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A Circular Economy Approach to Omega-3 Extraction

Rosaria Ciriminna,[1] Antonino Scurria,[1] Giuseppe Avellone,*[2] Mario Pagliaro*[1]

[1]*Istituto per lo Studio dei Materiali Nanostrutturati, CNR, via U. La Malfa 153, 90146 Palermo, Italy*
[2]*Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche Università degli Studi di Palermo, via Archirafi 32, 90123 Palermo, Italy*
*Corresponding authors: mario.pagliaro@cnr.it; beppe.avellone@unipa.it

ABSTRACT

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Fish oil rich in polyunsaturated omega-3 fatty acids is extracted in high yield from anchovy filleting waste using *d*-limonene as green biosolvent in a simple solid-liquid extraction performed by mechanically stirring and maceration followed by limonene removal via evaporation under reduced pressure. As *d*-limonene is renewably obtained from waste orange peel, this protocol establishes a circular bioeconomy method to obtain valued omega-3 extracts from biowaste available worldwide in several million t/year amount. The method closes the materials cycle and opens the route to full valorisation of an important biological resource so far mostly discarded as waste. Significant economic opportunities benefiting local communities, the ecosystem and public health are anticipated.

1. **Introduction**

Consumption of omega-3 long chain polyunsaturated fatty acids (PUFA) abundant in oily fish is critical for both physical and mental health of both adults and children.[1] Health authorities across the world generally recommend healthy adults increase their intake of omega-3 fats by regularly eating fish twice a week or, in case of lack of regular consumption of fish, by assuming a 2 g fish oil supplement several times a week. Fish oil, indeed, is a primary source of omega-3 PUFA.[2] Depending on the position of the first double bond from the methyl end group (ω end) of the fatty acid, the main long-chain PUFA belong to ω-6 (*n*-6) or to ω-3 (*n*-3) families.[3] In order to reestablish a better balance between ω-3 and ω-6 fats, the World Health Organization, recommends a daily intake of eicosapentaenoic acid (EPA, 20:5 ω-3) plus docosahexaenoic acid (DHA, 22:5 ω-3) of 250 mg in primary prevention of coronary hearth disease and 2 g in secondary prevention.[4] The European Food Safety Authority recommends a daily intake of 250 mg for EPA plus DHA.[5]

Omega-3 nutrients are fundamental hormone precursors which moderate the inherent propensity for arachidonic acid cascade overreactions when *n*-6 mediators dominate.[6] The efficiency of the tissue to defend itself against oxidative stress depends upon its ω-6:ω-3 composition. High percentage of omega-6 leads to persistent inflammation reinforced by the action of free radical continuously generated. Omega-3 fats reduce the concentrations of prostaglandins 2-series PG, a potent mediator of inflammation and cell proliferation, and increase the synthesis of much less inflammatory 3-series PG.

In a recent study devoted to enhanced methods for the extraction omega-3 fats from fish oil, we concluded that practically useful advances in sustainable extraction and sourcing of marine omega-3 nutrients are urgent,[7] since new health benefits of omega-3 assumption extending to the prevention of many pathologies are continuously reported,[8] while non sustainable fishing of anchovy (a preferred source of omega-3 fats) has, for example, led the Peruvian government to first suspend in 2014 one of two annual fishing seasons due to concerns about the number and size of the available anchovy, and in 2017 to set quota for first anchovy fishing season, while the previous one was still underway.[9]

Fish oil is the most popular dietary supplement in numerous countries with consumers globally spending $31 billion (in 2016) on products with added EPA and DHA.[10] Driven by rapidly growing demand in Asia, the global EPA/DHA ingredient volume has gone from 87,925 tonnes in 2015 to 91,321 tonnes in 2016. Most of the oil used to produce dietary supplements originate from Peruvian anchovies, even though about 5% of world's fish oil production is used to extract omega-3 nutrients for use in food and dietary supplement products, and most fish oil being rather employed as fishmeal for fish farming.[7]

In the conventional fish oil omega-3 concentrate production process, once caught anchovies are cooked and pressed still on board the shipping vessel. The omega-3 extraction process is carried out at industrial sites where the water-oil mixture obtained onboard is freed from water with a three-phase centrifuge, undergoing refinement in several consecutive steps including neutralization with alkalis, bleaching, deodorization,

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and degumming. The refined fish oil thereby obtained typically contains about 30% omega-3 fatty acids (18% in EPA and 12% in DHA). Omega-3 supplements use either 55% omega-3 ethyl ester oil in which the triglycerides comprising natural fish oil are transesterified with ethanol followed by molecular distillation or, even better, highly concentrated fish oil extracts providing 70% active ingredients. The latter are obtained either via supercritical fluid extraction combined with supercritical fluid chromatography or via enzyme-assisted concentration for the conversion of the omega-3 ethyl esters back into triglyceride form. Today's best omega-3 dietary supplements contain EPA and DHA in triglyceride form as this leads to a 70% higher increase in the omega-3 index when the ingredients are consumed as triglycerides rather than as ethyl esters.[11]

Extending the production of omega-3 from blue fish to fishery by-products so far mostly discarded as waste, would enable to recover and transfer key essential nutrients from the sea to the human food chain with significant economic, environmental and health benefits. Only in the process of fish filleting up to 60% of the fresh fish is cut off and generally treated as waste. A large amount of blue fish and seafood industry leftovers such as head, skin, trimmings and bones is mostly thrown away back into the sea, even though the huge potential of marine processing byproducts for the production of omega-3 has been long recognized.[12]

In the following, we describe how to obtain fish oil with abundant omega-3 nutrients directly from anchovy processing waste using *d*-limonene as extraction biosolvent.

2. Extraction and analysis

Anchovies are the world's largest fish catches with overfishing threatening their overall population.[13] The European anchovy (*Engraulis encrasicolus*) is particularly abundant in the Sicilian Channel, and its capture to produce anchovies filleted, salt-cured, and stored in olive oil is a key economic asset of southern Sicily's urban centres, including the city of Sciacca from where the anchovy fillet leftovers (Figure 1) used in this study were kindly made availably by a company selling anchovy fillets worldwide.

Figure 1. Frozen leftovers of anchovy fillets used throughout this study.

An electric blender was used to mix and homogenize the frozen leftovers along with an aliquot of *d*-limonene (Figure 2). In detail, 204 g of frozen anchovy waste in the blender jar of the electric blender was added with a first aliquot of 106 g of *d*-limonene (Acros Organics, 96%) refrigerated at 4 °C. After grinding twice for 15 s each time (Figure 2), a semi-solid grey purées was obtained which was extracted with limonene.

Figure 2. The leftovers of fresh anchovy fillets mixed with limonene prior to grinding in an electric blender. Readers can watch the video by clicking on the image.

An aliquot (50.7 g) of this mixture was transferred in a glass beaker and added with 51.4 g *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 at room temperature under stirrring at 700 rpm 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, Figure 3) to remove the solvent under reduced pressure (40 mbar) at 90 °C.

Figure 3. Evaporation under reduced pressure of limonene biosolvent to obtain the anchovy leftover extract. Readers can watch a video by clicking on the image.

The limonene biosolvent could be almost entirely recovered via evaporation under reduced pressure (Figure 4), ready for use in subsequent extraction runs.

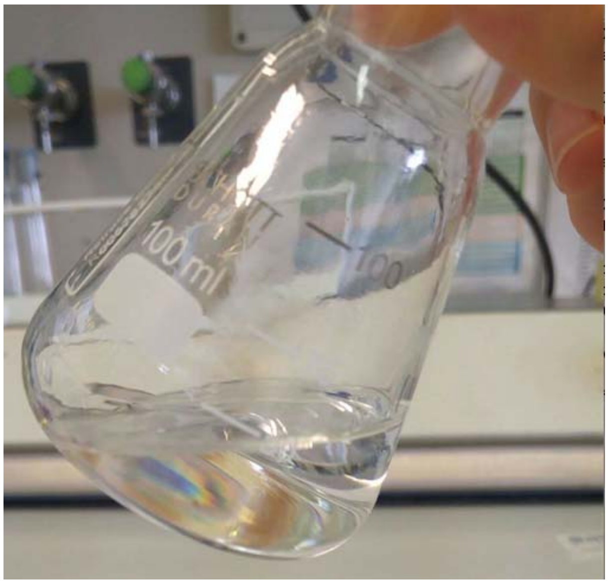

Figure 4. Limonene recovered after solvent evaporation under reduced pressure at the rotovapor.

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After evaporating limonene, we obtained 3.0 g of oily extract (Figure 5).

Figure 5. The anchovy leftover oily extract obtained after evaporating limonene biosolvent under reduced pressure.

A 100 mg sample of the latter oil was added with three consecutive aliquots of MeOH (0.5 mL┴3). Most residual limonene dissolved in the methanol liquid phase. The pale yellow fat precipitate was dried with a flux of nitrogen at room temperature obtaining 18 mg of solid fat. The fatty acids in triglyceride form were transesterified to obtain the fatty acid methyl esters (FAME) required for the GC-MS analysis by treating the solid fat residue with 0.5 mL of KOH dissolved in MeOH (2 N). The resulting FAME solution was extracted with 1 mL of *n*-hexane.

The GC-MS analysis was carried out using a ThermoScientific Trace 1310/ISQ LT single quadrupole GC/MS spectrometer. A 1 μL sample of the FAME solution was injected in the GC (split mode 1/100) using a ZB-5MS column (Zebron, 30 m 0.25 mm id 0.25 mm) with an autosampler Triplus RSH Thermo Scientific, using He (5.0) as gas carrier (flow-rate 0.95 mL/min). The temperature ramp used was as follows: the column was held for 2 min at T = 60 °C, after which temperature was raised at 20 °C/min rate up to 250 °C, with a final 5 min isotherm. The overall time for the analysis was 17 min. The injector and the transfer line temperature were 250 °C and 265 °C, respectively. Following automatic tuning, the electron multiplier voltage was set at 70 eV. Full scan data were acquired over the *m/z* range 35–500 at 0.73 s per scan, keeping the ion source at 260 °C. The retention times and molecular fragment mass data obtained were processed using the instrument's software. Each measurement was repeated three times. Both chromatographic and spectrometric results showed excellent reproducibility (SD = 4%). All FAME compounds were identified by critical comparison with mass spectral data from NIST/EPA/NIH Mass Spectral Library 2005.

Besides omega-3 PUFA, anchovies are a rich source of nutrients including saturated fatty acids (SFA), monounsaturated fatty acid (MUFA), vitamins including vitamin E (in α-tocopherol form), retinol (vitamin A), Vitamin D and D3 cholecalciferol, protein amino acids and minerals.[14]

Table 1 shows that in the leftovers of European anchovies catched in Sicily in early July, the major SFA was palmitic acid (33.55%), followed by myristic (6.98%) and pentadecanoic (1.2%) acid. Oleic acid is the most abundant MUFA with 23.97% relative abundance, whereas docosahexaenoic (12.38%) and eicosapentaenoic (5.4%) acid are the most abundant omega-3 PUFA, followed by valued stearidonic acid (1.04%).

The 1.80% fat content in the anchovy by-product oily extract is not far from the average 2.27% fat content found in the whole-body of anchovy caught in the Adriatic Sea.[15] Remarkably, scholars in Croatia discovered that the fat content in anchovy showed significant seasonal changes, being inversely correlated to water content, and varying between 0.86% in February and 4.47% in October.[15]

The n-3/n-6 ratio found for the anchovy by-product oil (10.04) is considerably higher than the n-3/n-6 ratio (6.29, 6.17 and 6.70 in November, December and January, respectively) found for anchovies catched in Turkey.[16]

Table 1. Main fatty acids relative abundance in the anchovy leftover extract upon esterification[a]

Acid (in lipid numbers)	Retention time (min)	Abundance (%)
Myristic acid (14:0)	9.95	6.98
Pentadecanoic (15:0)	10.38	1.2
Palmitic (16:0)	10.61	33.55
(6,Z)-7 methyl-6-Hexadecenoic	11.04	1.19
Margaric (17:0)	11.1	0.94
Stearic (18:0)	11.34	0.53
Oleic (18:1, n-9)	11.39	23.97
Linoleic (18:2, n-6)	11.6	1.97
alpha-Linolenic (18:3, n-3)	11.78	0.96
Stearidonic (18:4, n-3)	11.86	1.04
Gadoleic (20:1, n-11)	12.18	3.09
Eicosapentenoic (20:5, n-3)	12.07	5.4
11-Docosenoic (22:1, n-11)	13.02	4.66
Docosahexaenoic (22:6, n-3)	13.90	12.39

[a]Values include the retention time using using a ZB-5MS column, obtained upon trans-esterification reaction with potassium methylate.

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The use of *d*-limonene as a green solvent for the lipid extraction of food as an alternative to *n*-hexane has been extensively pioneered by Chemat's team in France since 2008.[17]

The same team has lately demonstrated how the terpene can be successfully used in the extraction of bioactive carotenoids from carrots, oils from rapeseed and terpenes from caraway seeds.[18]

In our case, the orange color of the anchovy leftover oily extract is due to α-tocopherol abundant in anchovies (0.57 mg/100 g),[19] namely the vitamin E form (labeled with the E307 E number in Europe) which is preferentially absorbed and accumulated in the human body, where it is responsible for several health beneficial effects including prevention of cancer and diseases of the central nervous system, the immune system, and the cardiovascular system.[20]

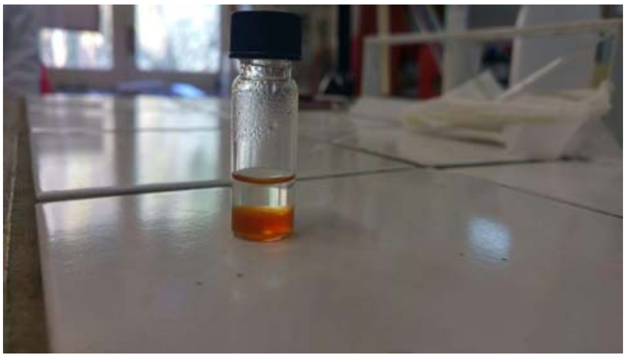

Figure 6. The glycerol/methanol layer formed at the bottom upon transesterification of the solid fat residue and extraction of FAME with *n*-hexane prior to the GC-MS analysis.

Remarkably, upon transesterification of the solid fat residue with potassium methylate and extraction of FAME with *n*-hexane for the GC-MS analysis, all α-tocopherol accumulated in the glycerol/methanol layer at the bottom (Figure 6) from which it can be readily recovered as we will report in a forthcoming study.

3. Conclusions

In conclusion, we have discovered that fish oil rich in omega-3 nutrients can be obtained in high yield from the discards of anchovy fillets using *d*-limonene as green biosolvent via simple solid-liquid extraction carried out at room temperature, followed by limonene removal via evaporation under reduced pressure.

First demonstrated with anchovies, *i.e.* the world's largest fish catches,[13] the method can be extended to all other fish processing waste, namely a resource available worldwide in several million t/year amount.[21] Besides European anchovy (*Engraulis encrasicolus*), widely catched examples of anchovies include Peruvian anchovy (*Engraulis ringens*), Japanese anchovy (*Engraulis japonicus*) and southern African anchovy (*Engraulis capensis*).

A potential new use of *d*-limonene in fish processing was discovered in 2015 reporting the significant activity of the terpene against *Anisakis* larvae when tested *in vitro*, suggesting its potential use in the industrial marinating process.[22] Its use to mask the unpleasant fishy odor co-encapsulated with fish oil in spray-dried and freeze-dried by milk protein microcapsules has been investigated by New Zealand's scholars who found that the limonene-containing microcapsules had much better flavor and odour profile than the fish oil microcapsules.[23]

Renewably obtained from waste orange peel, indeed, *d*-limonene has antimicrobial, antifungal, anti-oxidant, anti-inflammatory and anti-carcinogenic properties for which it is emerging as a key resource of the bioeconomy.[24] The terpene is also a safe food ingredient, being the main component of orange essential oil widely used in the food industry, making its use ideally suited to produce omega-3 extracts from fish and seafood processing waste.

As it happens in circular bioeconomy production processes,[25] the method closes the materials cycle and opens the route to full valorization of an important biological resource so far mostly discarded as waste. Significant economic opportunities benefiting local communities, the ecosystem and public health are anticipated.

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References

1. W. E. M. Lands, *Fish, Omega-3 And Human Health*, 2nd edition, AOCS Publishing, Urbana (IL): 2005.
2. V. Rizliya, E. Mendis, *Biological, Physical, and Chemical Properties of Fish Oil and Industrial Applications* In *Seafood Processing By-Products: Trends and Applications*, S.-K. Kim (Ed.), Springer Science+Business Media, New York: 2014.
3. A. C. Rustan, C. A. Drevon, Fatty Acids: Structures and Properties, *eLS* **2005**, 1-7. doi: 10.1038/npg.els.0003894
4. FAO/WHO Joint FAO/WHO Expert Consultation on Fats and Fatty Acids in Human Nutrition, Interim Summary of Conclusions and Dietary Recommendations on Total Fat & Fatty Acids, Geneva: 2008.
5. EFSA Panel on Dietetic Products, Nutrition and Allergies, Scientific Opinion on the substantiation of a health claim related to DHA and contribution to normal brain development pursuant to Article 14 of Regulation (EC) No 1924/2006, *EFSA J.* **2014**, *12* (10): 3840-3847.
6. W. E. M. Lands, Human Life: Caught in the Food Web, In *Lipids in Aquatic Ecosystems*, M. T. Arts, M. T. Brett, M. J. Kainz (Ed.s), Springer; New York: 2009; pp. 327-354.
7. R. Ciriminna, F. Meneguzzo, R. Delisi, M. Pagliaro, Enhancing and improving the extraction of omega-from fish oil, *Sustain. Chem. Pharm.* **2017**, *5*, 54-59.
8. F. Shahidi, P. Ambigaipalan, Omega-3 Polyunsaturated Fatty Acids and Their Health Benefits, *Ann. Rev. Food Sci. Technol.* **2018**, *9*, 345-381
9. M. Mereghetti, Peru sets quota for first anchovy fishing season, while previous one still underway, *Undercurrent News*, 12 January 2017. See at the URL: www.undercurrentnews.com/2017/01/12/peru-opens-first-anchovy-fishing-season-while-previous-one-still-underway/
10. A. Ismail (Global Organization for EPA and DHA Omega-3s), Threats to the Global EPA+DHA Omega-3 Industry, *Australasian Section of the American Oil Chemists' Society (AAOCS) Biennial Conference*, Château Tanunda, Barossa Valley, Australia, 11-13 September 2017.
11. J. Neubronner, J. P. Schuchardt, G. Kressel, M. Merkel, C. von Schacky, A. Hahn, Enhanced increase of omega-3 index in response to long-term n-3 fatty acid supplementation

from triacylglycerides versus ethyl esters, *Eur. J. Clin. Nutr.* **2011**, *65*, 247-254.

12. A. Khoddami, A. A. Ariffin, J. Bakar, H. M. Ghazali, Fatty Acid Profile of the Oil Extracted from Fish Waste (Head, Intestine and Liver) (*Sardinella lemuru*), *World Appl. Sci. J.* **2009**, *7*, 127-131.

13. G. Catanese, R. Watteaux, I. Montes, M. Barra, P. Rumolo, D. Borme, B. Buongiorno Nardelli, V. Botte, M. G. Mazzocchi, S. Genovese, I. Di Capua, M. Iriondo, A. Estonba, P. Ruggeri, V. Tirelli, V. Caputo-Barucchi, G. Basilone, A. Bonanno, D. Iudicone, G. Procaccini, Insights on the drivers of genetic divergence in the European anchovy, *Sci. Rep.* **2017**, *7*, Article number: 4180.

14. National Food Institute - Technical University of Denmark, Anchovy, pickled, canned, *2018.* See at the URL: https://frida.fooddata.dk/ShowFood.php?foodid=1034&2

15. V. Šimat, T. Bogdanović, Seasonal changes in proximate composition of anchovy (*Engraulis encrasicolus*, L.) from the central Adriatic, *Acta Adriat.* **2012**, *53*, 125-132.

16. Y. Kaya, H. Turan, Fatty acid composition of anchovy (Engraulis engrasicolis L. 1758) oil produced in Sinop-Turkey, *J. FisheriesSciences.com* **2008**, *2*, 693-697.

17. M. Virot, V. Tomao, C. Ginies, F. Chemat, Total Lipid Extraction of Food Using d -Limonene as an Alternative to *n*–Hexane, *Chromatographia* **2008**, *68*, 311-313

18. M. Aissou, Z. Chemat-Djenni, E. Yara-Varón, A.-S. Fabiano-Tixier, F. Chemat, Limonene as an agro-chemical building block for the synthesis and extraction of bioactive compounds, *C. R. Chim.* **2017**, *20*, 346-358.

19. A. Martins Delgado, M. D. Vaz Almeida, S. Parisi, *Chemistry of the Mediterranean Diet*, Springer, Cham: 2017; p.185.

20. K. M. Ranard, J. W Erdman, Jr, Effects of dietary *RRR* α-tocopherol *vs* all-*racemic* α-tocopherol on health outcomes, *Nutr. Rev.* **2018**, *76*, 141-153.

21. D. Zeller, T. Cashion, M. Palomares, D. Pauly, Global marine fisheries discards: A synthesis of reconstructed data, *Fish Fish.* **2018**, *19*, 30-39.

22. F. Giarratana, D. Muscolino, F. Panebianco, A. Patania, C. Benianti, G. Ziino, A. Giuffrida, Activity of R(+) Limonene Against *Anisakis* Larvae, *Ital. J. Food Saf.* **2015**, *4*, 5499.

23. Q. Chen, F. Zhong, J. Wen, D. McGillivray, S. Young Quek, Properties and Stability of Spray-Dried and Freeze-Dried Microcapsules Co-Encapsulated with Fish Oil, Phytosterol Esters, and Limonene, *Drying Technology* **2013**, *31*, 707-716.

24. R. Ciriminna, M. Lomelli, P. Demma Carà, J. Lopez-Sanchez, M. Pagliaro, Limonene: A Versatile Chemical of the Bioeconomy, *Chem. Commun.* **2014**, *50*, 15288-15296.

25. D. D'Amato, N. Droste, B. Allen, M. Kettunen, K. Lähtinen, J. Korhonen, P. Leskinen, B. D. Matthies, A. Toppinen, Green, circular, bio economy: A comparative analysis of sustainability avenues, *J. Clean. Prod.* **2017**, *168*, 716-734.