Discover Applied Sciences

Brief Communication

High stability of AnchoisOil extracted with limonene from anchovy fillet leftovers

Giuseppe Angellotti[1] · Daniela Maria Pizzone[2] · Mario Pagliaro[1] · Giuseppe Avellone[3] · Claudia Lino[3] · Francesco Mauriello[2] · Rosaria Ciriminna[1]

Received: 16 January 2024 / Accepted: 4 March 2024
Published online: 08 March 2024

Abstract
To investigate the vitamin content in an AnchoisOil sample stored at − 20 °C under N_2 for more than four years, we have developed a new HPLC method for simultaneous identification and quantification of vitamins in anchovy fish oil that is efficient and applicable in practice. Coupled to full retention of the original visual appearance (orange color and transparency), the relatively high concentrations of lipid-soluble vitamin Q (coenzyme Q10) and vitamin A (retinol) in AnchoisOil extracted with *d*-limonene from European anchovy fillet leftovers more than four years after extraction point to remarkable chemical stability of this marine oil. These findings further support the practical use of the "LimoFish" process to extract valued fish oil from the leftovers of the world's most caught fish species.

Graphical Abstract

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42452-024-05781-w.

✉ Mario Pagliaro, mario.pagliaro@cnr.it; Francesco Mauriello, francesco.mauriello@unirc.it; Rosaria Ciriminna, rosaria.ciriminna@cnr.it | [1]Istituto per lo Studio dei Materiali Nanostrutturati, CNR, Via U. La Malfa 153, 90146 Palermo, Italy. [2]Dipartimento di Ingegneria Civile, Energia, Ambiente E Materiali, Università Mediterranea di Reggio Calabria, Via Graziella, Feo Di Vito, 89122 Reggio Calabria, Italy. [3]Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche and ATeN Center, Università di Palermo, 90123 Palermo, Italy.

Highlights

- This work aims at studying the stability of AnchoisOil investigating the vitamin content in an AnchoisOil sample stored at − 20 °C under N_2 for more than four years.
- We have developed a new HPLC method for simultaneous identification and quantification of vitamins in anchovy fish oil that is efficient and applicable in practice.
- AnchoisOil extracted with *d*-limonene from European anchovy fillet leftovers after more than four years of storage retains its color, transparency and high concentration of vitamins Q and A.
- These findings further support the practical use of the "LimoFish" process to extract valued fish oil from the leftovers of the world's most caught fish species.

Keywords AnchoisOil · Circular economy · Fish oil · Coenzyme Q10 · Green extraction

1 Introduction

Widely carried out on industrial scale to obtain omega-3 enriched food supplements [1], the extraction of fish oil relies on many different marine species, including oily blue fish and, more recently, also krill [2]. The process is followed by extensive refining of the extracted oil and generally affords fish oil enriched in long chain polyunsaturated fatty acids (PUFAs) docosahexaenoic acid (DHA, C22:6*n*-3) and eicosapentaenoic acid (EPA, C20:5*n*-3) in ethyl ester form, rather than in the natural triglyceride form [1]. Determining a truly global "hidden hunger" [3], diets prevalent in most industrialized countries since several decades are poor in anti-inflammatory DHA and EPA omega-3 (or *n*-3) abundant in fish oil. The latter "hunger" significantly contributes to overfishing, with even krill now being threatened by overfishing aimed at omega-3 food supplement production [2].

Plentiful research has been devoted in the last two decades to replace the current, unsustainable fish oil production routes with circular economy processes from seafood processing by-products [4], preferably using greener, intensified processes [5]. Recent estimates suggest that a combined optimization strategy of omega-3 production (supply) based on replacement of marine species with others that have the highest omega-3 content, direct wild fish consumption, and improved by-product utilization might increase the availability of fish oil by 50% (reaching 630 kt y^{-1}) [6].

Amid the new circular economy extraction routes reported so far [4], the "LimoFish" process using biobased *d*-limonene as extraction solvent shifts the fish oil production from fish to fish processing waste employing a health beneficial and environmentally friendly solvent that is nearly entirely recovered after biowaste defatting [7]. First applied to European anchovy (*Engraulis encrasicolus*) fillet leftovers, the process is economically and technically viable [8]. The method, furthermore, closes the fishing material cycle for anchovy fishing because the solid residue of anchovy fillet leftovers extracted with antibacterial limonene [9], is an highly effective organic fertilizer dubbed "AnchoisFert" [10].

These discoveries hold significant potential to dramatically increase the sustainability of anchovy fishing, omega-3 lipid and fertilizer production. Anchovy indeed is by far the most caught fish species in the world amounting to 11% of the world's total landings [11].

Rapidly perishable leftovers of salted anchovy fillets (after evisceration, blood and other organic matter are spread over the surface, activating the peroxidase of blood leucocytes in the presence of NaCl and oxygen) [12], indeed, are generally dealt with by anchovy fillet manufacturers as biowaste. Disposal of biowaste bears relatively high economic cost (€400/t), but the high economic value of salted anchovy fillets (sold for instance in Italy at €60–70/kg) largely allows producers to bear said costs. In brief, an abundant potential source of fish oil and fertilizer of exceptional quality is generally used, at best, as feed for compost fertilizer and biogas production.

Another major issue affecting current fish oil refining for omega-3 food supplement production is that refining removes lipid-soluble marine polyphenols and valued vitamins that protect the omega-3 lipids from quick oxidation, leading manufacturers to add expensive natural antioxidants such as tocopherols or olive biophenols [13]. Besides *n*-3 PUFAs and oleic acid in natural triglyceride form [7], AnchoisOil contains also the three isomers of vitamin D (cholecalciferol, calcidiol, and calcitriol) in relatively good amount (81.5 µg/kg) [14]. Anchovy oil, however, contains also other fat-soluble vitamins that impart the oil its distinctive orange color. As recently shown by South Korea's scholars using Japanese anchovies (*Engraulis japonicus*), said vitamins are vitamin A, vitamin E and vitamin Q (coenzyme CoQ_{10}) [15]. This work

aims at studying the stability of AnchoisOil by investigating the vitamin content in a AnchoisOil sample kept under N_2 stored at − 20 °C for more than four years. To achieve this aim, we developed a novel HPLC method for the simultaneous identification and quantification of vitamins in anchovy fish oil.

2 Materials and methods

2.1 AnchoisOil extraction and storage

A fresh sample of AnchoisOil was extracted from anchovy fillet leftovers kindly donated by a anchovy fillet manufacturer based in Sicily (Agostino Recca Conserve Alimentari Srl, Sciacca, Italy) using the procedure previously described [7]. The anchovies were caught in the Strait of Sicily. A AnchoisOil sample extracted in 2019 (AnchoisOil 2019 in the Supplementary Information files) stored at -20 °C under a N_2 atmosphere for more than four years was also analyzed.

2.2 HPLC method

The analysis of liposoluble vitamins (vitamin A, E, D and Q) in all AnchoisOil samples was carried out using High-Performance Liquid Chromatography with Diode Array Detection (HPLC–DAD). The HPLC system utilized was an Agilent 1260 Infinity (Agilent Technologies, Palo Alto, USA) equipped with a Binary Pump G1312C, a Diode Array Detector G7115A, and a column oven G1316A. Chromatographic separation was successfully achieved using a Chromolith Performance RP-18e 100 mm × 4.6 mm column (Merck KGaA, Darmstadt, Germany) kept at 30 °C throughout the analysis. Prior to analysis, 200 µL of each sample was supplemented with 100 µL of 2-propanol and subsequently filtered through a 0.45 µm PTFE filter to ensure sample purity.

The injection volume was 20 µL and the mobile phase consisted of MilliQ water (solvent A) and a mixture of acetonitrile and 2-propanol in a 50:50 ratio (solvent B), both supplemented with 0.1% (v/v) trifluoroacetic acid (TFA). Solvents were purchased from Sigma Aldrich (Milan, Italy), TFA was supplied from VWR International (Milan, Italy). The flow rate was set to 1.5 mL/min, and the DAD was configured to cover a wavelength range from 200 to 800 nm.

Chromatographic elution was programmed as follows: an initial isocratic condition of A:B = 80:20 for the first 2 min, followed by a gradient from 2 to 42 min from A:B = 80:20 to A:B = 10:90. Subsequently, isocratic elution was maintained at A:B = 10:90 from 42 to 45 min, with a final gradient from 45 to 50 min returning to the initial run condition.

Coenzyme Q10 ((2,3 dimethoxy-5 methyl-6-decaprenyl benzoquinone, purity, 98%), vitamin A (retinol, purity, 97.5%), vitamin D_3 (cholecalciferol, purity, 98%), and vitamin E (α-tocopherol, purity, 96%) were purchased from Sigma Aldrich (today Merck Life Science, Milan, Italy). Retention times for the analytes were as follows: coenzyme Q10 at 16.74 min, vitamin A (retinol) at 28.30 min, vitamin D_3 (cholecalciferol) at 38.53 min, and vitamin E (tocopherol) at 39.16 min. To enable vitamin quantification, accurate calibration curves were measured for both coenzyme Q10 and vitamin A. For coenzyme Q10, λ_{max} was 275 nm (linearity range: 0.1375–13.75 µg/mL, regression equation: Area = 23.992 + 59.34 × [µg mL^{-1}], R = 0.999). For vitamin A, λ_{max} was 325 nm (linearity range: 0.1–10 µg/mL, regression equation: Area = 20.132 + 90.762 × [mg mL^{-1}], R = 0.998). Quantification data are expressed as µg of each analyte in 100 g of the respective oil and reported as means of nine measurements ($n = 9$) ± SE.

Additional materials of chromatograms (Figure S1: HPLC chromatogram recorded with diode array detector at 275 nm of AnchoisOil 2019 (A) and vitamins standard (B); Figure S2: Chromatogram recorded at 265 nm of AnchoisOil 2019 (A) and vitamins standard (B); Figure S3: Chromatogram recorded at 298 nm of AnchoisOil 2019 (A) and vitamins standard (B); Figure S4:. Chromatogram recorded at 325 nm of AnchoisOil 2019 (A) and vitamins standard (B)).

3 Results and discussion

Figure 1a shows samples of AnchoisOil aged for more than 4 years. Figure 1b shows a fresh AnchoisOil sample, using the same procedure (see Supplementary Information) extracted from heads and tails of anchovies purchased at a fishery store in Palermo, Sicily, in June 2022. In both cases, the amount of limonene residual in AnchoisOil after solvent removal under reduced pressure at 90 °C was ~ 8 wt%.

The bright color and transparency of AnchoiOil after more than 4 years storage (Fig. 1a) suggest limited degradation of the oil's orange-colored vitamin Q and vitamin A upon prolonged (> 4 years) storage. The visual appearance of both aged

and fresh AnchoisOil oils, furthermore, is nearly identical to that of the anchovy oil extracted from *Engraulis japonicus* with supercritical CO_2 [15].

The 3D plot derived from the HPLC analysis of the aged AnchoisOil sample in Fig. 2 vividly illustrates the extractive power of *d*-limonene, a broad-scope green solvent for the extraction of natural products [16].

The outcomes of the HPLC analyses for vitamins A and Q in the > 4 year old AnchoisOil sample were, respectively, 113.4 (± 4.1) μ g/100 g and 1015 (± 19.1) μg/100 g (each value is the average of HPLC measurements repeated 9 times). Vitamins D and E were not detected.

A first result of the analyses is that vitamin A degrades during storage of anchovy fillet leftovers for one year. Indeed, using ultra-high performance liquid chromatography (UHPLC) combined with heated electrospray ionisation mass spectrometry (HESI-MS) analytical technique, we could not detect neither vitamin A (all-*trans*-retinol) nor vitamin E (α-tocopherol) in a AnchoisOil sample extracted from a one year old sample of frozen fillet leftovers [14]. A second outcome is that limonene is particularly effective in extracting vitamin A from fresh fillet leftovers since its amount (113.4 μg/100 g) in AnchoisOil obtained from *Engraulis encrasicolus*, even after > 4 four year storage was nearly five times higher than in anchovy oil extracted from *Engraulis japonicus* with supercritical CO_2 (22.51 µg/100 g) or with *n*-hexane (21.92 µg/100 g) [15].

On the other hand, the anchovy oil extracted from *Engraulis japonicus* using hexane at 40 °C contains δ-tocopherol in plentiful amount (< 9,000 μg/100 g) [15]. The absence of vitamin E in the aged AnchoisOil suggests that tocopherols, that dissolve in limonene under reflux (at ~ 176 °C) [17], are either not effectively extracted by limonene at low temperatures used in the LimoFish process; or that vitamin E degrades after prolonged storage of the oil.

Similar degradation after said prolonged storage may have concerned vitamin D_3 detected in the oil obtained from anchovy fillet leftovers aged one year [14], but not detected in the aged AnchoisOil via the HPLC method developed for this work.

Finally, the concentration of vitamin Q (ubiquinone or coenzyme Q10) in the aged AnchoisOil extracted with limonene from *Engraulis encrasicolus* fillet leftovers, exceeding 1015 μg/100 g, is high and slightly lower than the amount (1260 μg/100 g) found in anchovy oil extracted from *Engraulis japonicus* wtih *n*-hexane [15]. This may be due both the excellent solubility of CoQ_{10} in limonene [18], as well as to the fact that extraction from anchovy leftovers takes place from regions of the anchovy (such as the head and viscera) particularly rich in this electron carrier involved in mitochondrial energy production and synthesis. Widely used as an adjunctive therapy in cardiovascular and neurodegenerative diseases and mitochondrial myopathies [19], ubiquinone is the active nutraceutical ingredient in a large number of food supplements.

4 Conclusions

In conclusion, coupled to full retention of the original visual appearance (orange color and transparency), the relatively high concentration of lipid-soluble vitamin Q (coenzyme Q10) and vitamin A in AnchoisOil extracted with *d*-limonene from European anchovy fillet leftovers more than four years after extraction point to remarkable stability of this marine oil (stored under nitrogen at -20 °C).

Comparing oils extracted with non-toxic CO_2, *n*-hexane and a commercial anchovy oil, Park and co-workers lately found that the anchovy oil extracted with $scCO_2$ had a 1.23 higher brightness value compared to the dark colored oil conventionally extracted with *n*-hexane at 40˚C [15]. The team concluded that fish oil extracted with sc-CO_2 is "expected to be used as a functional material, which could lead to economic benefits through the high valorization of anchovies" [15]. Though using a non-toxic and entirely recyclable solvent such as supercritical CO_2, extraction with $scCO_2$ requires substantially higher capital and operating expenditure costs than extraction with *d*-limonene at low temperature [20].

Combined with the present findings, the lack of vitamin A detected in AnchoisOil extracted from leftovers aged for one year at − 20 °C [14], suggests that for practical applications the extraction of best AnchoisOil will take place from freshly obtained fillet leftovers, and thus preferably directly at the anchovy fillet manufacturing company, as required by bioeconomy productions at marine biorefineries [21].

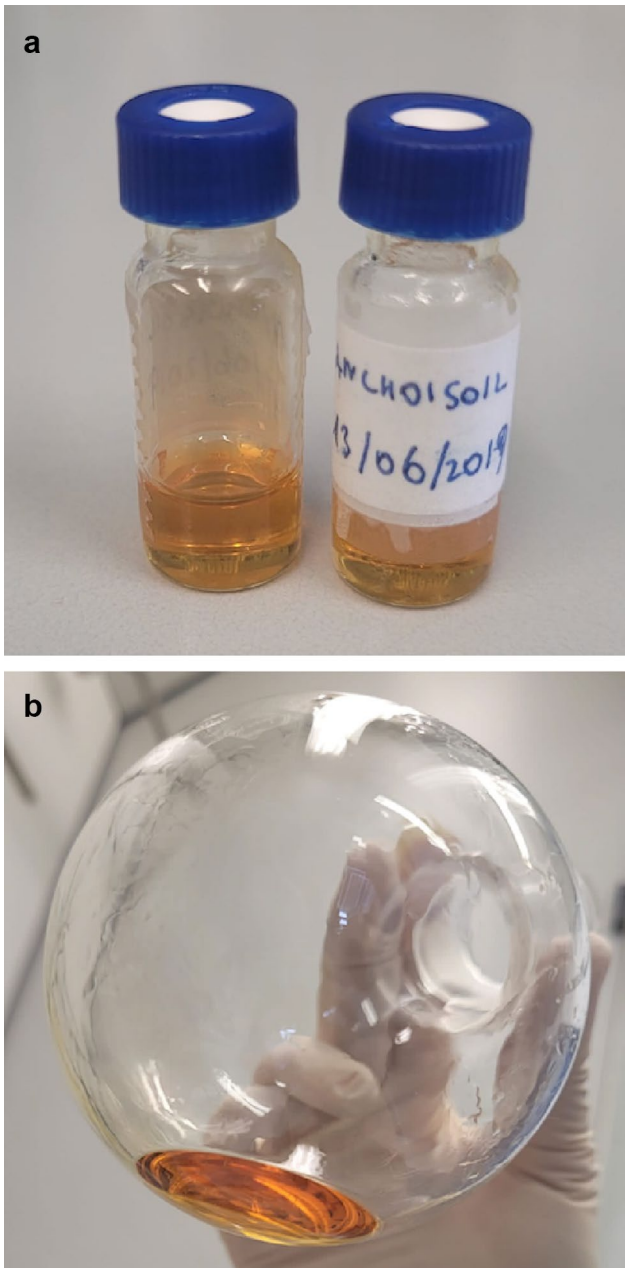

Fig. 1 **a** AnchoisOil samples after more than four years storage under N_2 at − 20 °C; **b** freshly extracted AnchoisOil

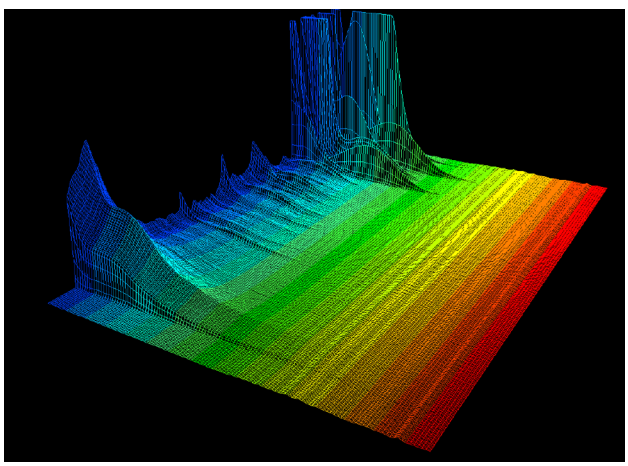

Fig. 2. 3D HPLC plot after injection of a AnchoisOil sample after > 4 year storage under N_2 at − 20 °C. Range from 200 nm (violet) to 500 nm (red)

Acknowledgements We thank Professors Adele Muscolo and Maria Teresa Russo, Università Mediterranea di Reggio Calabria, and Professor Fabrizio Passarini, Università di Bologna, for fruitful collaboration. Thanks to Agostino Recca Conserve Alimentari Srl (Sciacca, Italy) for kindly donating the anchovy fillet leftovers.

Author contributions G.A. (methodology, investigation, data curation); D.M.P. (methodology, investigation); M.P. (conceptualization, supervision, writing original draft, writing review and editing); G.A. (data curation, methodology); C.L. (investigation, formal analysis); F.M. (supervision, funding acquisition); R.C. (conceptualization, funding acquisition, supervision).

Funding This work was partly supported by SAMOTHRACE (Sicilian Micro and Nano Technology Research and Innovation Center) Innovation Ecosystem, European Union Next-Generation EU-PNRR—Mission 4 Component 2—Investment 1.5 (ECS00000022)-CUP B63C22000620005).

Data availability The data that support the findings of this study are available in the Supplementary Information that includes a PDF file embedding the chromatograms and selected photographs of the AnchoisOil extraction process displaying different steps of the process; alongside Excel files with (*i*) the HPLC calibration curves for vitamin A and vitamin Q, and (*ii*) the quantitative analysis of vitamin Q and vitamin A in the aged AnchoisOil.

Declarations

Competing interests The authors declare no conflict of interest.

References

1. Rubio-Rodríguez N, Beltrán S, Jaime I, de Diego SM, Sanz MTJ. Rovira carballido. Innov Food Sci Emerg Technol. 2010;11:1–12. https://doi.org/10.1016/j.ifset.2009.10.006.
2. Greenberg P. The Omega principle. New York: Penguin Press; 2018.
3. Winkler JT. The most hidden of all the hidden hungers: the global deficiency in DHA and EPA and what to do about it. In: Biesalski HK, Birner R, editors. Hidden hunger: strategies to improve nutrition quality. Basel: Karger Publishers; 2018. p. 123–30. https://doi.org/10.1159/000484645.
4. Šimat V, Vlahović J, Soldo B, Mekinić IG, Čagalj M, Hamed I, Skroza D. Production and characterization of crude oils from seafood processing by-products. Food Biosci. 2020;33:100484. https://doi.org/10.1016/j.fbio.2019.100484.
5. Monsiváis-Alonso R, Mansouri SS, Román-Martínez A. Life cycle assessment of intensified processes towards circular economy: Omega-3 production from waste fish oil. Chem Eng Process. 2020;158: 108171. https://doi.org/10.1016/j.cep.2020.108171.
6. Shepon A, Makov T, Hamilton HA, Müller DB, Gephart JA, Henriksson PJ, Troell M, Golden CD. Sustainable optimization of global aquatic omega-3 supply chain could substantially narrow the nutrient gap. Resour Conserv Recycl. 2022;181:106260. https://doi.org/10.1016/j.resconrec.2022.106260.
7. Ciriminna R, Scurria A, Avellone G, Pagliaro M. A circular economy approach to fish oil extraction. ChemistrySelect. 2019;4:5106–9. https://doi.org/10.1002/slct.201900851.
8. Ciriminna R, Scurria A, Fabiano-Tixier A-S, Lino C, Avellone G, Chemat F, Pagliaro M. Omega-3 extraction from anchovy fillet leftovers with limonene: chemical, economic, and technical aspects. ACS Omega. 2019;4:15359–63. https://doi.org/10.1021/acsomega.9b01168.
9. Gupta A, Jeyakumar E, Lawrence R. Journey of limonene as an antimicrobial agent. J Pure Appl Microbiol. 2021;15:1094–110. https://doi.org/10.22207/JPAM.15.3.01.
10. Muscolo A, Mauriello F, Marra F, Calabrò PS, Russo M, Ciriminna R, Pagliaro M. AnchoisFert: a new organic fertilizer from fish processing waste for sustainable agriculture. Global Chall. 2022;6:2100141. https://doi.org/10.1002/gch2.202100141.
11. In 2018, 7.045 million t of Peruvian anchovy (*Engraulis ringens*) and 0.957 million t of Japanese anchovy (*Engraulis japonicus*) were fished. See: Food and Agriculture Organization of the United Nations, *The State of world fisheries and aquaculture 2020*, Rome: **2020**. https://www.fao.org/publications/sofia/2020/en/. Accessed 4 Jan 2024.
12. Hernandez-Herrero MM, Roig-Sagues AX, Lopez-Sabater EI, Rodriguez-Jerez JJ, Mora-Ventura MT. Total volatile basic nitrogen and other physico-chemical and microbiological characteristics as related to ripening of salted anchovies. J Food Sci. 1999;64:344–7. https://doi.org/10.1111/j.1365-2621.1999.tb15897.x.
13. Hrebień-Filisińska A. Application of natural antioxidants in the oxidative stabilization of fish oils: a mini-review. J Food Process Preserv. 2021;45: e15342. https://doi.org/10.1111/jfpp.15342.
14. Scurria A, Lino C, Pitonzo R, Pagliaro M, Avellone G, Ciriminna R. Vitamin D3 in fish oil extracted with limonene from anchovy leftovers. Chem Data Coll. 2020;25: 100311. https://doi.org/10.1016/j.cdc.2019.100311.
15. Park J-S, Kim S-Y, Lee S-C, Jeong Y-R, Roy VC, Rizkyana AD, Chun B-S. Edible oil extracted from anchovies using supercritical CO_2: availability of fat-soluble vitamins and comparison with commercial oils. J Food Process Preserv. 2021;45:e15441. https://doi.org/10.1111/jfpp.15441.

16. Chemat S, Tomao V, Chemat F. Limonene as green solvent for extraction of natural products. In: Mohammad A, editor. Green solvents I. Dordrecht: Springer; 2012. https://doi.org/10.1007/978-94-007-1712-1_5.
17. Liu SX, Mamidipally PK. Quality comparison of rice bran oil extracted with d-limonene and hexane. Cereal Chem. 2005;82:209–15. https://doi.org/10.1094/CC-82-0209.
18. Palamakula A, Nutan M, Khan M. Response surface methodology for optimization and characterization of limonene-based coenzyme Q10 self-nanoemulsified capsule dosage form. AAPS PharmSciTech. 2004;5:114–21. https://doi.org/10.1208/pt050466.
19. Barakat A, Shegokar R, Dittgen M, Müller RH. Coenzyme Q10 oral bioavailability: effect of formulation type. J Pharm Investig. 2013;43:431–51. https://doi.org/10.1007/s40005-013-0101-4.
20. On the other hand, the economic value of fish oil enriched in omega-3 lipids is so high that that industrial extraction plants using $scCO_2$ combined with supercritical fluid chromatography for the production of highly concentrated omega-3 oily extracts already exist for example in Germany.Developed by Lembke in 1994 and later patented and trademarked by a company as the "kd-pur" technology. See: P. Lembke, Production Techniques for Omega-3 Concentrates. In: Omega-6/3 Fatty Acids. Nutrition and Health, F. De Meester, R. Watson, S. Zibadi, S. (Eds), Humana Press, Totowa (NJ): **2013**. https://doi.org/10.1007/978-1-62703-215-5_19
21. Bisgaard C, Collection of discard in European fishing ports challenges and opportunities, Pelagic AC Pelac Meetings, Dublin, 25 April 2017. https://effop.org/wp-content/uploads/2019/01/bodo_von_holten-iceland-2015.pdf. Accessed 4 Jan 2024.