending on the species) of the total catch, which is annually around 90.3 million tonnes, as estimated in the latest FAO report of 2022 (Ahuja et al., 2020; Monsivais-Alonso et al., 2020; Cooney et al., 2023; Fraterrigo-Garofalo et al., 2023). This amounts to approximately 31.6 to 63.2 million tonnes of underutilized biomass per year. This is mainly due to an increase in the average per capita consumption of fish and the fact that, as mentioned earlier, many anatomical parts of fish are removed because they are not commonly considered edible as such (Fraterrigo-Garofalo et al., 2023; Tola et al., 2023), e.g., due to social-ethical principles. But now, with the constant rising of the global population, associated increase food demand and stricter eco-

sustainability policies the world governments publish every year, it is crucial to reduce human impact, e.g., by trying to reach full utilization of available biological resources, including oil and other valuable ingredients from zooplankton, such as *C. finmarchicus*, obtained through sustainable pathways through pelagic fish processing.

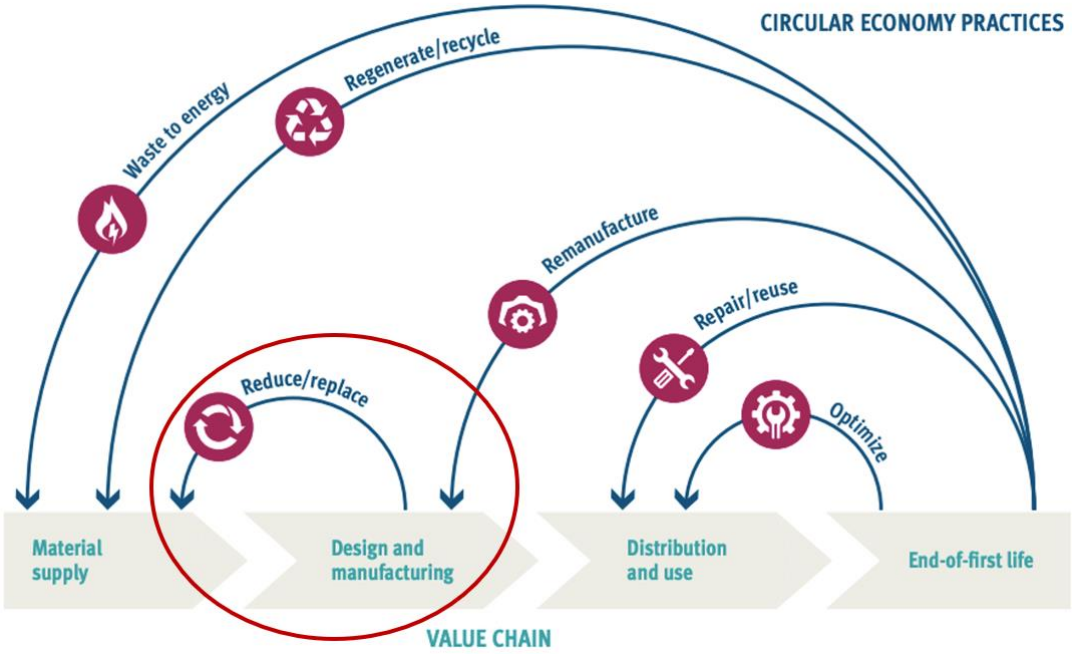

Figure 1. Circular economy scheme associated with the value chain; United Nation Industrial Development Organization (UNIDO), 2021.

3.2. *Calanus finmarchicus* generics

C. finmarchicus (Gunnerus 1764) is one of the main organisms contributing to the zooplankton biomass that characterizes the entire Arctic Ocean, and the northern part of the Atlantic Ocean. Its global distribution is reported in **Figure 2** (Pedersen, 2016; Razouls et al. 2005). Classified in the Crustacea phylum, this copepod (**Figure 3**), not exceeding 4 mm in size forms a significant part of the diet of many mysticete cetaceans, as well as small to medium-sized pelagic fish, such as herring (*Clupea harengus*) and Atlantic mackerel (*Scomber scombrus*) (Pedersen, 2016). The reason for its prevalence in these diets lies in the fact it is a rich and easily accessible source of fatty acids, which constitute approximately 60% of its dry matter (Vosskötter et al. 2023). Lipids are 90% represented by wax esters (WE) of polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA, 20:5

n-3) and docosahexaenoic acid (DHA, 22:6 n-3), which accumulate in storage sacs in the prosome (Eilertsen et al., 2012; Petersen et al., 2014; Vang, 2015).

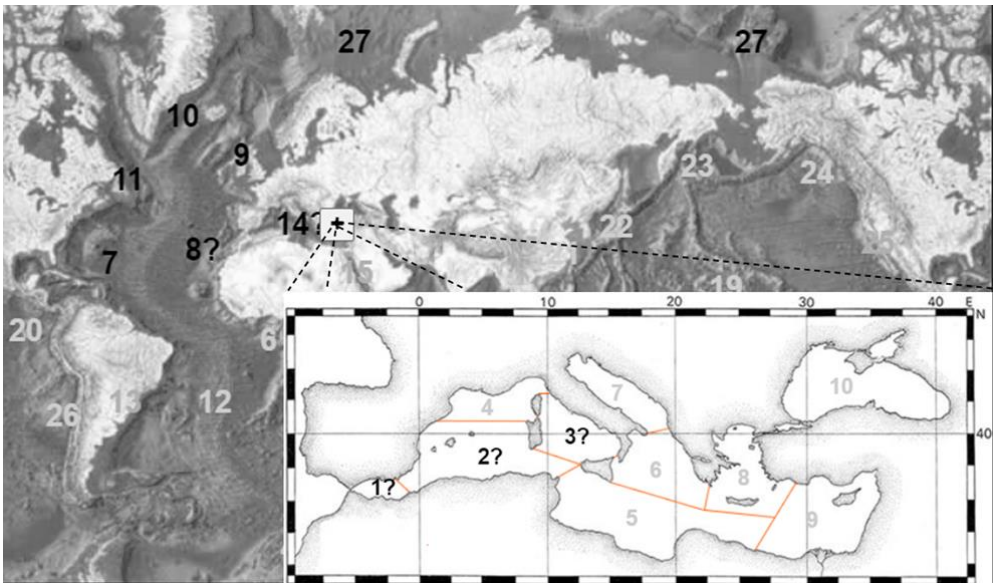

Figure 2. Distribution map of Calanus finmarchicus in the oceansand seas zones and the possible ones in which it may reside in the Mediterranean Sea (Razouls et al., 2005) represented by the black marked numbers.

These lipids originate from the phytoplankton the *Calanus* feeds on, especially the fast-growing diatoms which are its most preferred food source (Daase et al., 2021). The *Calanus* also hav*e* an important role in controlling the population of phytoplankton and are vital to pass on the carbon fixed through photosynthesis up the food chain (Falk-Petersen et al., 2008; Eysteinsson et al., 2018; Stefánsdóttir, 2020).

Figure 3. Microscopic view (10x) of Calanus finmarchicus

The high concentration of fatty acids in this copepod can be attributed to two survival mechanisms, in addition to considering that they are an indispensable energy source and a fundamental resource during eggs spawning (Gasmi et al., 2020). Firstly, these lipids act as a natural antifreeze, enabling the *Calanus* to thrive in Arctic and sub-Arctic waters and climates (Head et al., 2000). The second reason pertains to its primary escape strategy against predators, but during the late autumn, when the *C. finmarchicus* reaches the final juvenile stages of its life cycle, it utilizes specific lipolytic enzymes to esterify most of its polyunsaturated fatty acids (PUFAs) into waxes, which are incompressible (Eysteinsson et al., 2018). This adaptation allows the *Calanus* to submerge to great oceanic depths, ranging from 500 to 2000 meters. By doing so it avoids pursuit by most of its predators, who are typically limited to maximum depths of about 300 meters. This technique is known as vertical migration. Throughout the winter period, it remains on the seabed in a state of dormancy called diapause. In early spring, the *Calanus* then resurfaces to complete the reproductive cycle and releases eggs for the new generation (Vang, 2015; Petersen, 2016).

3.3. Atlantic Mackerel (*Scomber scombrus*)

Due to overall warming of the oceans an abundance of zooplankton is now surrounding Iceland and the subarctic regions of the globe during the summer months (Astthorsson et al. 2012; Daase et al., 2021; Razouls. et al., 2005).
These areas are invaded by an immense quantity of fish species that feed on the zooplankton, including the Atlantic mackerel (*Scomber scombrus*) (**Figure 4**), which first appeared in these waters as recently as 2006 (Eysteinsson, 2020). Although the mackerel inhabits Icelandic waters from May to September, it reaches its peak of zooplankton consumption during the months of June and July (Oskarsson et al., 2016), coinciding with the period of its maximum lipid accumulation (Lee et al. 2006). Indeed, during this period (the summer months), *C. finmarchicus* constitutes the vast majority of the Atlantic mackerel's diet, accounting for values between 53% and 98% of its stomach content upon capture, with a lipid percentage of the total bolus mass ranging from 13.7% to 23.7%

(Baldursson, 2020; Oskarsson et al., 2016; Stefánsdóttir, 2020). However, from August until the vertical migration of *Calanus* in September, the mackerel competes for the *Calanus* with herring, compensating for its diet by increasing consumption of euphausiids, pteropods, arrow-worms, sea butterflies, fish larvae, and small fish (Stefánsdóttir, 2020).

Figure 4. Side-view of Atlantic Mackerel (Scomber scombrus)

The Atlantic mackerel is a pelagic fish belonging to the category of bluefish, which also includes other species like herring (*Clupea harengus*) and yellowfin tuna (*Thunnus albacares*). A distinctive feature of these species is their ability to accumulate lipids not only subcutaneously and within myosepta (bundles of muscle fibers typical of bony fish), but also in the liver, similar to white-fleshed fish and demersal species like the classic Atlantic cod (*Gadus morhua*). This renders the mackerel as a highly lipid-rich species, especially in terms of polyunsaturated fatty acids Omega-3 (PUFA), and it can withstand temperatures as low as 5°C (Astthorsson et al. 2012; Eysteinsson, 2020).

3.4. *Calanus finmarchicus* chemical composition

3.4.1. Lipid composition

In recent years, several studies and research have proven that the lipid composition in *C. finmarchicus* depends on various factors such as diet, developmental stage, collection period, and location (Daase et al., 2021). Analyzing the individual characteristics that determine the fatty acid content, it has emerged that the developmental phase of the copepod at the time of capture is crucial. Considering that they can remain in diapause in the ocean depths for up to 9 months, with only part of their development occurring at the surface, the moment when *C.* finmarchicus reaches its highest lipid content is obtained during late summer, when the *Calanus* is between stages IV and V of the juvenile phase, just before vertical migration, (**Figure 5**) (Lee et al., 2006; Pedersen, 2016; Vang, 2015). As mentioned earlier, this content can exceed 60% of the total dry weight (Lee et al., 2006; Daase et al., 2021).

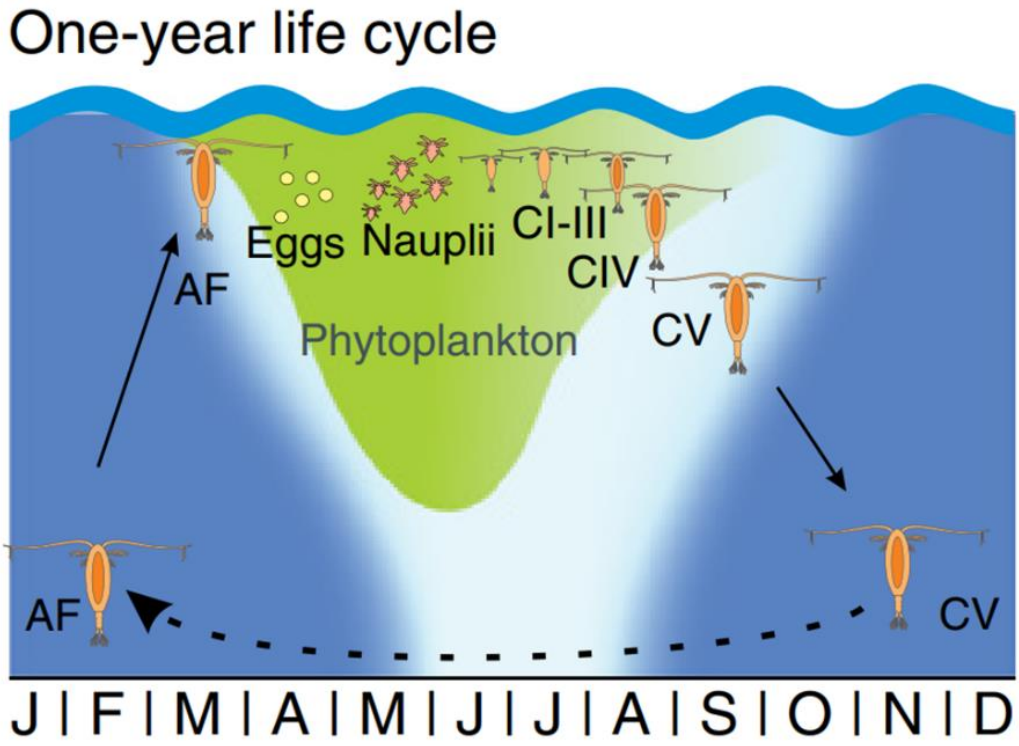

Figure 5. one-year life cycle of Calanus finmarchicus (Daase et al., 2021).

Most lipids found in the *C. finmarchicus* consist of wax esters, while phospholipids (PL), free fatty acids (FFA), and triglycerides (TAG) are minor components (Miller et al., 1998, **Table 1**).

Table 1. Lipid class composition of late copepodite stages and adult C. finmarchicus sampled in different periods and presented as % total lipids (Miller et. al, 1998).

	% of total lipid			
Lipid class	*June*[1]	*October*[1]	*March*[1]	*March*[2]
Triacylglycerols	8.9	1.3	nd	3.1
Sterols	1.2	2.6	3.2	1.4
Free fatty acids	0.2	nd	1.7	nd
Wax esters	85.4	88.1	84.9	73.8
Phospholipids	4.2	7.3	10.3	21

nd = not detected. *Source*: [1]Falk-Petersen et al. (1987); [2]Fraser et al. (1989).

Although the TAG increase during emergence as the reverse process of their esterification occurs during this phase (Falk-Petersen et al., 1987).

Moreover, it has been observed that this process takes place when the *Calanus* contains a minimum concentration of 17 µg of TAG and these esters are then converted back into triglycerides because they are essential for egg formation (Miller et al., 1998). As mentioned in the copepod generalities section, these wax esters play a key role in the species' survival. However, it should be noted that, depending on the latitude, *Calanus* exhibits different percentages of wax esters in terms of both quantity and type, as they act as excellent antifreeze agents (Eilertsen et al., 2012; Petersen et al., 2014; Vang, 2015). The composition of these wax esters generally reflects the diet that supports the *C. finmarchicus* and may vary between ocean zones and feeding periods (Daase et al., 2021). Transcending these differences, the waxes of the copepod in question are mainly composed of monounsaturated fatty alcohols, derived from de novo biosynthesis (Eysteinsson et al., 2020). As shown in **Table 2 and Table 3**, the range of fatty acids and alcohols constituting the lipid fraction of *Calanus* varies depending on the collection period (Eysteinsson et al., 2020).

Regarding unsaturated alcohols, the main constituents are 20:1n-9 and 22:1n-11, which can represent from 62% to 82% of the total lipids. Concerning the

polyunsaturated fatty acids front (PUFA), there is a prevalence of stearidonic acid (SDA, 18:4 n-3), and eicosapentaenoic acid (EPA, 20:5 n-3), which can make up to over 30% of the total fatty acids (Eysteinsson et al., 2020). Among other noteworthy acids, we find the monounsaturated palmitoleic acid (16:1n-7), oleic acid (18:1n-9), gondoic acid (20:1n-9), and cetoleic acid (22:1n-11), which reach overall concentrations of up to 50% (Miller et al., 1998; Eysteinsson et al., 2020).

Table 2. Fatty alcohol composition of wax esters in Calanus finmarchicus, during late copepodite stages and in adults (Petersen et, al, 2014).

Location	*Fram Strait*	*Balsfjord*			*Loch Thurnaig*
Fatty alcohols	*June–August*[1]	*June*[2]	*October*[2]	*March*[2]	*March*[3]
14:0	3.9	6.4	1.3	0.5	1.2
16:0	14.6	17.8	8.9	7.9	9.8
16:1n-7	3.4	2.5	2.6	1	1.5
18:0	nd	1.1	0.6	nd	0.7
18:1n-9	nd	3.7	2.7	2.2	4.9
18:1n-7	nd	1.6	1.7	1.1	nd
18:2n-6	nd	4.7	1.4	0.9	nd
18:3n-3	nd	2.6	1.3	1	nd
20:1n-9	39.3	40.5	34.1	33.8	23.3
22:1n-11	38.8	17.7	40.6	48.6	45.3
22:1n-9	nd	1.2	1.4	1.1	nd
Others	nd	0.2	3.4	1.9	13.3

nd = not detected; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids. *Source*: [1]Albers et al. (1996); [2]Falk-Petersen et al. (1987); [3]Fraser et al. (1989).

Among the saturated ones, there is an abundant presence of myristic acid (14:0) and palmitic acid (16:0), constituting 20-35% of the total fatty acids present in the wax esters (Fraser et al., 1989; Pedersen et al., 2014; Eysteinsson et al., 2018).

The phospholipids of C. finmarchicus are characterized by a very high content of Omega 3 fatty acids (Eysteinsson et al., 2021; Remadi, 2021). As reported in **Table 4**, EPA and DHA (**Figure 6**) constitute more than 50% of the fatty acids in phospholipids.

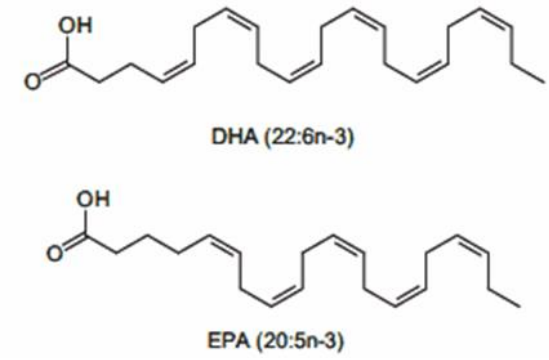

Figure 6. Molecular structure of DHA and EPA.

Table 3. fatty acid profiles (% of total fatty acids) of zooplankton rich side-stream samples collected from headed and gutted mackerel in the summers of 2016-2017 (late July, early August) and 2018 (early and late August and September). Values are presented as means ± SD (Eysteinsson et al., 2020).

SFA (saturated fatty acids), MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids).a–cDifferent lowercase superscript letters in each row denote significant difference between samples within the same year ($p < 0.05$).

	2016			2017			2018			
n =	early July 6	early August 12	late August 9	early July 3	early August 9	late August 9	early August 3	late August 12	September 3
Fatty acid	mean	mean	mean	Mean	mean	mean	mean	Mean	mean
C11:0	0.2 ± 0.1^{a}	0.1 ± 0.4^{a}	0.3 ± 0.5^{a}	0.1 ± 0.2^{a}	0.2 ± 0.3^{a}	0.3 ± 0.2^{a}	0.2 ± 0.1^{a}	0.2 ± 0.4^{a}	0.1 ± 0.4^{a}
C14:0	6.8 ± 0.6^{a}	6.2 ± 0.7^{a}	6.5 ± 1.2^{a}	7.9 ± 0.8^{ab}	7.1 ± 1.1^{b}	9.2 ± 0.6^{a}	8.9 ± 0.6^{a}	7.3 ± 1.4^{a}	5.3 ± 0.3^{b}
C15:0	0.4 ± 0.4^{a}	0.4 ± 0.4^{a}	0.4 ± 0.3^{a}	0.5 ± 0.1^{a}	0.4 ± 0.3^{a}	0.3 ± 0.1^{a}	0.2 ± 0.6^{a}	0.4 ± 0.3^{a}	0.4 ± 0.8^{a}
C16:0	**12.7 ± 0.5^{a}**	**11.5 ± 0.7^{a}**	**11.1 ± 3.2^{a}**	**14.9 ± 0.3^{a}**	**14.2 ± 0.6^{a}**	**14.6 ± 0.9^{a}**	**17.8 ± 0.1^{a}**	**15.2 ± 1.5^{b}**	**11.1 ± 0.6^{c}**
C16:1n7	3.1 ± 0.3^{a}	3.5 ± 0.5^{a}	3.4 ± 0.7^{a}	2.6 ± 0.5^{b}	3.4 ± 0.4^{ab}	3.7 ± 0.7^{a}	3.3 ± 0.2^{a}	3.1 ± 0.5^{a}	3.1 ± 0.2^{a}
C16:2n4	0.2 ± 0.3^{a}	0.2 ± 0.3^{a}	0.2 ± 0.4^{a}	0.2 ± 0.7^{a}	0.2 ± 0.4^{a}	0.2 ± 0.4^{a}	0.2 ± 0.1^{a}	0.2 ± 0.5^{a}	0.2 ± 0.8^{a}
C16:3n4	0.4 ± 0.4^{a}	0.3 ± 0.4^{a}	0.4 ± 0.3^{a}	0.4 ± 0.3^{a}	0.4 ± 0.1^{a}	0.4 ± 0.5^{a}	0.2 ± 0.3^{a}	0.3 ± 0.2^{a}	0.3 ± 0.1^{a}
C17:0	0.3 ± 0.2^{a}	0.3 ± 0.2^{a}	0.3 ± 0.5^{a}	0.3 ± 0.8^{a}	0.2 ± 0.3^{a}	0.3 ± 0.3^{a}	0.2 ± 0.2^{a}	0.3 ± 0.2^{a}	0.3 ± 0.1^{a}
C18:0	2.1 ± 0.7^{a}	1.9 ± 0.5^{a}	1.7 ± 0.8^{a}	2.7 ± 0.4^{a}	2.5 ± 0.5^{a}	2.5 ± 0.4^{a}	3.4 ± 0.2^{a}	2.9 ± 0.3^{a}	1.9 ± 0.1^{b}
C18:1n11	0.3 ± 0.4^{a}	0.3 ± 0.4^{a}	0.3 ± 0.2^{a}	0.4 ± 0.2^{a}	0.4 ± 0.6^{a}	0.3 ± 0.4^{a}	0.2 ± 0.1^{a}	0.3 ± 0.1^{a}	0.4 ± 0.1^{a}
C18:1n7	1.7 ± 0.4^{a}	2.1 ± 0.4^{a}	1.8 ± 0.5^{a}	1.6 ± 0.2^{ab}	2.1 ± 0.3^{a}	1.5 ± 0.5^{b}	1.9 ± 0.4^{a}	2.2 ± 0.5^{a}	2.2 ± 0.7^{a}
C18:1n9	**7.3 ± 0.9^{b}**	**9.2 ± 1.4^{a}**	**8.1 ± 0.7^{ab}**	**7.4 ± 0.1^{b}**	**9.7 ± 1.8^{a}**	**7.8 ± 1.0^{b}**	**8.2 ± 0.5^{a}**	**10.1 ± 2.2^{a}**	**11.6 ± 0.7^{a}**
C18:2n6	1.4 ± 0.2^{a}	1.5 ± 0.3^{a}	1.5 ± 0.5^{a}	1.5 ± 0.7^{a}	1.5 ± 0.3^{a}	1.4 ± 0.4^{a}	1.5 ± 0.8^{a}	1.5 ± 0.3^{a}	1.4 ± 0.4^{a}
C18:3n3	1.2 ± 0.6^{a}	1.3 ± 0.5^{a}	1.3 ± 0.2^{a}	1.1 ± 0.2^{a}	1.2 ± 0.3^{a}	1.3 ± 0.3^{a}	1.4 ± 0.1^{a}	1.3 ± 0.1^{a}	1.4 ± 0.1^{a}
C18:3n4	0.2 ± 0.2^{a}	0.1 ± 0.2^{a}	0.1 ± 0.4^{a}	0.1 ± 0.2^{a}	0.1 ± 0.5^{a}	0.1 ± 0.3^{a}	0.1 ± 0.3^{a}	0.2 ± 0.2^{a}	0.2 ± 0.3^{a}
C18:4n3	**4.6 ± 0.6^{a}**	**4.5 ± 0.9^{a}**	**5.1 ± 1.2^{a}**	**4.2 ± 0.7^{b}**	**3.9 ± 0.5^{b}**	**5.8 ± 0.8^{a}**	**4.0 ± 0.3^{b}**	**4.1 ± 0.3^{b}**	**4.8 ± 0.3^{a}**
C20:1n11	0.1 ± 0.3^{a}	0.1 ± 0.2^{a}	0.1 ± 0.2^{a}	0.1 ± 0.3^{a}	0.1 ± 0.6^{a}	0.1 ± 0.2^{a}	0.1 ± 0.4^{a}	0.2 ± 0.4^{a}	0.2 ± 0.3^{a}
C20:1n9	**9.9 ± 0.5^{a}**	**9.7 ± 0.4^{a}**	**9.9 ± 1.0^{a}**	**10.7 ± 0.3^{a}**	**9.8 ± 0.8^{a}**	**8.9 ± 0.6^{b}**	**9.0 ± 0.6^{a}**	**8.6 ± 1.4^{a}**	**9.3 ± 0.6^{a}**
C20:1n7	0.1 ± 0.4^{a}	0.1 ± 0.4^{a}	0.1 ± 0.4^{a}	0.1 ± 0.5^{a}	0.2 ± 0.5^{a}	0.1 ± 0.6^{a}	0.2 ± 0.6^{a}	0.2 ± 0.5^{a}	0.2 ± 0.6^{a}
C20:4n3	1.1 ± 0.1^{a}	1.2 ± 0.3^{a}	1.1 ± 0.3^{a}	1.2 ± 0.3^{a}	1.1 ± 0.6^{a}	1.1 ± 0.4^{a}	1.2 ± 0.5^{a}	1.3 ± 0.5^{a}	1.4 ± 0.6^{a}
C20:4n6	0.5 ± 0.3^{a}	0.6 ± 0.4^{a}	0.5 ± 0.4^{a}	0.4 ± 0.4^{a}	0.5 ± 0.1^{a}	0.5 ± 0.4^{a}	0.4 ± 0.5^{a}	0.4 ± 0.5^{a}	0.6 ± 0.6^{a}
C20:5n3 EPA	**8.5 ± 0.5^{a}**	**9.4 ± 0.6^{a}**	**8.7 ± 1.2^{a}**	**7.3 ± 0.5^{c}**	**8.9 ± 0.8^{b}**	**10.1 ± 1.1^{a}**	**7.8 ± 0.4^{b}**	**8.1 ± 0.6^{b}**	**9.0 ± 0.3^{a}**
C22:1n11	**16.9 ± 1.1^{a}**	**14.8 ± 0.5^{a}**	**15.3 ± 2.7^{a}**	**18.5 ± 0.3^{a}**	**14.7 ± 1.7^{b}**	**12.8 ± 0.7^{c}**	**13.9 ± 0.9^{a}**	**13.5 ± 1.8^{a}**	**12.6 ± 0.8^{a}**
C22:1n9	0.5 ± 0.6^{a}	0.8 ± 0.3^{a}	0.8 ± 0.3^{a}	0.9 ± 0.1^{a}	0.9 ± 0.5^{a}	0.7 ± 0.5^{a}	0.9 ± 0.3^{a}	0.8 ± 0.5^{a}	0.8 ± 0.7^{a}
C22:5n3	1.3 ± 0.2^{a}	1.5 ± 0.3^{a}	1.2 ± 0.4^{a}	1.2 ± 0.4^{a}	1.4 ± 0.3^{a}	1.2 ± 0.4^{a}	1.3 ± 0.8^{a}	1.5 ± 0.6^{a}	1.7 ± 0.9^{a}
C22:6n3 (DHA)	**13.7 ± 0.7^{a}**	**14 ± 0.7^{a}**	**12.9 ± 1.1^{a}**	**11.7 ± 0.8^{a}**	**12.7 ± 0.8^{a}**	**12.6 ± 0.6^{a}**	**12 ± 0.1^{a}**	**13.3 ± 2^{a}**	**14.7 ± 0.3^{a}**
C24:1n9	0.8 ± 0.3^{a}	0.8 ± 0.3^{a}	0.8 ± 0.3^{a}	0.8 ± 0.2^{a}	0.9 ± 0.2^{a}	0.8 ± 0.3^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
SFA	22.5 ± 0.2^{a}	20.4 ± 0.9^{a}	20.3 ± 5.6^{a}	26.3 ± 0.7^{a}	24.7 ± 1.5^{b}	27.0 ± 1^{a}	30.6 ± 0.9^{a}	26.3 ± 3.1^{b}	19.1 ± 1.2^{c}
MUFA	40.7 ± 0.9^{a}	41.4 ± 1.7^{a}	40.6 ± 3.0^{a}	43.1 ± 0.7^{a}	42.0 ± 2.7^{a}	36.8 ± 1.9^{b}	37.6 ± 1.1^{a}	39.0 ± 1.5^{a}	40.4 ± 2.1^{a}
PUFA	33.2 ± 0.3^{a}	34.6 ± 1.6^{a}	33.1 ± 3.4^{a}	29.4 ± 1.2^{c}	31.8 ± 2.2^{b}	34.7 ± 2.6^{a}	30.2 ± 1.2^{a}	32.2 ± 3.1^{a}	35.9 ± 2.1^{a}

This is similar to the composition of phospholipids in cold-water marine fish (Pedersen et al., 2014). Their percentage remains relatively stable as they primarily serve as structural lipids in cellular membranes (Albers et al., 1996; Fraser et al., 1989; Eysteinsson et al., 2018). These phospholipids are mainly in the form of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), bound to the membranes (Fraser et al., 1989). However, after death or in rare cases due to intensive feeding sessions, these compounds are enzymatically cleaved by phospholipase A2 into free fatty acids (FFA) (Pedersen et al., 2014). Furthermore, Overrein (2010) observed that when captured before diapause, *Calanus finmarchicus* undergoes rapid post-mortem degradation of phospholipids, with degradation rates varying with the storage temperature. When captured after diapause, lower phospholipase activity was recorded, indicating that they are less stable in spring than in winter.

Table 4. Fatty acid composition (mass%) of triacylglycerol and phospholipids in C. finmarchicus, late copepodite stages and adults (Pedersen et al., 2014).

	Triacylglycerol		*Phospholipids*	
Fatty acids	*June–August*[1]	*March*[2]	*June–August*[1]	*March*[2]
14:0	12	1.2	3.3	0.8
16:0	30.4	27.6	25.8	20.9
16:1n-7	3.6	4	1.1	0.7
18:0	6.1	2.1	3.6	2.8
18:1n-9	10.4	10.3	2.5	1.8
18:1n-7	1.9	5.6	1	2.2
18:2n-6	2.7	7.5	1.5	2.6
18:3n-3	2.3	6.1	0.6	1.6
18:4n-3	5.9	7.2	2.5	3.2
20:0	4.2	nd	nd	nd
20:1n-9	nd	4.8	0.2	0.3
20:4n-6	2.5	1.4	0.2	0.3
20:5n-3	8.7	7.4	19.2	23.1
22:1n-11	2.2	4	0.2	nd
22:1n-9	nd	nd	nd	nd
22:5n-3	1.2	0.3	0.2	0.3
22:6n-3	5.8	5.1	37.4	30.9
∑ SFA	52.7	30.9	32.7	24.5
∑ MUFA	18.1	28.7	5	2.8
∑ n-3 PUFA	23.9	26.1	59.9	59.1

nd = not detected; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids. *Source*: [1]Albers et al. (1996); [2]Fraser et al. (1989).

3.5. Astaxanthin in *Calanus finmarchicus*

An important component in *Calanus finmarchicus* that has been increasingly studied in recent years is the content of astaxanthin (**Figure 7**), a lipophilic carotenoid pigment of the xanthophyll group, which gives an orange red colour. *Calanus* synthesizes astaxanthin from β-carotene present in the phytoplankton and microalgae it feeds on (Daase et al., 2021). Astaxanthin can constitute up to 90% of all pigments present (ranging between 550 and 850 ppm and can reach concentrations up to 1600 ppm in extracted oils from the species). Chemically, astaxanthin can be present in a free form, as fatty acid monoesters, as diesters, or bound to proteins, such as carotenoproteins.

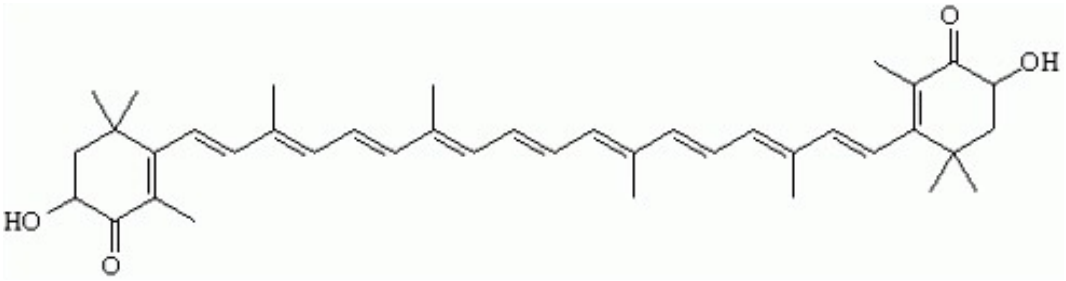

Figure 7. Molecular structure of astaxanthin

Among the hypothesized functions, it mainly serves as a photoprotective antioxidant for essential lipid reserves during vertical migration. Additionally, it may play a role in lipid metabolism and aid in camouflage against predators for the *Calanus* (Pedersen et al., 2014; Eysteinsson et al., 2018). As showed in the **Figure 8**, astaxanthin is sensitive to air exposure, causing its oxidation, reducing its antioxidant power. This point is significant because it means the astaxanthin, when isolated must be rapidly stored in a modified atmosphere bottle and shielded to light to preserve it (Pedersen, 2016).

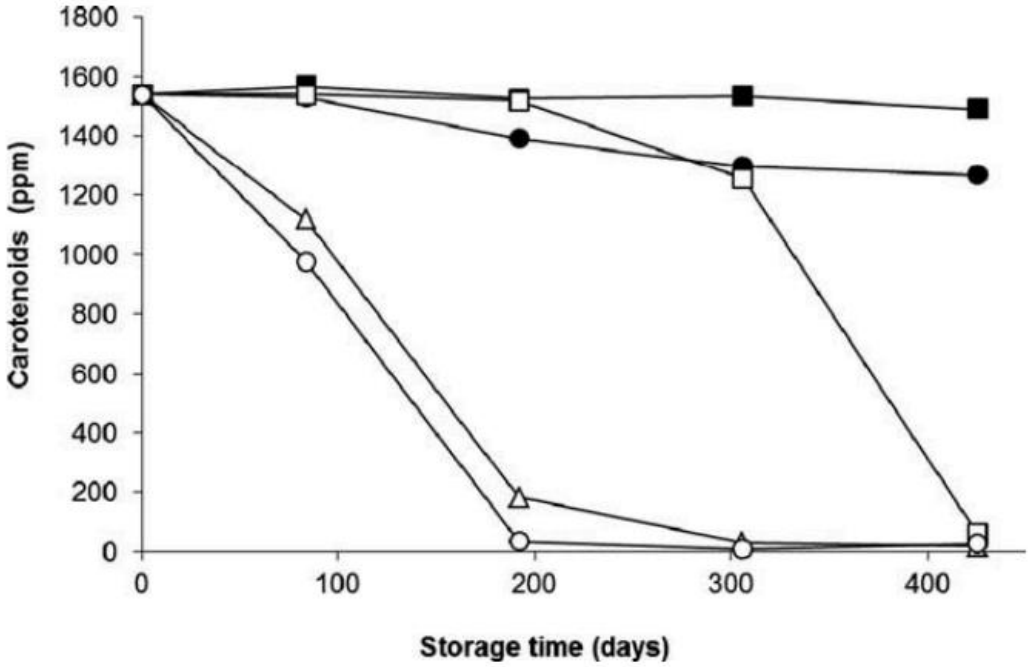

Figure 8. Stability of astaxanthin in oil extracted from Calanus finmarchicus. The oil was stored for 425 days either in the dark or exposed to daylight in closed transparent bottle with an inert atmosphere (N2; closed symbols) or in bottles exposed to air (open symbols). The bottles were kept in the dark at 4°C in a refrigerator (■/□) or in the laboratory (22°C) exposed to light (●/○) or in the darkness (Δ). (Pedersen, 2016).

3.6. Heavy metals in *Calanus finmarchicus*

Heavy metals have always been a concern in the seafood industry due to the fact that the aquatic food chain is more extensive than the terrestrial one, leading to a greater biomagnification of heavy metals (AMAP, 2002). Biomagnification refers to the increased accumulation of these compounds as they move up the food chain. Fortunately, *Calanus* is found at the lower levels of the Arctic food chain, but it is not exempt from containing some heavy metals (AMAP, 2002). Some of the metals that used to be present in alarming quantities in seafood products, such as mercury and lead, are now found at lower levels, thanks to social and economic policies, as showed in **Table 5** (Zauke et al., 2006). These policies include discontinuing the discharge of toxic waste and eliminating lead from gasoline, which used to be transported by air currents and deposited in the waters and ice of the polar regions following the release of automobile exhaust (although leaded gasoline is still used as fuel for snowmobiles in some northern populations) (AMAP, 2002; AMAP, 2021).

Table 5. Mean trace metal concentrations in copepods from different regions of the world (mg/kg DW). (Zauke et al., 2006).

**: Mixed collectives of C. finmarchicus and C. helgolandicus.*

Species	Region	Cd	Cu	Pb	Zn
Calanus finmarchicus	Greenland Sea	0.3	4	<0.3	86
Calanus finmarchicus	Fram Strait	0.3	5	<0.3	93
Calanus finmarchicus	Greenland Sea	7.7	8	1.6	164
Calanus finmarchicus	Fram Strait	6.8	5	0.7	192
*Calanus finmar/helgol.**	German Bight	0.7	4	1.0	110
*Calanus finmar/helgol.**	Southern North Sea	1.8	7	1.0	129
*Calanus finmar/helgol.**	Central North Sea	3.2	7	1.0	123
*Calanus finmar/helgol.**	Northern North Sea	5.0	8	1.0	98
*Calanus finmar/helgol.**	N. North Sea/Atlantic	10.9	7	1.0	70

The heavy metals weaknesses of copepods, such as *Calanus*, lie in cadmium (Cd) and arsenic (Ar), which are assimilated by the *Calanus* from diatoms and microalgae, respectively (AMAP, 2021; Daase et al. 2021). Indeed, these autotrophic microorganisms filter and accumulate these heavy metals, which are harmless to them and most predatory species (Daase et al. 2021). These heavy metals would normally be slowly disposed of by the periodic phytoplankton cell turnover, but they are actually assimilated by zooplankton and then stored in their lipid reserves (Zauke et al., 2006).

It is worth noting that Pickharrdt et al. (2002) demonstrated that as the population of diatoms and microalgae increases during blooming, the total accumulated heavy metal content decreases, and conversely, as the microalgae population decreases, individual heavy metal accumulation increases. This means that as microalgae blooming increases, there are more zooplankton feeding on them, and with the increase in "predators," the number of autotrophic species decreases (Razouls et al., 2005). As a result, there is a greater accumulation of heavy metals in late-season *Calanus* compared to early-season *Calanus* (Zauke et al., 2006).

3.7. Collection and processing of *Calanus finmarchicus*

C. finmarchicus can be collected under three different conditions: through direct fishing using fine-mesh pelagic trawl nets (**Figure 9**); as a bycatch during the capture of other aquatic animals (mainly during the herring or mackerel season); or indirectly as they are ingested by fish, from which they can be isolated soon after fish caught (which is made using pelagic or purse seine nets) (Eysteinsson et al., 2020; Geściak, 2019; Stefánsdóttir, 2020). Focusing on the third method, central to the thesis, the zooplankton collection process is described roughly as follows: during the mackerel fishing season, ships hoist aboard nets filled with the catch, pouring the contents into below-deck tanks filled with chilled seawater, to keep the majority of specimens alive, and/or preserve those already lifeless. At this point, the ships return to port and discharge the tanks directly to the processing companies, where the decapitation and gutting process takes place (while avoiding breaking the guts to prevent contamination from gastric fluids that could compromise the freshness and quality of the flesh due to the contained proteases and lipases) (Eysteinsson et al., 2020). Finally, the digested *Calanus* is squeezed out from the stomach of the mackerel and stored in a lightproof bottle and saved in a freezer at -28°C for at least 72 hours before oil extraction.

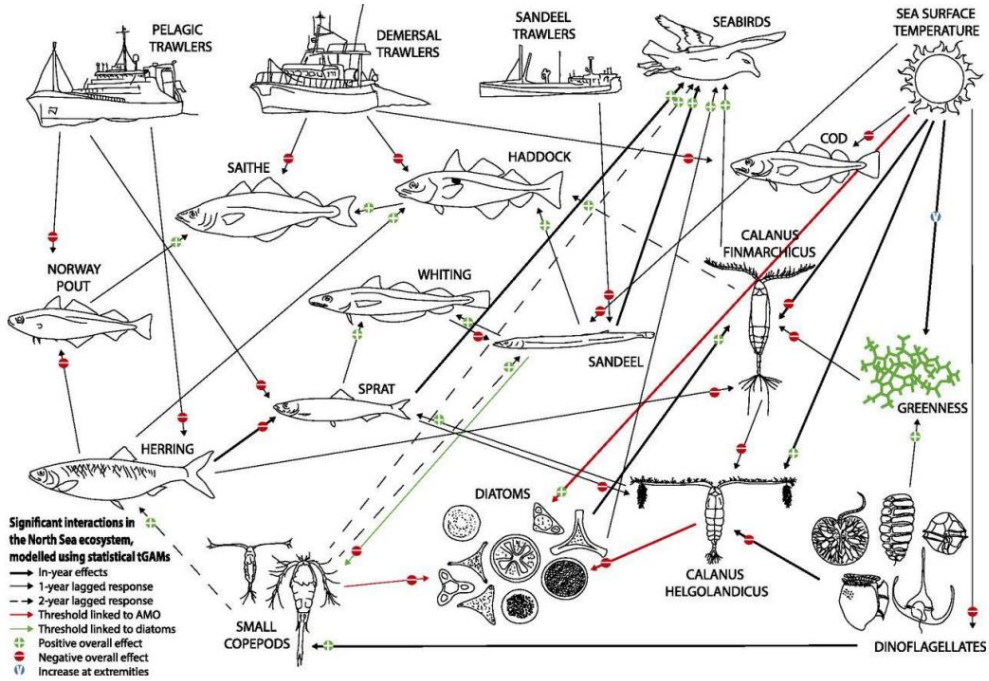

Figure 9. The most significant trophic interactions in the North Sea ecosystem according to Lynam et al. (2016).

4. Materials and Methods

4.1 Commercial marine oils

Five commercial marine oils from ready to eat pills were analysed within this research work for comparison with the oils extracted from fish viscera. These commercial oils were the following:

1. *C. finmarchicus* oil encapsulated in fish gelatin softgel pills;
2. *C. finmarchicus* oil encapsulated in fish gelatin pills, enriched in Vitamin D3 from algae;
3. Krill (*Euphausia superba*) oil encapsulated in bovine gelatin pills;
4. Fish (*Gadus morhua*) oil encapsulated in bovine gelatin soft pills;
5. Microalgae (*Haematococcus pluvialis)* oil encapsulated in fish gelatin pills, enriched with astaxanthin and Vitamin E.

The declared nutritional value label for these commercial oils is reported in **Table 6**, with specified the content of total n-3 fatty acids, of SDA, EPA, DHA and of astaxantin.

Table 6. Nutritional label of the commercial Calanus, krill, fish and astaxantin rich- oils under exam (data expressed as mg/500 mg of oil).

	Total Omega-3	SDA	EPA	DHA	Astaxanthin
Calanus oil 1	90	20	15	20	0.300
Calanus oil 2	105	38	32	28	0.476
Krill oil	110	nd	60	27	0.050
Fish oil	168	nd	80	53	nd
Astaxanthin enriched oil	nd	nd	nd	nd	4

4.2 *Calanus* collection from caught Atlantic mackerel viscera

Three different batches of Atlantic mackerel (*Scomber scombrus*) were caught between July 24th and July 26th, 2023, in the Norwegian Sea by a fishing vessel owned by Síldarvinnslan, a company specializing in processing pelagic fish for food and feed purposes, and located in Neskaupstaður, a small port town on the east coast of Iceland. The catch, collected using purse seine nets, was stored in below-deck tanks containing seawater at a temperature of 4-5°C. Once the vessel docked, the catch was immediately processed by removing the head, tail, and viscera into separate processing streams. The viscera, once manually collected, were pressed, and the contents were placed in plastic bottles and stored in a walk-in freezer room at -25°C. Approximately 72 hours after manual pressing, the samples were shipped to the Matís laboratories via a domestic flight. The samples were kept in insulated polystyrene containers to minimize temperature fluctuations during the transport. Upon reaching the laboratories, the samples, previously separated by batches of approximately 300 grams weigh, were stored in a freezer set at -80°C until the time of analysis (which began on September 7th, 2023).

4.3. Calanus sample preparation for oil extraction

Samples of the *C. finmarchicus* rich- stomach content, weighing approximately 300 grams per batch, were removed from the -80°C freezer and allowed to thaw overnight in a refrigerator at 0-1°C. The morning after, the samples were blended in a classic kitchen blender to obtain a homogeneous paste (note that some stomachs also contained nematodes, constituting approximately 1-6% of the sample, depending on the batch and homogeneity, that were not ground during this process). The paste was immediately used for moisture content evaluation or extraction of the oils as described in Par. 4.4 and 4.5.

4.3.1 Moisture content

The moisture content (MC) of the Atlantic mackerel stomach blended content samples was determined by calculating the weight loss of the samples during drying (ISO6496, 1999).

Around 3 ± 1.5 g of raw material, emulsion, and solids from the different extraction experiments were weighed, each in a porcelain bowl. The samples were weighed on an analytical scale with 4 decimals, and then placed in glass bowls which had previously been weighed while empty. The samples were spread evenly around the bowl to increase the surface area for evaporation. The bowls were then put into an oven (Gallenkamp hot box oven with a fan, Gallenkamp & Co. Ltd, UK) at 105°C for one night, before being taken out and cooled down in desiccators for a period of 30 minutes. The final weight of the dried samples was then recorded, and the moisture content calculated according to the equation:

$$\text{Moisture content: } \frac{m2-m3}{m2-m1}$$

where m1 = mass of an empty porcelain bowl (g), m2 = mass of the porcelain bowl and wet sample(g), and m3= mass of the porcelain bowl and dried sample (g).

4.4 Preparation of *Calanus* oil by the cold extraction method

This method was chosen based on a previous analysis conducted by Remadi in 2021. *Calanus* samples obtained as described in Par. 4.3 (approximately 10 g) were added to a 50 mL plastic vial with a red cap, and the weight was recorded. Initially, only one trial was conducted per catching date due to the limited material, but since three catching dates were included, this formed a sample triplicate representative of the catching season. The samples were kept on ice until they were centrifuged at 5100 rpm at 4°C for 12 minutes in a centrifuge (model TJ-25, Beckman Coulter, USA). The centrifugation produced 3-4 phases depending on the extraction method. The samples were centrifuged three times to ensure optimal separation and isolation of the phases. The weight of the collected oil was measured and recorded, and the samples were placed in a freezer at -18°C, awaiting further analysis.

4.4.1 Evaluation of the cold-extracted oil yield

The oil yield from each extraction was calculated considering the weight of oil collected, and the initial mass of zooplankton sample, following the formula reported below. Data are expressed as g/100 g of sample.

$$\text{Oil yield (g/100 g sample)} = \frac{\text{Weight of oil collected from each extraction method (g)}}{\text{Initial mass of zooplankton sample (g)}} \times 100 \text{ g}$$

4.5 Preparation of *Calanus* oil by the Bligh & Dyer method

An extraction of lipids based on the Bligh & Dyer method from 1959 was also performed on the *Calanus* samples to allow comparison of the two extraction methods. The samples were added to 250 mL centrifuge bottles, and the weight was adjusted based on the moisture content: the samples contain approximately 80% moisture, so the method suggests adding approximately 25 g of the *Calanus* paste into the bottles. To the samples, 25 mL of chloroform (Sigma-Aldrich Company Ltd, Germany) and 50 mL of methanol (Sigma-Aldrich Company Ltd, Germany) were added, and the mixture was homogenized using a homogenizer (IKA T25 Ultra-Turrax, IKA®-Werke GmbH & Co, Germany) for 2 minutes. Then, additional 25 mL of chloroform were added and mixed for 1 minute, followed by the addition of 25 mL of 0.88% KCl, and the mixture further homogenized for another minute. The samples were then centrifuged for 20 minutes at 2500 rpm at 4°C (model TJ-25, Beckman Coulter, USA). The lower chloroform phase containing the lipids was pipetted and filtered using a Whatman GF/C microfiber filter paper under suction. The contents of the suction flask were transferred to a 50 mL volumetric flask, and any trace of the upper methanol phase was removed. The volumetric flask was filled with chloroform to the 50 mL mark. The contents were poured into 50 mL plastic vials and stored at -80°C awaiting further analysis.

4.5.1 Evaluation of total lipid content

The total lipid content of *Calanus* oil, obtained as above described, was estimated by pouring the lower phase previously recovered, into a screw-top tube. The weight of the tube before and after the addition of the sample was recorded. Any solvent

present was removed on a heating element at 55°C with a stream of nitrogen. The samples were cooled, and their weight was recorded. The weights measured, which corresponds to the g of lipid per 25 g of the homogenized sample were normalized to g/100 g, by multiplying per 4.

4.6.1 Fatty acid composition analysis of marine oils (AOCS Official Method Ce 1b-89)

The fatty acid composition (FAC) analysis was performed on the oils from each extraction and for the commercial competitors' samples, following the AOCS Official Method Ce 1b-89 with minor adjustments. The samples were separated on a Varian 3900 GC equipped with a fused silica capillary column (HP-88, 100 m x 0.25 mm x 0.20 µm film), a split injector and a flame ionization detector, fitted with the Galaxie Chromatography Data System, Version 1.9.3.2 software. The oven was programmed as follows: hold at 100°C for 4 min, increase to 240°C at a rate of 3°C/min, then hold at 240°C for 15 min. The injector and detector temperature were set to 225°C and 285°C, respectively. Helium was used as a carrier gas at the column flow of 0.8 mL/min, with a split ratio of 200:1. The program used is based on the AOAC 996.06 method, and the peaks were identified by comparison with known fatty acid methyl standards (supelco37; Sigma Chemical Co, Ltd).

4.6. Total astaxanthin content evaluation

The total astaxanthin content was measured in the cold extracted *Calanus* oil samples (Par. 4.4), in the Blighand Dyer extracted oils (Par. 4.5) as well as in the five commercial oils. For the Bligh-dyer extracted oils two samples were used: one sample obtained after the evaporation of the chloroform (made as described in section 4.5), performed the same day of the total astaxanthin analysis; a second sample where the evaporation was performed four days earlier, to see any potential astaxanthin variation. The astaxanthin was extracted directly from the oils. After tempering the frozen samples to room temperature, between 0.002 and 0.500 g of sample were added to a 14 mL glass tube, which had been previously marked (two duplicates per the three sampling days). Depending on the colour of the sample, 1.5

to 22.5 ml of acetone were added to dissolve the oil. The absorbance of the solution was then measured in a glass cuvette at 470 nm in a spectrophotometer (Pharmacia Biotech Ultrospec 3000 Pro).

The total astaxanthin content (%) in the samples was obtained using the following equation:

$$\%\ Astaxanthin\ in\ sample = \frac{\text{Absorbance 470 nm}}{\epsilon\ \%}$$

where Є % is the extinction coefficient of astaxanthin, given as Є % = 2100.

The total astaxanthin content expressed in ppm (mg/kg) was obtained by the following equation:

$$Astaxanthin\ in\ sample\ (g/kg) = \frac{(\text{mL acetone} \times \rho\ \text{of solvent x \% astaxantin})}{\text{100 x kg sample}}$$

4.7. Heavy metals and oxidation products evaluation

Heavy metals, especially mercury (Hg), cadmium (Cd), arsenic (As) and lead (Pb), were determined following the NMKL186, 2007 mod. 186 method which is based on an inductively coupled plasma-mass spectrometry (ICP-MS) after pressure digestion.

Peroxide value (PV) and anisidine value (AV) were determined for the extracted and commercial oils based on the ISO 3960:2010 II.D.26 method. The test sample was dissolved in isooctane and glacial acetic acid, and potassium iodide was added. The iodine liberated by the peroxides was determined iodometrically (visually) with a starch indicator and a sodium thiosulfate standard solution. The endpoint of the titration was determined iodometrically (visually).

5 Results and discussion

5.1 Moisture content

The moisture content was assessed for the stomach contents of the Atlantic mackerel following mild blending (Par. 4.3.1). Due to the lack of raw material, only one trial per sampling date was possible. However, the variation in moisture content between sampling days was lower than the variation caused by the analytical method. The thee sampling days were thus considered as triplicates of the analyzed processing stream. As it can be calculated from the results reported in **Figure 10**, the average moisture content of the samples was found to be 79.3 ± 1.0%. It is important to note that these values do not rely on the actual moisture content of ingested *Calanus* but may also originate from residues of stomach fluids (Eysteinsson et al., 2020; Khiari, 2010). The slight differences in moisture content among samples could be attributed to the fact that the viscera processing stream is often treated with excess water. However, it could also be due to the fact that, during the squeezing process, some of the viscera fluids from the stomach surface may have flowed into the samples.

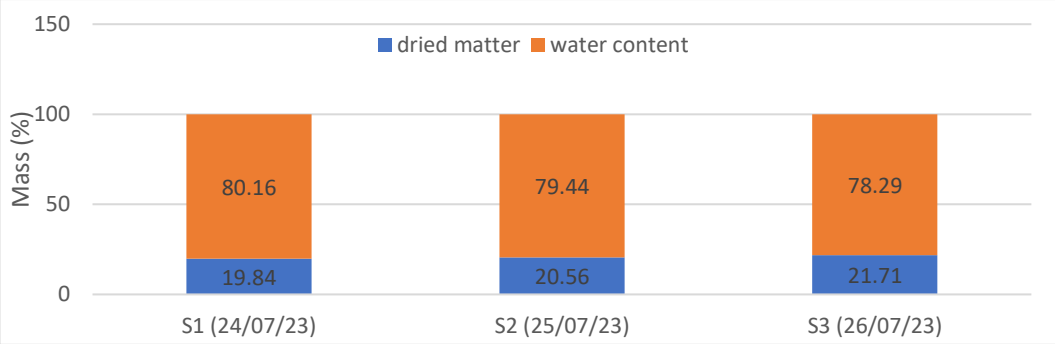

Figure 10. water content % compared to dried matter in the samples after desication.

5.2 Oil and lipid yield from the cold and Bligh and Dyer extraction methods

The samples were extracted from the stomach content mixture using two different extraction methods, the cold and the Bligh and Dyer extraction methods. After separation, the samples obtained with the cold extraction method contained four phase layers: an upper layer of oil, an emulsion layer, an aqueous layer, and a lower layer containing solids (as seen in **Figure 11**).

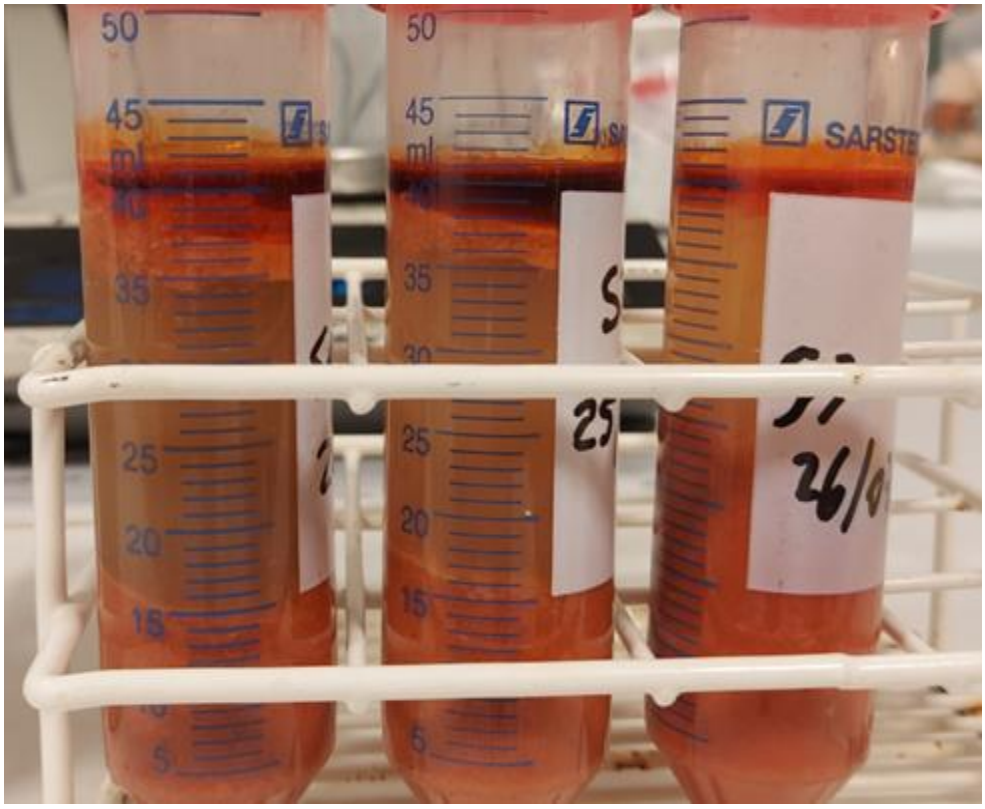

Figure 11. Different layers in Calanus oil obtained from mackerel viscera, using the cold extraction method.

Five centrifugations were performed to separate as much oil as possible from the emulsion, with the understanding that a portion would remain trapped within the solid phase and emulsified phase.

On the other hand, for the Bligh and Dyer extraction method, three distinct layers were obtained: a superficial layer of methanol-water and proteins, an intermediate layer of discolored solid substance, and a bottom layer of oil-chloroform emulsion. From the results obtained from both extractions, the Bligh and Dyer method extracted almost triple the amount of oil compared to the cold extraction (**Figure 12**). In fact, the average value of the three replicates, reported in **Figure 12**, was 6.3 ± 1.3 g for the Bligh and Dyer extraction and 2.2 ± 0.9 g for the cold extraction method. This difference can be attributed to the fact that the Bligh and Dyer method was able to extract oils present in the emulsion layer and, to a lesser extent also from the solid bottom layer.

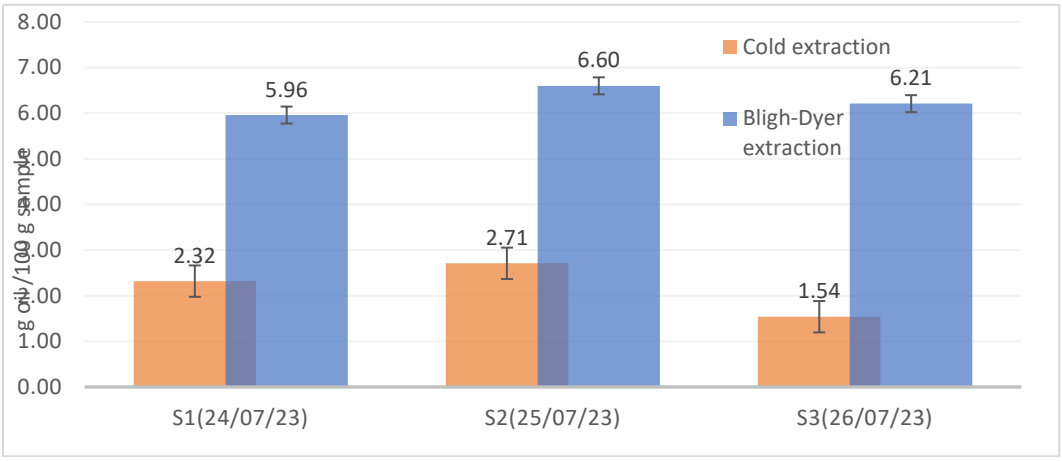

Figure 12. Oil yield from the cold extraction samples and from the Bligh-Dyer extraction samples. Results expressed as g per 100 g of sample.

In terms of time and consumption, the solvent extraction using the Bligh and Dyer method was proven to be more resource-intensive, despite yielding over twice as much as the cold extraction (Remadi, 2021).

This can be attributed to the fact that chloroform and methanol were effective in breaking down the membrane and structural proteins around the copepod's adipose reserves, thereby releasing a greater quantity of lipids from the mixture (Baldursson, 2020; Remadi, 2021). It is worth noting, however, that even the Bligh and Dyer solvent extraction method resulted in oil losses due to oil adhering to the evaporating flasks in the rotary evaporator. All in all, the results, although not many previous studies have been conducted on this particular feedstock (ingested *C. finmarchicus*), the results are highly promising. Unfortunately, the Bligh and Dyer method uses a couple of dangerous compounds, such as methanol and chloroform, which they could remain in the final product, causing several damages to the final consumer. The Bligh and Dyer extraction method can therefore not be used for human consumption applications.

Another observation that can be made on the results of **Figure 12** is that the quantity of oils extracted from the samples varies significantly, particularly when using the cold extraction method. In fact, with the latter, the average percentage of extracted oil (2.19 ± 0.65%) indicates an absolute variability around 7% higher than that measured for the oil extracted by the Bligh and Dyer method (6.17 ± 1.38%). This variability on the results indicates that the extraction method has a huge impact on the total oil extracted, but also seasonal feeding changes have an important cause

on these differences, and, obviously, there is also to consider the unpredictability of the stomach content. Indeed, the first and the third sets of stomach content samples contained the highest amount of other species, meanly represented by 4-8 cm white nematodes, but they reduced the final amount of oil obtained (one of them can be spotted in the bottom right of the third bottle in **Figure 11**).

5.3 Fatty acid composition (FAC)

The fatty acid composition (FAC) of the samples, obtained using both the cold extraction and the Bligh and Dyer method, was evaluated by the GC-FID method (Par. 4.6), and was also compared with the FAC of the commercial oils obtained from various marine sources (**Table 7**).

An example of chromatogram in available in **Figure 13**.

From the analysis conducted, it was observed that the major fatty acids (comprising more than 3% of the total content) of the *C. finmarchicus* based oils were myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1n7), oleic acid (C18:1n9 and the n7 isomer), gadoleic acid (C20:1n11), EPA (C20:5n3), a mix of cetoleic and erucic acid (C22:1n11+9), DPA (C22:5n3), and DHA (C22:6n3). It is quite evident from data reported in the **Table 7** that there is an abundance of polyunsaturated fatty acids (PUFAs), which represent, as a percentage of the total lipid content, approximately 49.4% ±2.2% for cold-extracted samples, and 50.1% ±0.3% for samples extracted using the Bligh and Dyer method. The cold extracted oil had the highest average content of EPA (14.97±0.84%) among all the samples, and the commercial products based on *C. finmarchicus*, while the Bligh and Dyer extracted oil excelled in DPA content (11.86±1.19%). It is interesting to note that the cold-extracted oil has the lowest content of saturated fatty acids (SFAs) (20.9±1.6%) among the animal-derived oils.

Table 7: Average fatty acid content % in the samples and in the commercial oil pills.

Fatty acid %	Industrial oil Bligh-Dyer extr. (S BD)	Industrial oil Cold extr. (S COLD)	Calanus oil 1 (C)	Calanus oil 2 (Z)	Fish oil (F)	Krill oil (K)	Astaxanthin rich oil (A)
C8:0	nd	nd	nd	nd	0.36±0.10	0.49±15	0.35±0.06
C10:0	nd	nd	nd	nd	0.27±0.08	0.33±0.09	0.27±0.05
C11:0	1.87±0.25	2.32±0.51	2.54±0.48	2.30±0.41	nd	nd	nd
C14:0	8.03±0.51	6.89±0.13	8.85±1.21	10.47±0.22	7.35±0.16	9.11±0.53	0.13±0.02
C14:1	0.49±0.02	0.57±0.02	0.55±0.01	0.52±0.01	0.23±0.01	nd	nd
C15:0	0.38±0.04	0.29±0.04	0.41±0.01	0.51±0.01	0.45±0.02	0.29±0.05	nd
C16:0	9.42±0.45	7.12±0.43	6.83±0.49	6.41±0.47	16.22±0.15	19.95±0.95	9.96±0.20
C16:1n7	3.60±0.41	3.97±0.38	2.45±0.05	2.67±0.08	8.88±0.31	4.92±0.82	0.45±0.01
C16:2n4	0.42±0.04	0.47±0.02	0.25±0.01	0.30±0.01	1.18±0.01	0.57±0.07	0.16±0.01
C16:3n4	0.79±0.07	0.84±0.05	0.40±0.03	0.47±0.02	1.84±0.02	0.24±0.09	nd
C17:0	nd	nd	nd	nd	0.17±0.02	nd	nd
C17:1	1.86±0.12	2.16±0.08	0.66±0.04	0.93±0.06	nd	1.23±0.14	0.27±0.01
C18:0	3.06±0.12	2.04±0.38	4.89±0.07	2.04±0.84	3.10±0.11	1.02±0.03	2.87±0.05
C18:1(n9+n7)	3.72±0.23	4.09±0.11	2.22±0.10	2.84±0.29	8.49±0.06	14.62±0.77	37.33±0.21
C18:2n6	0.78±0.07	0.82±0.03	0.73±0.03	0.59±0.03	1.03±0.05	1.72±0.30	40.21±0.14
C18:3n6	0.22±0.01	0.26±0.01	0.23±0.02	0.23±0.01	nd	0.19±0.01	0.18±0.03
C18:3n3	nd	nd	nd	nd	0.25±0.01	1.82±0.26	3.93±0.20
C18:3n4	1.29±0.09	1.40±0.06	1.64±0.09	1.37±0.07	0.74±0.06	nd	nd
C18:4n3	7.75±0.53	8.81±0.90	10.99±0.56	10.79±0.51	3.04±0.31	5.12±0.84	0.40±0.02
C20:0	nd	nd	nd	nd	0.17±0	nd	0.31±0.01
C20:1(n11+n9)	1.86±0.13	2.18±0.16	2.34±0.85	1.98±0.40	0.86±0.06	0.63±0.14	0.12±0.01
C20:2	nd	nd	nd	nd	0.13±0.01	nd	0.61±0.02
C20:3n6	nd	nd	nd	nd	0.15±0	0.06±0.11	nd
C20:3n3	nd	nd	nd	nd	nd	nd	nd
C20:4n6	0.49±0.12	0.59±0.31	1.42±0.32	0.99±0.24	0.77±0.03	0.34±0.08	0.31±0.01
C20:4n3	0.67±0.02	0.75±0.02	0.81±0.04	0.91±0.12	0.69±0.05	0.40±0.03	nd
C20:5n3(EPA)	14.72±1.02	14.97±0.84	9.41±0.75	10.37±0.67	18.87±0.40	20.93±1.18	0.17±0.01
C22:0	2.67±0.19	2.95±0.03	2.08±0.05	2.13±0.05	nd	nd	0.55±0.01
C22:1n (11+9)	8.12±0.89	8.78±0.46	11.65±0.46	12.27±0.57	0.13±0.12	0.62±0.10	nd
C22:2	0.49±0.02	0.50±0.05	0.33±0.02	0.41±0.01	0.75±0.06	0.72±0.15	nd
C22:4n6	nd	nd	nd	nd	0.31±0.01	nd	nd
C22:5n3(DPA)	10.74±0.84	8.60±1.46	6.96±0.86	6.27±0.65	1.87±0.02	0.40±0.02	nd
C22:6n3(DHA)	11.87±1.19	11.48±1.11	13.88±0.59	14.82±0.51	13.02±0.50	10.13±0.05	nd
C24:1n9	1.18±0.21	1.75±0.12	2.78±0.07	3.04±0.05	0.33±0.01	0.12±0.10	nd
SFA	25.4±0.3	20.9±1.6	25.6±1.8	23.9±0.3	28.10±0.23	37.05±1.95	15.15±0.34
MUFA	20.4±0.6	23.5±0.6	22.7±1.1	24.3±0.9	21.58±0.60	16.85±0.81	37.62±0.21
PUFA	50.1±0.3	49.4±2.2	47.0±1.6	47.3±1.7	44.63±0.33	42.06±1.93	45.80±0.13

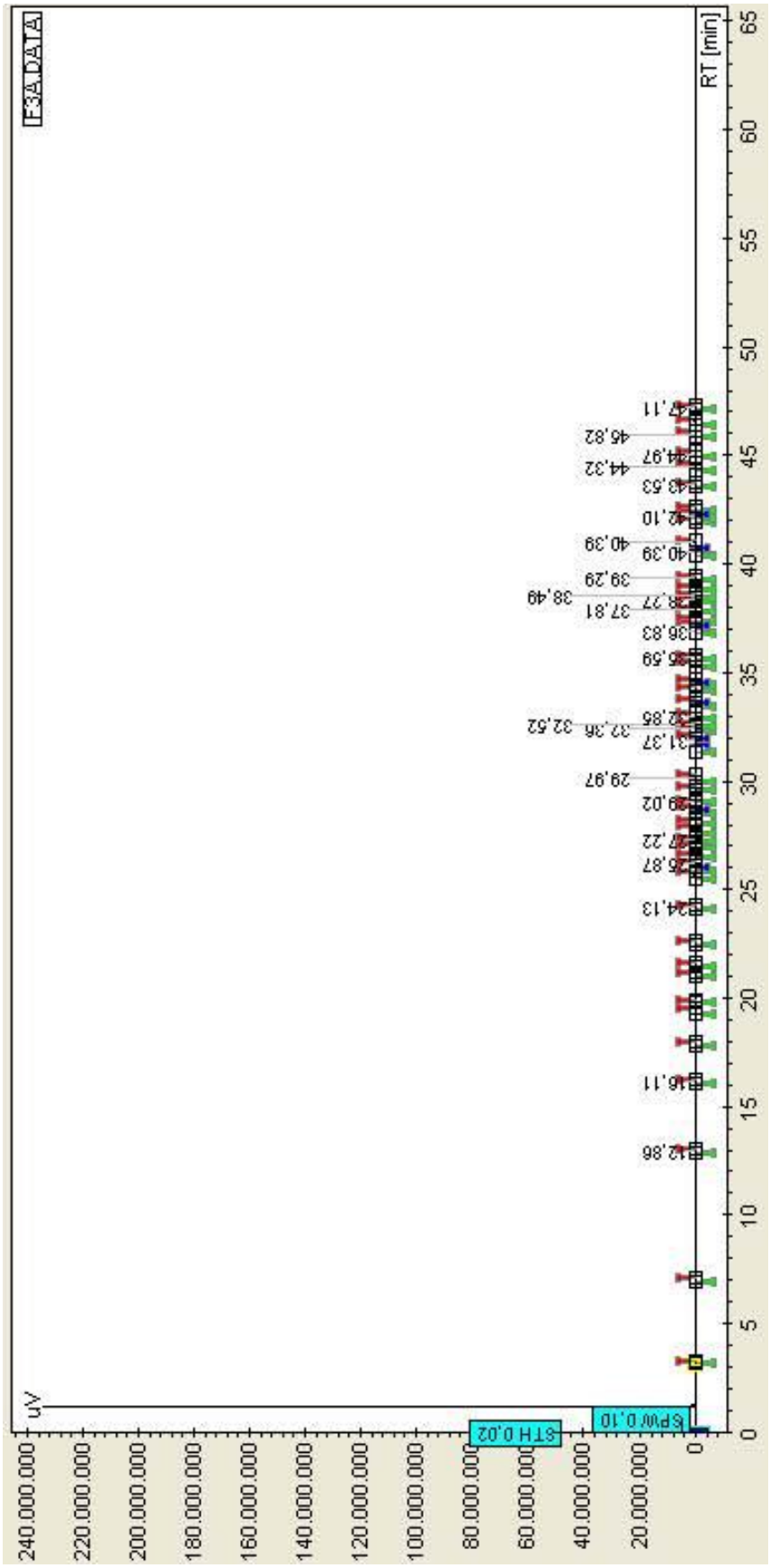

Figure 13. Example of GC-FID of marine oil

5.4 Total astaxanthin

The total astaxanthin content was measured for both the oils extracted using different methods, as well as the previously mentioned for the commercial oils reported in **Table 6** (see **Figure 14, Table 8**). Concerning the initial coloration, the extracted oils and *Calaunus* oil 1 appeared with a dark orange color, while the *Haematococcus pluvialis* oil displayed an intense red colour, due to the significant addition of astaxanthin. The *Calanus* oil 2 and the Krill oil had a lighter orange colour, and finally, the fish oil appeared with a pale yellow colour.

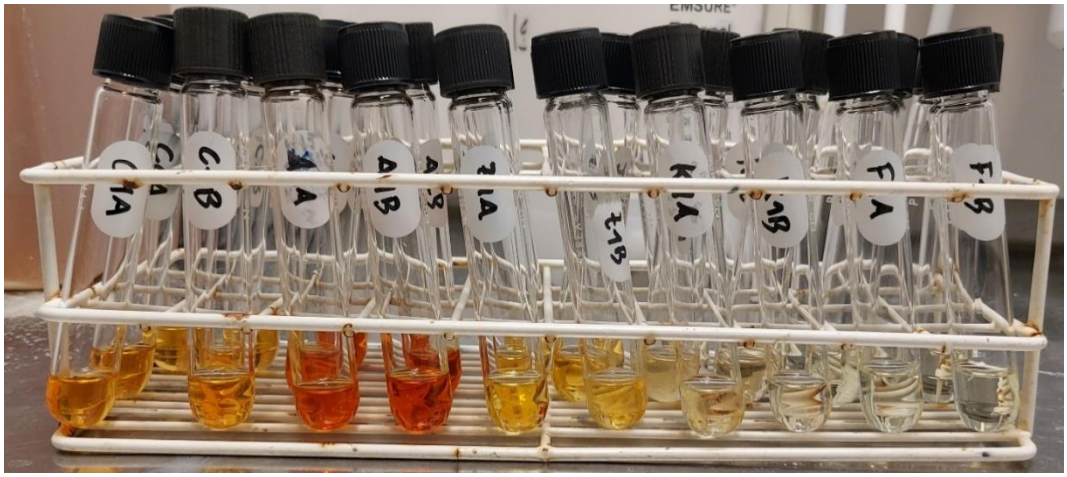

Figure 14. Glass tubes containing a small amount of oil mixed with acetone.

Table 8. Astaxantin content in the extracted oils (S samples) and in the commercial ones.

Compound	Industrial oil Bligh-Dyer extr.	Industrial oil Cold extr.	Calanus oil 1	Calanus oil 2	Krill oil	Fish oil	Astaxanthin rich oil
Astaxanthin (µg/g)	888±145	912±138	741±100	442±49	135±37	81±13	5706±720
Astaxanthin (µg/500 mg capsule)	444±73	456±69	371±50	221±24	67±19	41±7	2853±360
Claimed astaxanthin content per capsule (500 mg)	-	-	300	476	50	nd	4000

Excluding the enriched oil, which had a significantly higher average astaxanthin content of 2853 ± 360 µg/500 mg, the other oils generally reflected their natural coloration, as you can compare the **Figure 14** with the **Table 8**. Naturally, fish oil could not have very high astaxanthin concentrations, as it is not naturally associated

with this compound. Indeed, fish oil had the lowest quantitative astaxanthin content, at 41 ± 7 µg/500 mg It should be considered that the main fish used to obtain liver oil is cod which eats other fish during the adult phase, instead of zooplankton (Cohen et al., 1990). This was followed by Krill oil with 67 ± 19 µg/500 mg, which falls perfectly within the values claimed on the label, like the *Calanus* oil 1. However, the *Calanus* oil 2 and the Krill oil did not reflect the declared data. Indeed, after 2 different runs using each time two duplicates, the results showed that these two oils had between 28.7 and 45.8% less astaxanthin than claimed on the packaging. The other oils stood out, with the Bligh and Dyer extracted oil on the same day as the astaxanthin assessment having an average value of 444 ± 73 µg/500 mg. It is worth noting that samples extracted with the Bligh and Dyer method five days before the astaxanthin analysis had a lower astaxanthin content of about 15%, possibly due to sensitivity to light and oxidation at high temperatures during extraction and storage (Pedersen, 2016). Nonetheless, the content obtained from the cold-extracted samples remained high at 456 ± 69 µg/500 mg, more than the other *Calanus* oils.

5.5 Heavy metals

Heavy metal amounts (mg/kg), evaluated by the method described in Par. 4.8, and reported in **Table 9**, indicate that both the cold-extracted and the Bligh and Dyer extracted oils have nearly double the amount of arsenic compared to the two commercial products based on *C. finmarchicus* (*Calanus* oil 1 and *Calanu*s oil 2). While these levels may seem relatively high, it is important to consider that if each pill contains one gram of oil and one pill is taken daily, the estimated daily intake of arsenic is around 22 µg. This value remains below the daily limit set by the European Food Safety Authority (EFSA) of 15 µg per kilogram of body weight (EFSA, 2021). A similar observation applies to cadmium, with the levels in both extraction methods being equal to, and lower than, those found in the commercial products. This suggests that the consumption of these oils is unlikely to result in cadmium intake exceeding established safety limits. It should be noted that the oil extracted using the Bligh and Dyer method and *Calanus* 1 oil have lead levels nearly forty to fifty times higher than the other two *Calanus* oils. This could be

explained by the fact that, during the Bligh and Dyer extraction, the heavier solution, composed of oil and chloroform, is gathered, and during centrifugation, lead may accumulate in this phase due to its high density or, maybe, the Bligh and Dyer method breaks some metallothioneins, specific metal-binding proteins, usually stored in the muscular fibers. The difference among the two commercial samples might be simply due to natural variability in the zooplankton stocks.

Table 9. Heavy metals amount in the extracted oils (Bligh-Dyer and cold) and in the five commercial ones (mg/kg).

Heavy Metals (mg/kg)	Industrial oil Bligh &Dyer extr.	Industrial oil Cold extr.	Calanus oil 1	Calanus oil 2	Krill oil	Fish oil	Astaxanthin rich oil
Mercury (Hg)	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
Cadmium (Cd)	0.180±0.061	0.033±0.011	0.413±0.016	0.292±0.020	<0.005	<0.005	<0.005
Lead (Pb)	0.143±0.06	<0.03	0.113±0.039	<0.03	<0.03	<0.03	<0.03
Arsenic (As)	21.960±3.425	22.010±7.841	10.420±0.046	11.290±0.147	<0.03	5.730±0.730	<0.03

5.6 Oxidation products

The oxidation status of the seven oils were analyzed for p-anisidine value (AV), peroxide value (PV) and total oxidation (TOTOX). The PV evaluates the primary oxidation products, while AV estimates the secondary oxidation products. Results are reported in **Table 10**.

Table 10. Oxidation status in the Cold extracted oil, in the Bligh-Dyer one and in the other five commercial oils.

Oil	Peroxide value mEq/kg (PV)	p-Anisidine value (AV)	Total Oxidation (TOTOX)
Industrial oil Bligh &Dyer extr.	3.07±0.22	12.47±2.83	18.59±2.41
Industrial oil cold extr.	Not detected	2.80±0.20	2.80±0.20
Calanus oil 1	1.17±0.47	6.37±1.23	8.71±0.97
Calanus oil 2	1.37±0.15	<0.05	2.73±0.31
Krill oil	2.49±0.36	4.57±0.77	9.55±1.21
Fish oil	Not detected	13.63±2.97	13.63±2.97
Astaxantin rich oil	1.97±0.03	12.77±4.47	16.71±4.41

From data of **Table 10**, it is easy to see that all the oils are within the limits the Codex Alimentarius commission recommends, which values for human consumption for PV and AV for fish oils are respectively less than 5 mEq/kg and less than 20 (Remadi, 2021). This indicates a low level of oxidation of the oils. The relatively high values of AV in the oils could be interfered by the high concentrations of astaxanthin. Perhaps, since there is no astaxanthin in fish oil and a high AV of the fish oil was still obtained, this is unlikely to apply to the fish oil. However, the astaxanthin may interfere with the AV measurement in the other samples, and the intense colour of astaxanthin might interfere with the measurement and results. Therefore, the p-anisidine test is not suggested for measuring secondary oxidation in oils with high levels of carotenoids like astaxanthin (Ismail et al., 2016).

6. Conclusions

Nowadays, the use of byproducts from fishing, derived from the capture of fish, is a highly debated and contentious issue concerning the reuse of waste materials in order to increase the sustainability of fisheries. An example comes from the oil obtained from *C. finmarchicus* ingested by caught specimens of Atlantic mackerel. In fact, studies involving this branch of zooplankton byproduct recovery are still underexplored and not widely disseminated on a global scale. The utilization of these byproducts can certainly represent a less impactful alternative to the direct caught of zooplankton for production of the oil.

This study has demonstrated how economical and fast methods for oil extraction, such as cold extraction and the Bligh and Dyer method, can lead to more than acceptable results in terms of the quantity of extracted oil. However, it should be noted that the latter extraction method resulted in a higher final oil amount but also contributed to the formation of increased oxidation compounds.

Cold extraction, while yielding a lower amount of oil, allowed for the extraction of significant quantities of mono and polyunsaturated fatty acids, especially EPA and DHA. Moreover, this method preserved the majority of lipids from oxidation, proving to be the best method for extraction without compromising the final quality.

This work also compared the extracted oils with other oils on the market, derived from different marine matrices. The results showed that the produced oils had compositional and qualitative characteristics similar to the commercial products, if not superior, especially concerning the oil extracted using the cold method.

Regarding the astaxanthin found in the oils, it emerged that the Bligh and Dyer method had minimal oxidative effects on the carotenoid. Surprisingly, the quantity of astaxanthin was higher than in the commercial *Calanus*-based products. It was also observed that two of the commercial products had lower levels of astaxanthin than those declared on the label.

A serious concern is the detection of heavy metals. Unfortunately, both the oils extracted in this study and the commercial *Calanus*-based oils had arsenic levels higher than the recommended levels, raising concerns for human health. The Bligh and Dyer extraction method also revealed extracted lead levels up to 50 times higher than with the cold method, but still in the allowed limit range.

Given the significant difference observed in the two extracted oils, the astaxanthin quantity in the oils marginally influences the total content of oxidation products, considering the residues in commercial products (especially fish and krill oils, which had virtually negligible astaxanthin levels). Further analysis is required to determine the feasibility and quality of the extracted oils for human consumption, especially concerning potential solvents and oxidation products, that may be present in the oil extracted using the Bligh and Dyer method, together with the presence and amount of relevant heavy metals.

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