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The LimoFish Circular Economy Process for the Marine Bioeconomy

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The outcomes of applying the zero-waste extraction "LimoFish" process based on defatting fish processing waste with limonene to leftovers of European sardine (*Sardina pilchardus*) and European anchovy (*Engraulis encrasicolus*), compared to conventional extraction with oil-derived solvents such as *n*-hexane and with petroleum ether, show that the process has general applicability. Meeting the principles of green extraction and those of the marine biorefinery requiring high process efficiency, the process establishes an "innovation through integration" circular economy production route enabling the marine bioeconomy.

Introduction

Omega-3 lipids (ω-3 or *n*-3: first double bond from the methyl end group, ω end, located on the third carbon atom) such as the triglycerides of eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:5 ω-3) are human essential nutrients abundant in marine oils.[1] These polyunsatured fats are key hormone precursors and moderate the propensity for arachidonic acid cascade overreactions when ω-6 (*n*-6, first double bond from the ω end between 6th and 7th carbon atom) lipids dominate, defending tissue against oxidative stress.[2,3] After over 30,000 studies since Bang's and Dyerberg's landmark paper on plasma lipid and lipoprotein patterns in Greenlandic Inuit in 1971,[4] the role of these long chain polyunsaturated fatty acid (PUFA) triglycerides in human health has been, at least in part, understood.[1,2,3]

Omega-6 lipids, in their turn, are abundant in most vegetable oils, pork, lard and turkey meat. So called "western" diets raised the ω-6/ω-3 fatty acid ratio from 1:1 in 1900 to today's 20:1 (15:1 in Europe and 25:1 in the United States of America) creating the conditions of chronic inflammation due to continuous production of pro-inflammatory lipid mediators.[5] Perhaps not surprisingly, therefore, fish oil, highly refined and generally made available as concentrate of marine omega-3 lipids in ethyl ester form,[6] has become one of the most popular dietary supplements in the world, particularly in Europe and North America.

Besides to dietary supplements, fish oil enriched in EPA and DHA is added to a number of finished products including food and beverage, pet food, infant formula, and pharmaceuticals, ultimately creating a global market that in 2021 reached $44.1 billion.[7] Health benefits of omega-3 supplementation include (but are not limited to) eye, brain, and heart protection. For instance, 38 randomized controlled trials support the hypothesis that omega-3 lipids reduce cardiovascular mortality and improve cardiovascular outcomes.[8]

Creating what Winkler termed "the most hidden of all hidden hungers",[9] more than 80% of the world's population in 2010 failed to meet even the lowest recommended daily intake of 250 mg.[10] Considering the latter, supplying said daily dosage 6.5 billion people would require a daily production of 1,625 t of EPA and DHA (> 593,000 t/a), not including the demand of fatty acids by hatcheries.[6] Current annual production of EPA and DHA enriched oils does not exceed 85,000 t, which explains why these long chain PUFAs need to be sourced by algae, land-based plants and from fish discards and no longer from fish only.[9]

Chiefly derived from the world's most fished species, the Peruvian anchovy (*Engraulis ringens*) yearly caught in over 7 million tonnes mostly to produce fishmeal for fish farming, fish oil for dietary supplements is also sourced from sardine, herring, mackerel, salmon and even krill (in phospholipid form, in this case). In general, about 5% of world's fish oil production is used to extract omega-3 nutrients for use in dietary supplement products.[6]

These demographic, health and food global trends are driving unsustainable anchovy fishing. Since 2014 the Peruvian government established numerous suspensions of one of two annual anchovy fishing seasons. In 2023, the same government cancelled its first of two anchovy fishing seasons "causing major supply constraints for the omega-3 fatty acids EPA and DHA".[11] For comparison, in 2023 only approximately 4,700 tonnes of crude Peruvian anchovy oil were produced ahead of the

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cancellation, namely 3% of the 155,000 t produced in the same area in 2021.[11]

In general, supply of EPA and DHA from aquatic environments is lower than global demand. Using a comprehensive optimization approach (model) of global aquatic omega-3 supply chain, Shepon and co-workers have lately shown that omega-3 supply could increase by 50% (reaching 630,000 t y^{-1}).[12] According to said model, feed inputs should shift to produce species that have the highest omega-3 content, diverting other production flows towards direct wild fish consumption, improving by-product utilization, and reducing waste at the retail and consumer level.[12]

The global need to shift production of fish oil from fish to fish by-products to recover and transfer key essential nutrients from the sea to the human and animal food chains has recently driven plentiful research efforts.[13,14,6] Reviewing the field, however, in 2014 Olsen and co-workers concluded that it is unlikely that fish processing by-products can be used to produce "high-priced products",[14] chiefly due to "high costs of isolating specific components often present in small amounts".[14] A few years later, though, companies in Germany and Japan started the successful production of fish oil and fishmeal (fish proteins) derived from the fish industry leftovers such as head, skin, and trimmings, chiefly based based on mechanical processes to separate the solid and liquid (oil) phases.[6]

The major advancement required for value-added bioproductions using fish waste requires "innovation through integration",[15] namely the ability to apply the best available manufacturing methods in plants able to collect and upgrade different types of fish discards. Such process versatility and high efficiency, we show herein, is shared by the "LimoFish" circular economy process based on defatting and stabilizing fish processing waste with limonene. In the following, indeed, we report the outcomes of applying the aforementioned zero-waste extraction process to leftovers of European sardine (*Sardina pilchardus*) and European anchovy (*Engraulis encrasicolus*) carried out independently in our laboratories.

Results and Discussion

Methods

Fish Oil Extraction Process with Limonene

In one experiment carried out in the CNR Labs in Palermo, anchovy oil was extracted from anchovies caught in Sicily in September 2023 using the same procedure reported in 2019.[16] In brief, an electric blender was used to mix and homogenize the frozen anchovy leftovers along with an aliquot of *d*-limonene. A sample (200 g) of anchovy leftovers previously frozen at −20 °C, was placed in the jar of an electric blender and added with limonene (200 mL, Acros Organics, 96% pure and stabilized) refrigerated at 4 °C. After a few min grinding, a semi-solid grey purée was obtained. The resulting mixture was poured in a beaker, added with another limonene aliquot (200 mL), sealed with aluminum foil, and left under stirring at room temperature (see video available online).[17] After 24 h stirring was stopped and the solid-liquid mixture added to centrifuge tubes for subsequent centrifugation at 10,000 rpm carried out at 4 °C.

The yellow supernatant liquid of each tube was collected to recover the biobased solvent via evaporation under reduced pressure (40 mbar) using a rotary evaporator with the water bath temperature set at 90 °C. Eventually, we obtained 3.0 g of a transparent oil ("AnchoisOil") colored in orange containing a 8 wt% residue of limonene.

In another experiment series carried out at the University Mediterranea of Reggio Calabria with anchovy fillet leftovers obtained in Calabria, the extraction was performed with a frozen anchovy leftover:limonene ratio (w/w) of 1:2. An electric blender was again used to mix and homogenize the frozen leftovers along with an aliquot of *d*-limonene. In detail, 100 g of frozen anchovy waste in the blender jar of the electric blender was added with a first aliquot of 100 g of limonene (Acros Organics, 96%) refrigerated at 4 °C. After grinding twice a semi-solid grey purée was obtained which was extracted with second aliquot of 100 g of *d*-limonene.

A simple solid-liquid extraction was performed by magnetically stirring the mixture kept in the beaker sealed with aluminum further coated with parafilm and left under stirring at 700 rpm at room temperature for 21 h. The yellow supernatant thereby obtained was transferred to the evaporating balloon of a rotary evaporator (Büchi Rotovapor R-200 equipped with a V-700 vacuum pump and V-850 vacuum controller) to remove the solvent under reduced pressure (40 mbar) at 90 °C. Limonene solvent could be almost entirely recovered via evaporation under reduced pressure, ready for use in subsequent extraction runs. After evaporating limonene, we obtained 4 g of AnchoisOil. The same procedure was performed for the lyophilized anchovy leftovers, in this case the lyophilized leftover:solvent ratio (w/w) was set at 0.3:2 (ratio based on water percentage in anchovy fillet leftovers (equal to 70%), that affords a 0.3:1 weight ratio between non lyophilized and lyophilized leftovers).

Soxhlet Extraction Procedure With Petroleum Ether

Soxhlet extraction was performed using a 20 g portion of frozen anchovy fillet leftovers mixed and homogenized with an electric blender. The mixture was transferred into a cellulose extraction thimble and inserted into a Soxhlet assembly (Behr Labor Technik behrotest R104 S Soxhlet Extractor Unit) fitted with a 250 ml flask. A 150 g portion of petroleum ether was added and the mixture heated for 4 h at 50 °C using an isomantle. The extract was concentrated using a rotary evaporator under reduced pressure (40 mbar) at 60 °C and then placed in a oven (set at 100 °C) overnight. We obtained 0.2 g of oily extract. The same procedure was performed for the Soxhlet extraction of fish oil from lyophilized leftovers, using a 3.5 g portion of lyophilized anchovy waste.

Fish Oil Extraction Process With Hexane

The solid-liquid extraction was performed with a frozen anchovy leftovers:hexane ratio (w/w) of 1:2. In detail, 100 g of frozen anchovy waste were mixed with an electric blender. After grinding twice a semi-solid grey puree was obtained that was extracted with 200 g of high purity *n*-hexane (Honeywell Chromasolv, for HPLC, ≥95.0%). A simple solid-liquid extraction was performed by magnetically stirring the mixture kept in the beaker sealed with aluminum, further coated with parafilm and left under stirring at 700 rpm for 21 h at room temperature. The supernatant thereby obtained was transferred to the evaporating balloon of a rotary evaporator (Büchi Rotovapor R-200 equipped with a V-700 vacuum pump and V-850 vacuum controller) to remove the solvent under reduced pressure (40 mbar) at 60 °C, eventually obtaining 4 g of anchovy oily. The same procedure was performed for the lyophilized leftovers, using a 2 g portion of lyophilized anchovy fillet leftovers with a frozen leftover:hexane ratio (w/w) of 1:20.

GC-MS Analysis

Using a gas chromatograph equipped a flame induction detector (GC-FID) we carried out the analysis of different fish oils obtained starting from European anchovy (*Engraulis encrasicolus*) fillet leftovers, European sardines (*Sardina pilchardus*) leftovers. We also analyzed the refined fish oil contained in commercial omega-3 capsules (OMEGOR Vitality 1000, containing 80% EPA/DHA in triglyceryde form in 2:1 ratio) made with fish oils extracted from anchovies and sardines caught in open water and not from farmed fish.[18] We briefly remind that a refined fish oil used to manufacture omega-3 capsules typically contains about 30% omega-3 fatty acids (18% in EPA and 12% in DHA) in ethyl ester form obtained after trans-esterification reaction of the oil in natural form with ethanol, followed by molecular distillation.[6] Today's best omega-3 dietary supplements, however, contain EPA and DHA in triglyceride form in highly concentrated 70% active ingredient concentration.[19]

The sample preparation was carried out according to the standard method for olive oil analysis, as reported below. A 0.1 g sample of the oil was placed in a 5 mL screw-capped vial. The vial was thus added with a 2 mL aliquot of heptane followed by mixing, after which a 0.2 mL aliquot of a methanolic solution of KOH was added. The vial was tightly closed with a cap equipped with a PTFE gasket, and shaken vigorously for 30 s. The mixture was allowed to settle until the top of the solution became clear. The upper layer containing the fatty acid methyl esters (FAMEs) was decanted affording a FAME solution in heptane ready for the analysis.

The composition of fatty acids was examined using a gas chromatography mass spectrometry (GC-MS) and gas chromatography with ionization flame detector (GC-FID) both from Shimadzu. In the qualitative analysis a QP2010 Ultra GC-MS column was used, whereas for chromatographic separation a capillary column SP-2380 (Supelco, 100 m×0.25 mm×0.2 μm) was employed. In each case, He of chromatographic grade purity (>99.999%) was used as carrier gas. The oven temperature was initially maintained at 165 °C for 8 min, followed by a gradual increase at 2° C/min to reach 210 °C. This temperature was kept for 45 min. The total run time for the analysis was set at 75 min. The injection temperature was set at 250 °C, and the injection mode to split with a split ratio of 38. Mass scan spectra were recorded in the range of 20–500 amu using the electron ionization (EI) source at 70 eV. The MS ion source and interface temperature were both kept at 270 °C, and a solvent cut time of 0.5 min was selected. The control of equipment and data acquisition was managed through the Shimadzu GC-MS Solution software. The identification of compounds was conducted comparing the mass spectra of unknown peaks with those stored in databases such as the National Institute of Standards and Technology library (NIST, version 2011), with a similarity threshold of over 80%. A solution of 26 FAMEs in 2,2,4-trimethylpentane (NIST) was also used to confirm the peak identification.

For the quantitative analysis a GC-FID Tracera (Shimadzu) was used. The separation was conducted using a MEGA-10 capillary column (100 m×0.25×0.2 μm, from Mega Srl, Legnano, Italy) with the same technical and analytical specifications mentioned above. Hydrogen (flow of 40 mL/min) and air (flow of 400 mL/min) were used to feed the flame detector. Compared to the GC-MS protocol, the GC-FID analytical procedure was retained nearly unvaried: the oven temperature was initially kept at 165 °C for 8 min, followed by a gradual increase at 2° C/min rate to reach the 210 °C temperature that was kept for 35 min. The total run time for the analysis was set at 65 min. The injection temperature was set at 250 °C, and the injection mode to split with a split ratio of 150. The Shimadzu GC solution software was used to operate the instrument and perform the data analysis. Quantification of each analyte was carried out using the internal normalization method, expressing the result as a percentage of the area of each individual compound to the total area obtained by summing the individual areas of all the eluted compounds.

Peroxide and Acid Value Assessment

Measurement of peroxide value (PV) was carried out by the iodine titration method. In brief, a 0.2 g sample of AnchoisOil extracted from anchovy fillet lefotvers after brief (24 h) storage at −20 °C was dissolved in 25 mL of acetic acid:chloroform (3:2 v/v) solution, added to 1 mL of saturated KI solution and kept in the dark for 10 min. The mixture was added with 30 mL ultrapure water and the resulting solution titrated with 0.01 N sodium thiosulfate, using 1 mL of starch solution (1% w/v) as indicator until the dark blue colour disappeared. A blank test was conducted using ultrapure water. The peroxide value (PV) is reported as meqO_2/kg of oil calculated according to Eq.1:

$$PV = \frac{(V_{tit} - V_{bl}) \times Fx1000}{W} \quad (1)$$

where V_{tit} and V_{bl} are the volume of 0.01 N sodium thiosulfate necessary to titrate the oil and the blank solution, respectively, F is concentration of sodium thiosulfate solution and W the weight of oil expressed in g.

The analysis was performed in triplicate and results reported as means ± SD ($n = 3$). For the assessment of acid value (AV) 0.1 g of AnchoisOil were dissolved in 10 mL of *n*-hexane. The resulting mixture was titrated with a 0.02 N potassium hydroxide solution in ethanol using 1% phenolphthalein as indicator. Titration continued until the violet colour lasted for 30 s. A blank test was conducted using pure ethanol. The acid value, in mg_{KOH}/g of oil, was calculated according to Eq.2:

$$AV = \frac{(V_{tit} - V_{bl}) \times Fx56.11}{W} \quad (2)$$

where V_{tit} and V_{bl} are the volume of 0.02 N KOH necessary to titrate the oil and blank solution, respectively, F is the titer of KOH solution, 56.11(g/mol) is the molecular weight of KOH, and W the weight of oil in g. The analysis was performed again in triplicate and results reported as means ± SD ($n = 3$).

Comparative Outcomes

Results in Table 1 show the outcomes of the comparative fatty acid analyses of a commercial omega-3 food supplement, as well as of fish oils extracted from European sardine (*Sardina pilchardus*) and European anchovy (*Engraulis encrasicolus*) leftovers (including anchovies caught in different months of the year) using *d*-limonene as green extraction solvent in our Labs in Sicily and in Calabria. Scheme 1 illustrates the LimoFish process on laboratory scale.

The analysis confirmed that the commercial omega-3 dietary supplement is a fish oil with 82% content in EPA (entry 24 in Table 1) and DHA (entry 31), with EPA:DHA ratio of ~ 2 (1.93). The results of the independent analysis of AnchoisOil carried out in Calabria confirm the composition measured in our Labs in Sicily in 2019,[16] with DHA (13%) and EPA (7%) relative amounts substantially similar to those (12.39% and 5.4%, respectively), and virtually the same relative amount of palmitic acid (C16:0) (30% measured in Calabria and 33.5% measured in Sicily).

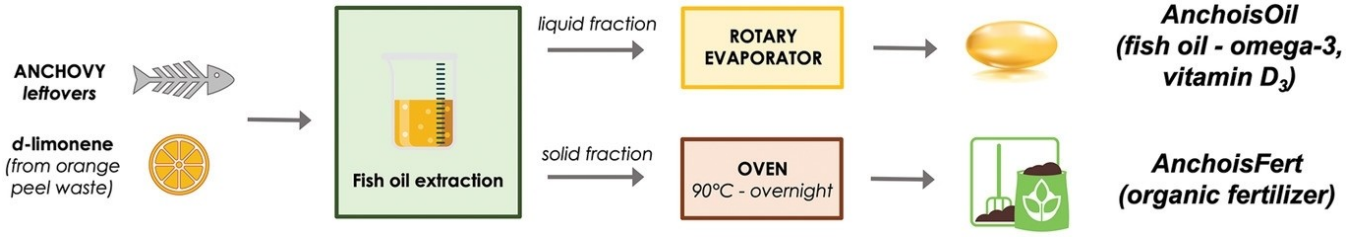
Extracting the AnchoisOil with the orange-derived terpene from anchovies caught in December, March and April resulted in substantial changes in the oil fatty acid composition. The amount of EPA nearly doubled from 7% in December to 11% in April. That of DHA peaked at 20% for the oil from anchovies caught in March and decreased to 15% for those caught one month later. The amount of palmitic acid for anchovies caught in Calabria in March (18%) was nearly half of that measured in anchovies caught in Sicily on late July (30%). An inverse trend was observed for myristic acid (C14:0), which peaked in anchovies caught in Calabria in March (12%). Finally, the amount of oleic acid (C18:1*n*–9) varied in a relatively narrow range between 15% for anchovies caught in December and 11% for the fish caught on April.

Showing the broad applicability of the method, extraction of sardine leftovers with *d*-limonene resulted in a fish oil even richer in DHA (24%) and EPA (13%) which also contained docosapentaenoic acid (DPA, entry 30) omega-3 fatty acid in 2% amount, namely half the value of sardine oil enriched in DPA obtained via multiple fractionation using supercritical CO_2 of hexane-extracted oil (adsorbed on silica) derived from sardine heads and tails.[20]

The sardine oil obtained with limonene also contains substantial amounts of palmitic (21%) and oleic (8%) acids. Finally, both sardine (3%) and anchovy oils (2–3%) extracted with limonene at room temperature contain relatively good amounts of vaccenic (C18:1*n*–7) and palmitoleic (C16:1*n*–7) acids, two monounsaturated fatty acids that, likewise to oleic acid, provide substantial health benefits. Oleic acid regulates the underlying causes of insulin resistance and type 2 diabetes mellitus.[21] Palmitoleic acid lowers blood lipid, enhances the body's sensitivity to insulin, reduces immunometabolic disorders, and promotes collagen synthesis.[22] Vaccenic acid (C18:1*n*–7t), a positional and geometric *trans* isomer of oleic acid, is epidemiologically associated with lower adiposity, diabetes risk, and systemic inflammation.[23]

Results in Table 2 summarize the results of the GC-FID fatty acid analyses for anchovy oil (D010 Hex) extracted on April 2021 with *n*-hexane at room temperature from the same anchovy leftovers of sample PD010, as well as on May 2021 from anchovies via the Soxhlet method with petroleum ether (PD010 Sox). Finally, "Lyo-Lim", "Lyo-Hex" and "Lyo-Sox" refer to anchovy oils extracted at room temperature from lyophilized anchovies with, respectively, *d*-limonene (on May 2021), *n*-hexane (on February 2021) and via Soxhlet method using petroleum ether.

Confirming the outcomes of calculations showing that *d*-limonene is a better solvent for extraction of triglycerides of oleic acid,[24] extraction of anchovy leftovers with *n*-hexane



Scheme 1. The LimoFish process on laboratory scale.

Table 1. Fatty acids (in methyl ester form for the analysis) in commercial omega-3 dietary supplement and in anchovy and sardine oils extracted from anchovy or sardine leftovers using *d*-limonene in different months of the year.*

Entry	Compound	Capsule (%)	PD000 (%)	PD006 (%)	PD009 (%)	PD010 (%)	Sardine (%)
1	Myristic acid, methyl ester	–	3	11	12	7	6
2	Pentadecanoic acid, methyl ester	–	2	2	1	1	1
3	Palmitic acid, methyl ester	–	30	22	18	23	21
4	Methyl palmitelaidate	–	1	–	–	1	1
5	Methyl petroselinate	–	–	–	–	–	–
6	Palmitoleic acid, methyl ester	–	6	3	5	6	5
7	Margaric acid methyl ester	–	2	2	2	1	1
8	6-Hexadecenoic acid, 7-methyl,methyl ester (*Z*)	–	1	1	1	1	1
9	Stearic acid, methyl ester	–	6	5	4	4	–
10	Oleic acid, methyl ester	1	14	15	10	11	8
11	*trans*-Vaccenic acid, methyl ester	–	3	2	3	2	3
12	Nonadecanoic acid, methyl ester	–	–	–	–	–	–
13	Linoleic acid, methyl ester	1	3	2	3	2	2
14	9,12-Hexadecadienoic acid, methyl ester	–	–	–	–	–	–
15	Arachidic acid methyl ester	–	–	–	–	1	1
16	Gondoic acid, methyl ester	–	3	1	1	1	1
17	Linolenic acid, methyl ester	1	2	1	1	2	2
18	Methyl stearidonate	2	1	1	3	3	2
19	*cis*-11,14-Eicosadienoic acid, methyl ester	–	–	–	–	–	–
20	Behenic acid, methyl ester	–	–	–	–	–	–
21	*cis*-10-Nonadecenoic acid, methyl ester	–	3	–	2	1	–
22	Arachidonic acid methyl ester	2	–	2	1	1	1
23	Methyl 8,11,14,17-eicosatetraenoate	2	–	1	1	1	1
24	5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-*Z*)	54	7	7	10	11	13
25	Methyl (*Z*)-5,11,14,17-eicosatetraenoate	–	–	–	–	–	–
26	15-Tetracosenoic acid, methyl ester, (Z)-nervonic	–	1	1	1	1	1
27	*cis*-7,10,13,16-Docosatetraenoic acid, methyl ester	–	–	–	–	–	–
28	Methyl 6,9,12,15,18-heneicosapentaenoate	2	–	–	–	–	–
29	Methyl 4,7,10,13,16-docosapentaenoate	1	–	1	–	1	1
30	Methyl 7,10,13,16,19-docosapentaenoate	4	–	1	–	1	2
31	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-*Z*)-	28	13	17	20	15	24

* Capsule refers to the commercial omega-3 dietary supplement. Sardine indicates fish oil extracted from sardine heads and tails in our Labs in Sicily on September 2020. PD000 indicates a AnchoisOil sample extracted in our Labs in Sicily on June 2019. PD006, PD009 and PD010 indicate AnchoisOil samples extracted in our Calabria's Labs, respectively, on December 2020, March 2021, and April 2021.

afforded lower yields even compared to limonene for both oleic (10%) and myristic (22%) acids. Yet, being an excellent solvent for omega-3 lipids, extraction with hexane afforded high yields of both DHA (22%) and EPA (10%). The Soxhlet extraction with petroleum ether with its typical long extraction time (4 h) and extraction temperature (50 °C) was virtually unfeasible as anchovy oil extraction method, affording poor EPA (3%) and DHA (5%) extraction yields due to rapid oxidation of PUFAs at high temperatures. Similar results were reported in the extraction of fish oil from lyophilized salmon with *d*-limonene under reflux affording a dark and highly degraded (oxidised) fish oil.[24]

Lyophilization of the anchovy leftovers substantially improves the amount of DHA extracted with *d*-limonene (20% *vs.* 15% when compared to the non-lyophilized leftovers), and slightly worsens (11% *vs.* 10%) the amount of EPA extracted. No significant changes, on the other hand, were observed when the extraction was carried out with *n*-hexane, pointing once again to the excellent solvation and extraction power of hexane for omega-3 lipids.[24] On the other hand, lyophilization of the anchovy leftovers, resulted in a substantial improvement in the amount of PUFAs extracted using the Soxhlet with petroleum ether (16% *vs.* 5% for DHA and 8% *vs.* 3% for EPA).

Table 2. Fatty acids (in methyl ester form for the analysis) in anchovy oils extracted with *n*-hexane and via the Soxhlet method with petroleum ether from the anchovy leftovers of sample PD010 in Table 1, and from lyophilized anchovies using *d*-limonene, *n*-hexane and petroleum ether via the Soxhlet method.*

Entry	Compound	PD010 Hex (%)	PD010 Sox (%)	Lyo-Lim (%)	Lyo-Hex (%)	Lyo-Sox (%)
1	Myristic acid, methyl ester	6	9	7	6	7
2	Pentadecanoic acid, methyl ester	1	2	1	1	1
3	Palmitic acid, methyl ester	22	32	20	20	23
4	Methyl palmitelaidate	1	1	1	1	1
5	Methyl petroselinate	–	–	–	–	–
6	Palmitoleic acid, methyl ester	5	8	6	6	7
7	Margaric acid methyl ester	1	2	1	1	1
8	6-Hexadecenoic acid, 7-methyl,methyl ester (*Z*)	1	1	1	1	1
9	Stearic acid, methyl ester	4	6	4	4	5
10	Oleic acid, methyl ester	10	16	9	8	9
11	*trans*-Vaccenic acid, methyl ester	2	3	3	3	3
12	Nonadecanoic acid, methyl ester	1	–	–	–	–
13	Linoleic acid, methyl ester	2	2	2	1	2
14	9,12-Hexadecadienoic acid, methyl ester	–	–	–	–	–
15	Arachidic acid methyl ester	1	1	1	1	1
16	Gondoic acid, methyl ester	1	1	3	2	2
17	Linolenic acid, methyl ester	2	1	–	1	1
18	Methyl stearidonate	2	1	2	2	2
19	*cis*-11,14-Eicosadienoic acid, methyl ester	–	–	–	–	–
20	Behenic acid, methyl ester	–	–	–	–	–
21	*cis*-10-Nonadecenoic acid, methyl ester	–	1	3	4	4
22	Arachidonic acid methyl ester	1	–	1	1	1
23	Methyl 8,11,14,17-eicosatetraenoate	1	–	–	1	–
24	5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-*Z*)	10	3	10	11	8
25	Methyl (*Z*)-5,11,14,17-eicosatetraenoate	–	–	–	–	–
26	15-Tetracosenoic acid, methyl ester, (Z)-nervonic	1	2	1	1	1
27	*cis*-7,10,13,16-Docosatetraenoic acid, methyl ester	–	–	–	–	–
28	Methyl 6,9,12,15,18-heneicosapentaenoate	–	–	–	–	–
29	Methyl 4,7,10,13,16-docosapentaenoate	1	–	–	–	–
30	Methyl 7,10,13,16,19-docosapentaenoate	1	–	1	1	1
31	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-*Z*)-	22	5	20	22	16

* D010 Hex and PD010 Sox stand, respectively, for anchovy oil extracted with *n*-hexane at room temperature and via the Soxhlet method with petroleum ether from the same anchovy leftovers of sample PD010 in Table 1. Lyo-Lim, Lyo-Hex and Lyo-Sox refer to anchovy oils extracted from lyophilized anchovies with, respectively, *d*-limonene, *n*-hexane and using petroleum ether via the Soxhlet method.

Industrially Suitable Circular Chemistry Process

AnchoisOil extracted from anchovy leftovers using *d*-limonene at room temperature is a transparent, clean oil colored in orange (Figure 1) due to the presence of relatively high amounts of vitamin A (retinol) and vitamin Q (coenzyme Q_{10}).[26] Vitamin D in its most bioactive form of vitamin D_3 (cholecalciferol) is also present.[27]

The oil is clearly similar to that obtained extracting lyophilized anchovies fished in Korea's sea (*Engraulis japonica*) using supercritical carbon dioxide ($scCO_2$), and very different from that extracted by the same South Korean team using *n*-hexane for 24 h at 40 °C.[28] In the latter case, indeed, the organic solvent non selectively extracts numerous other compounds which darken the oil, requiring further purification. Compared to the oil extracted with $scCO_2$, however, the AnchoisOil extracted from fresh anchovy leftovers with *d*-limonene has a substantially lower peroxide value (13.35 *vs.* 28.92 meq/kg, respectively). On the other hand, the acid value of AnchoisOil (31.40 mg_{KOH}/g) is significantly higher than that of the oil extracted with supercritical CO_2 (11.06 mg_{KOH}/g) likely due to the substantially higher dissolution power of limonene for partly hydrophilic compounds when compared to carbon dioxide.[29]

The use of biobased limonene as natural product extraction solvent, however, goes much beyond its largely enhanced

Figure 1. Sample of AnchoisOil following limonene removal.

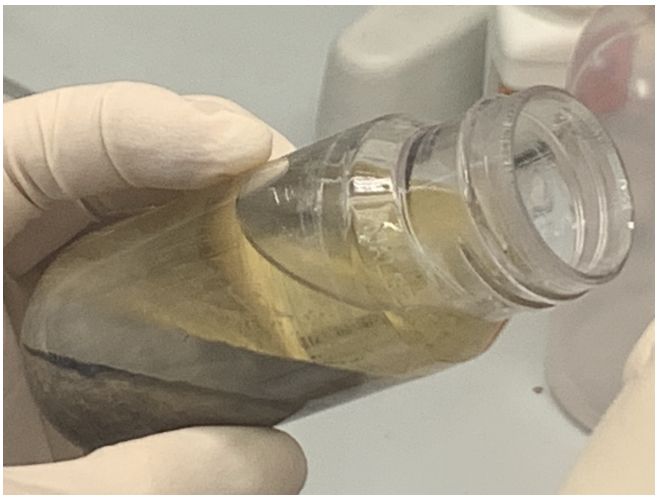


Figure 2. AnchoisFert at the bottom of a centrifuge tube. The liquid surnatant contains the AnchoisOil dissolved in limonene.

sustainability profile when compared to toxic (and highly volatile) *n*-hexane.[30] Limonene, indeed, is both a antioxidant and bactericidal agent,[31] ensuring protection of PUFAs in AnchoisOil during the solvent removal at 90 °C. The peroxidation-prone double bonds of omega-3 lipids are protected from oxidation also by lipophilic polyphenols such as the phlorotannins naturally present in certain fish and seafood species,[32] as well as by the antioxidant coenzyme Q_{10} and retinol abundant in anchovy, that are transferred to the AnchoisOil imparting it with a distinct orange color (Figure 1).[26]

The presence of limonene residual in the AnchoisOil at 8–10 wt%, is beneficial from the health and safety viewpoint. We remind that limonene is an edible, safe natural product widely employed by the food industry in many food and beverage products.[33] With numerous different tradenames (*GerdLi*, *Orange Burps* etc.) or with the simple chemical name, numerous dietary supplement products are successfully commercialized consisting either of softgels containing *d*-limonene derived from the orange peel or simply of orange oil in small dark vessels.

Following mild drying overnight in a oven at 90 °C, the solid residue of the oil extraction (Figure 2), comprising 90 wt% of the original anchovy leftovers, is an exceptional organic fertilizer.[34] Free of antibiotics, and of antibiotic resistance genes, but rich in flavonoids, organic carbon, phosphate, sulphate, magnesium, potassium and essential aminoacids,[35] when compared to conventional organic (manure) and chemical NPK commercial fertilizers, the new organic fertilizer "AnchoisFert" gives significantly better results for example in promoting the growth of Tropea's red onion (*Allium cepa*).[34]

Overall, thus, the use of limonene under mild and safe extraction conditions (low temperature, lack of heating, flame or pressurized environment) directly converts "in one pot" the by-product of fish filleting (comprising >50% of the fresh fish and generally disposed of as biowaste at €300–400/t cost) in two highly valued bioproducts ready for advanced uses, closing the fishing material cycle. Besides use as active nutraceutical ingredient in new generation omega-3 dietary supplement products, AnchoisOil may soon find therapeutic applications. Encapsulated in mesoporous silica, indeed, it has shown pronounced *in vitro* anticarcinogenic activity against non-small cell lung cancer cells).[36] AnchoisFert, in its turn, also when derived by other species such as sardines and other blue fish species, will likely find utilization in high-value crops cultivation, especially in greenhouse cultivations getting rid of soil contamination.[37]

Conclusions

In summary, the LimoFish process diverts fish biowaste from landfills and biogas-compost production plants for the world's most caught species (the anchovy) eliminating the environmental burden of fish waste, conserving and upgrading biological resources into valued bioproducts that can be commercialized in the nutraceutical, pharmaceutical and organic fertilizer markets. Here demonstrated also for European sardine (*Sardina pilchardus*), the process can be applied to any fish and seafood processing by-products including those of shrimp.[38] This means, *inter alia*, that a single bioeconomy plant using the LimoFish process will be able to process different fishery biowaste streams in different part of the fishing season. Said distinctive advantages of the circular economy process allow to address the point raised by Olsen for whom it would be unlikely that fish processing by-products can be used "to any large extent to produce high-priced products".[14]

The life cycle assessment of the process in the scaled-up configuration in which the laboratory scale solvent recovery step using a rotovapor is replaced by industrial distillation clearly points to its environmental sustainability.[39] Being easily

adopted at fish processing plants or at new sites located near (30 km away) the fish factory, the process addresses the two main difficulties in implementing bioeconomy productions based on circular industrial chemistry applied to fish biowaste, namely physical proximity and process efficiency.[40]

Using a health beneficial natural solvent under ultramild conditions (between 4 °C and room temperature under atmospheric pressure) to convert animal biowaste into two highly valued bioproducts and generating no waste, the process indeed meets the green extraction principles of reduction in energy consumption by low energy input demand, reduction of unit operations, and safe and easily controlled process conditions.

Working in close partnership with industry throughout his professional life dedicated to the science and technology of natural product green extraction, Chemat has shown that industry will quickly uptake new green technology when clear economic and technical benefits are clearly demonstrated. "The R&D, industrial, and cost control departments", he wrote, "are constantly looking for more effective and cost-efficient ways"[41] to extract valued natural products. It is also noteworthy that also limonene is obtained via a green extraction technology (cold pressing) from oranges and lemons prior to fruit industrial squeezing. Only about 30 per cent of the world's oranges harvested yearly are actually used to make juice. This creates a large, and so far mostly untapped potential for orange oil production from collected waste orange peel of freshly consumed fruits. This is especially true today as consumers around the world, looking for healthier and more natural products, are shifting consumption from reconstituted frozen concentrate orange juice (FCOJ, the main orange industry product) to NFC (not from concentrate) juice.[42] For instance, one company in the Netherlands commercializes both crude orange oil (and limonene) from orange peel collected from restaurants, supermarkets, catering companies and even offices.[43] To realize the scope of the value adding extent, the resulting orange oil recovered from waste orange peel by late 2023 was sold online in 20 L batch at € 35.3/L rate (€ 706).[44]

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Conflict of Interests

The Authors declare no conflict of interest.

Data Availability Statement

All data supporting the findings are available upon reasonable request to the corresponding Authors.

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RESEARCH ARTICLE

The outcomes of applying the zero-waste extraction process based on defatting fish processing waste with limonene to leftovers of European sardine (*Sardina pilchardus*) and European anchovy (*Engraulis encrasicolus*), compared to conventional extraction with oil-derived solvents such as *n*-hexane and with petroleum ether, show that the circular economy "LimoFish" process has general applicability and meets the principles of green extraction and those of the marine biorefinery requiring high process efficiency.

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The LimoFish Circular Economy Process for the Marine Bioeconomy