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Fatty Acids from *Paracentrotus lividus* Sea Urchin Shells Obtained via Rapid Solid Liquid Dynamic Extraction (RSLDE)

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Abstract: Sea urchins (Echinodermata, Echinoidea) are good a source of bioactive compounds belonging to different classes of natural substances. The edible Mediterranean sea urchin *Paracentrotus lividus* is a renowned animal model for study in different fields of biology, but it is intensively harvested for high commercial value due to the delicacy of its gonads. Most studies have focused on the composition and the nutritional value of *P. lividus* gonads (the edible part), but little interest has been taken in the other body parts, such as the shells and spines, which are generally considered waste material. The purpose of this study was to obtain an extract from sea urchin shells, with a green methodology of extraction, and to characterize the lipophilic components for potential applications. The shells of *P. lividus* were extracted via a very well performing technology based on rapid solid liquid dynamic extraction (RSLDE) implemented via an automated device (Naviglio Extractor®). The obtained extract shows the presence of fatty acids and their esters (methyl, ethyl and 1-glycerol esters). Gas chromatography-mass spectrometry (GC-MS) measurements were used to determine fatty acid abundance in the chromatographic fractions of the extract. Arachidonic acid (ARA), 5,8,11,14,17-eicosapentanoic acid (EPA), and 11-eicosenoic acids and their esters are the most abundant components. The presence of many polyunsaturated fatty acids (PUFA) in the extract, even in low percentages allows a future application in nutrition or medical use.

Keywords: fatty acids; sea urchin; *Paracentrotus lividus*; Naviglio Extractor; EPA; ARA

1. Introduction

Food wastes are produced by many sources, ranging from agricultural operations to household consumption. In particular breeding, seafood, and dairy processing are the main source of animal-derived wastes [1–3].

Food industry wastes derive from the processing of raw materials into foodstuffs for human consumption, which consists in the extraction or separation of the nutritional part from the remains, having scarce nutritional value or inedible components [4]. Industrial processing of vegetable and animal materials produces tons of by-products and the non-utilization of those materials not only leads to the loss of potential profits, but the bio-waste generated raised serious concerns about the cost of disposal of these products considering the environmental impact. Therefore, converting food wastes to higher value products not only reduces the environmental impact but also provides potential revenue for industry in various fields, such as nutraceutical, cosmeceutical, and agrifood [5,6]. The recovered

biomolecules and by-products can be applied in nutraceutical and pharmaceutical fields. In fact, waste material can be valorized through the extraction of high-value components, which can be re-used as additives, adjuvants, and pharmacologically functional ingredients. The choice of the extractive technique depends on the type of compounds and matrix. In recent years the aim of the scientific community has been the optimization of the extraction conditions using conventional or advanced techniques and the treatment of food waste [7].

The huge amount of marine food waste has generated significant activity on its alternative utilizations to turn these products into some economic and commercial value. Marine bio-waste includes unwanted materials derived from the fish processing industry, such as skin, bones, and shells. The global market demand for edible sea urchins is estimated at several tons annually [8]. The Mediterranean species *Paracentrotus lividus* is strongly harvested for processing and consumption of the gonads, which are considered a luxury sea food. Consequently, a significant portion of the raw material, essentially shells and spines, is produced as waste.

The aim of this study is to obtain extracts of *P. lividus* shells using the rapid solid liquid dynamic extraction (RSLDE) implemented via Naviglio Extractor®, as this technique has already successfully applied in the pharmaceutical, cosmetic, herbal, food and beverage fields [9–15]. Here we present our results on the qualitative and quantitative analysis via gas chromatography-mass spectrometry (GC-MS) of lipophilic components of the crude extract. Obtained results could be generalized to other species and may help to understand the use of sea urchin shell by-product in nutraceutical and cosmeceutical fields.

2. Materials and Methods

2.1. General Experimental Procedures

Nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz in $CDCl_3$ on a Bruker spectrometer (Ascend^{TM}400) (Bremen, Germany) and the same solvent was used as an internal standard. All reagents and solvents were analytical grade and purchased from Carlo Erba (Milan, Italy), Sigma-Aldrich (Saint Louis, MO, USA), and Fluka (Buchs, Switzerland).

An Agilent 6850 GC equipped with an HP-5MS capillary column (5% phenyl methyl polysiloxane stationary phase) and the Agilent 5973 Inert MS detector (Milan, Italy) (used in the scan mode) was used for GC-MS analysis, with ionization achieved by electron impact (EI) at 70 eV and 200 °C. Helium was employed as the carrier gas (flow rate of 1 mL min^{-1}). The operating conditions were set with the injector temperature at 250 °C and during the run a temperature ramp raised the column temperature from 50 °C to 240 °C: starting with 50 °C for 2 min, 10 °C min^{-1} until reaching 180 °C, 180 °C for 5 min, 5 °C min^{-1} until reaching 240 °C, and 240 °C for 25 min. For identification purposes, the mass spectrum of each peak was recorded in the total ion current mode of the mass spectrometer, within a *m/z* range of 45 to 550 at a frequency of 3.9 Hz.

Solutions of standard compounds were prepared in *n*-hexane at a concentration range of 50–200 mg L^{-1} and analyzed by GC-MS. Methyl esters of nonanoic and pentadecanoic acids (Sigma-Aldrich, Saint Louis, MO, USA) were used as internal standards, a stock solution at concentration of 1 g L^{-1} was prepared and stored at −20 °C.

Analytical thin layer chromatography (TLC) was performed on silica gel plates (Kieselgel 60, F_{254}, 0.25 mm) purchased from Merck (Darmstadt, Germany). The spots were visualized by exposure to UV radiation (253 nm), or by spraying with 10% H_2SO_4 in MeOH followed by heating at 110 °C for 10 min. Column chromatography (CC) was performed using silica gel (Kieselgel 60, 0.063–0.200 mm) purchased from Merck (Darmstadt, Germany).

2.2. Sea Urchins Collection

Specimens of sea *Paracentrotus lividus* were hand collected by scuba-diving from rocky shores of the Gulf of Naples, along the southern Tyrrhenian coasts of Italy. Animals were placed in a cooler

and carried to the laboratory under moist conditions within 2 h. Sea urchins were held in suspended baskets in a semi-closed recirculating system as described in [16].

Sea urchin shells were collected from individuals processed for harvesting of gonads and gametes. Shells with spines were emptied of body residues and washed in sea water. Samples which were not immediately processed for extraction were stored frozen at −20 °C.

2.3. Shell Extraction and Purification Process

Before extraction, sea urchin shells obtained as described above were air dried for 24 h. Eighty grams of dried shells (*P. lividus*) were gently broken into big pieces and subsequently extracted. The rapid solid liquid dynamic extraction (RSLDE) was carried out using Naviglio Extractor® (Atlas Filtri Engineering S.r.l., Padova, Italy). This is an apparatus that allows a rapid solid–liquid extraction maintaining the liquid in contact with the solid in a programmable pressurization cycle between atmospheric pressure and 8–9 bar [17]. The Naviglio Extractor Mod. 500 cc was used in these experiments. An ethanol/water mixture (50:50, v/v) was used as the solvent for an extraction time of 70 h. Ultrapure water was obtained from a Milli-Q system (Millipore, Burlington, MA, USA). The hydro-alcoholic extract (500 mL) was evaporated under reduced pressure to remove the organic solvent. The aqueous phase (250 mL) was acidified to pH 2 with 2 M HCl and extracted three times with the same volume of ethyl acetate (EtOAc). The EtOAc phases were combined, dried with Na_2SO_4, and evaporated under reduced pressure to give a brown-red oil extract (180.0 mg). The extract was fractioned by CC, eluted with $CHCl_3$/*i*-PrOH (95:5; v/v), originating six homogeneous fractions (frs) namely: A, 21.1 mg; B, 19.8 mg; C, 21.8 mg; D, 8.0 mg; E, 12.5 mg; and F, 11.8 mg (Figure 1).

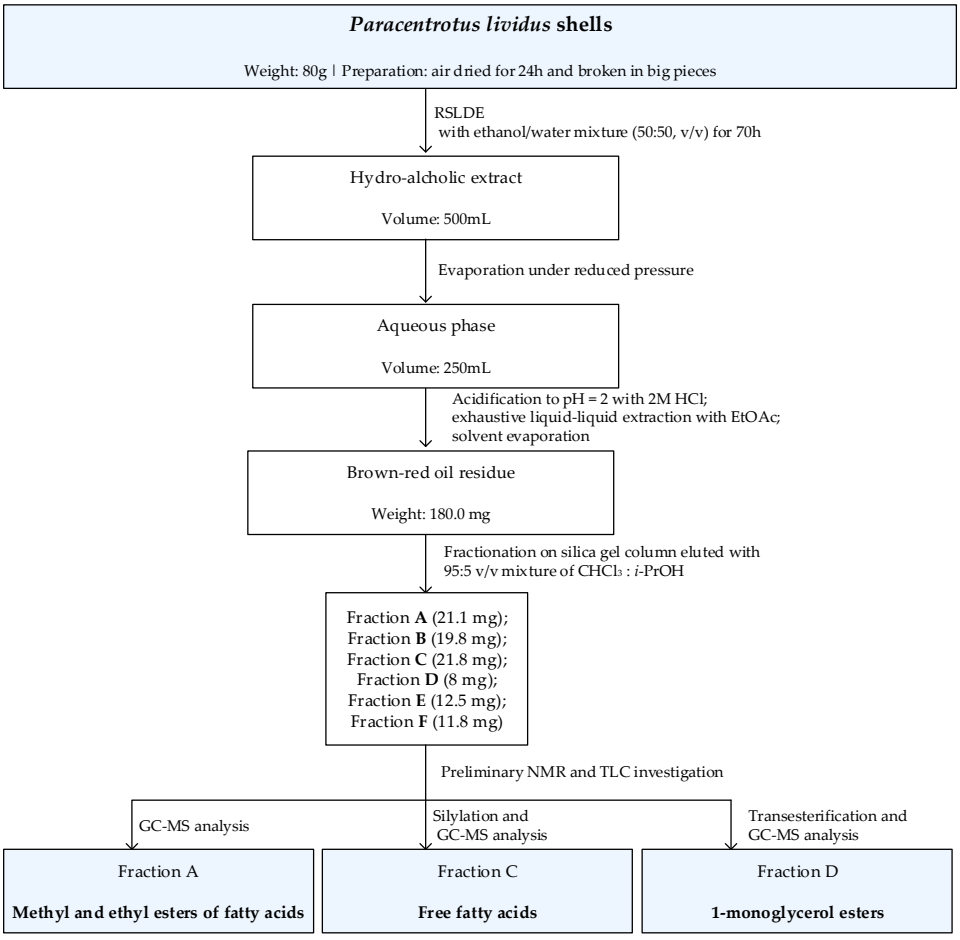


Figure 1. Schematic representation of extraction and purification processes of *Paracentrotus lividus* shells. Nuclear magnetic resonance (NMR), thin layer chromatography (TLC), gas chromatography-mass spectrometry (GC-MS).

2.4. GC-MS analysis of Chromatographic Fractions

2.4.1. Sample Preparation

The collected NMR data led us to analyze the CC fractions (frs A, C, and D) via GC-MS and according to the compounds potentially present in them, samples were treated in different ways. Hence, preliminary NMR data (see Results section) suggest the presence of methyl and ethyl ester of fatty acids in fr A (FAME, FAEE), free fatty acids in fr C (FA), and monoglycerides in fr D (MG). Due to the presence of methyl and ethyl esters of fatty acids, fr A was detected via GC-MS without preliminary treatment.

In order to detect free fatty acids in fr C via GC-MS, fatty acids were trimethylsilylated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) according to a previously reported method [18].

Fr D was analyzed via GC-MS after a transesterification process due to the presence of typical signals of monoglycerides in the NMR spectrum of this fraction. Three milligrams of the sample was dissolved in 1 mL of n-hexane and then 200 µL of KOH 2 M in MeOH was added to make the reaction happen. After thoroughly mixing the solution, a short period of rest was given to allow the separation into two phases, where the upper phase contains methyl esters of fatty acids [19].

2.4.2. Qualitative and Quantitative analysis of Fatty Acids

Fatty acids and their esters were identified by their Kovats non-isothermal retention index (KI) and by comparing their mass spectra with those present in databases by employing the National Institute of Standards and Technology (NIST) Mass Spectral Search Program v.2.0g which, among others, could explore the NIST 14 Mass Spectral library (2014) and the Golm Metabolome Database. To overcome problems caused by matrix components and compounds coelution, GC-MS data were analyzed by the deconvolution program Automated Mass Spectral Deconvolution and Identification System (AMDIS) distributed by NIST [20,21].

The quantification of individual silylated (TMS), methylated (ME), and ethylated (EE) fatty acids was obtained by linear regression of the chromatographic peak areas and known concentrations of certified standards (ranging from 50 to 200 mg L^{-1}). The response of each compound was normalized to the response of an internal standard (9:0 ME or 15:0 ME), which was added to the sample at the constant concentration of 100 mg L^{-1}. The concentrations of individual FAs in each sample were converted to percentage contributions of the total FAs [22,23].

3. Results

The chromatographic purification process of crude extract gave different fractions that were identified as mixtures of methylated and ethylated fatty acids, fatty acids, and monoglycerides by ^{1}H NMR. In particular, the protonic spectrum of fraction (fr) A showed the typical signals of a mixtuere of methyl and ethyl esters of sutured, monounsaturated, and polyunsaturated fatty acids (SFA, MUFA, and PUFA) [24]. The following are the chemical shifts, the multiplicity, and main signals: δ 5.46–5.33 (m) (–C<u>H</u>=C<u>H</u>–); 4.18–4.12 (dq, *J* = 2.3, 16 Hz) ($CH_3C\underline{H}_2O$–); 3.70 (s) ($C\underline{H}_3O$–); 2.87–2.80 (m) (=CH–C<u>H_2</u>–CH=); 2.37–2.29 (m) (–O–CO–C<u>H_2</u>); 2.16–1.70 (m) (C<u>H_2</u>–CH=CH–), 1.67–1.62 (m) (–O–CO–CH_2–C<u>H_2</u>–); 1.02–0.98 (t, *J* = 7.3 Hz) ($C\underline{H}_3CH_2$–); and 0.93–0.88 (m) ($C\underline{H}_3CH_2$–).

Fr C was identified as a mixture of fatty acids. In fact, the main difference from the fr A spectrum was the absence of signals relating to methoxy and ethoxy groups. Fr D was identified as a mixture of monoglycerides. In fact, its proton spectrum, in addition to the signals related to fatty acid residues, showed the typical signals of a residue of glycerol esterified in C-1 as reported below: δ 4.26–4.16 (ddd, *J* = 5, 6, 12 Hz, ROC<u>H_2</u>–CHOH–CH_2OH); 3.99–3.93 (m, $ROCH_2$–C<u>H</u>OH–CH_2OH); 3.74–3.60 (ddd, *J* = 4, 5, 12 Hz, ROC<u>H_2</u>–CHOH–CH_2OH).

Fatty Acids Composition

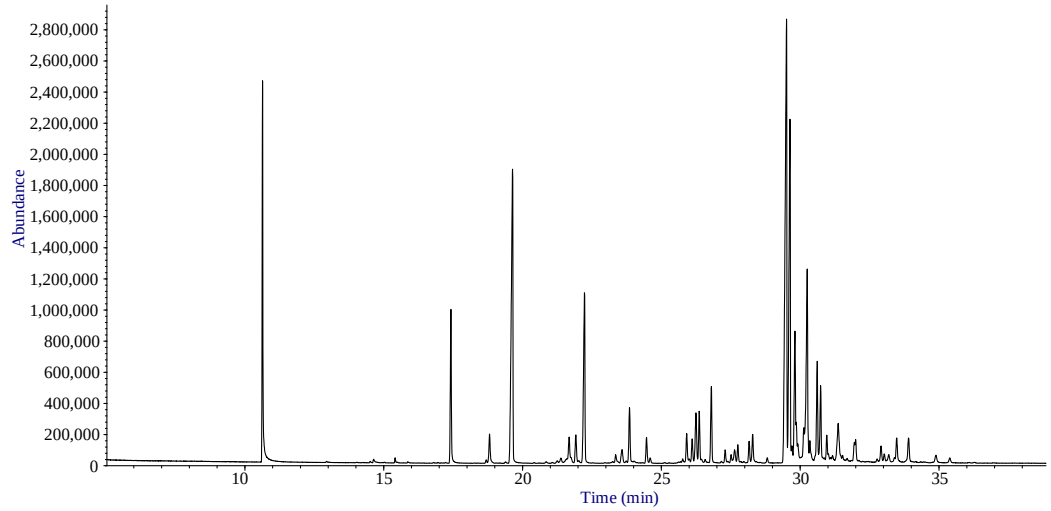
In all samples, FAs and their esters were identified and quantified via GC-MS. Figure 1 shows the total ion chromatograms (TICs) obtained from the fractions treated in different ways according to NMR data.

GC-MS analysis of fraction A (Figure 2A), without treatments, confirms the NMR results and revealed the presence of methyl esters and ethyl esters of fatty acids as listed in Table 1. 20:4n-6 EE (24.86%) and 20:5n-3 ME (26.51%) are the most abundant esters of fatty acids revealed in this fraction. TIC of fraction C (Figure 2B), acquired after trimethylsilylation, shows some peaks produced by the derivatization process and just two significant peaks which were identified as 20:4n-6 TMS (62.44%) and 20:5n-3 TMS (38.56%) (Table 1).

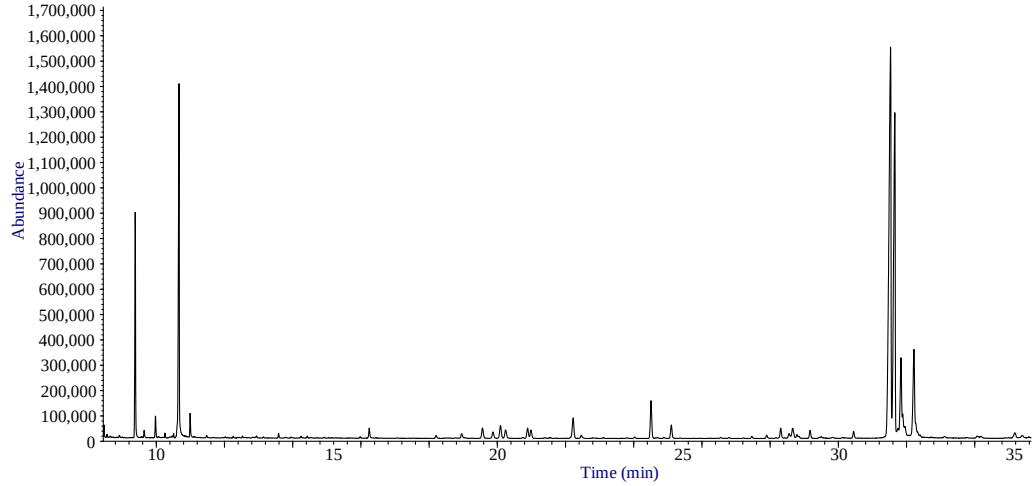
Table 1. Fatty acid profile of chromatographic fractions (frs) from the crude extract of *Paracentrotus lividus* shells.

Code *	Name	KI	Abundance (%)
	Fr A (Methyl and Ethyl Esters of Fatty Acids)		
14:0 ME	Myristic acid ME	1722	6.7
14:0 EE	Myristic acid EE	1791	6.1
16:1n-7 ME	Palmitoleic acid ME	1903	3.4
16:0 ME	Palmitic acid ME	1926	6.0
16:0 EE	Palmitic acid EE	1992	2.2
18:2n-6 ME	Linoleic acid ME	2092	1.4
18:1n-9 ME	Oleic acid ME	2103	0.7
18:0 ME	Stearic acid ME	2125	2.1
18:0 EE	Stearic acid EE	2191	0.8
20:4n-6 EE	Arachidonic acid EE	2262	24.9
20:5n-3 ME	5,8,11,14,17-Eicosapentanoic acid (EPA) ME	2267	26.5
20:1n-11 ME	11-Eicosenoic acid ME	2300	12.4
20:4n-6 ME	Arachidonic acid ME	2321	6.8
	Fr C (free fatty acids)		
20:4n-6 TMS	Arachidonic acid TMS	2376	61.4
20:5n-3 TMS	5,8,11,14,17-Eicosapentanoic acid (EPA) TMS	2400	38.6
	Fr D (1-monoglycerol esters)		
16:1n-7 ME	Palmitoleic acid ME	1903	5.0
16:0 ME	Palmitic acid ME	1926	7.8
18:2n-6 ME	Linoleic acid ME	2092	1.1
18:1n-3 ME	15-Octadecenoic acid ME	2012	0.4
18:0 ME	Stearic acid ME	2125	8.3
20:4n-6 ME	Arachidonic acid ME	2267	30.2
20:5n-3 ME	5,8,11,14,17-Eicosapentanoic acid (EPA) ME	2271	32.3
20:2n-6 ME	11,14-Eicosadienoic acid ME	2288	0.5
20:1n-9 ME	11-Eicosenoic acid ME	2300	14.4

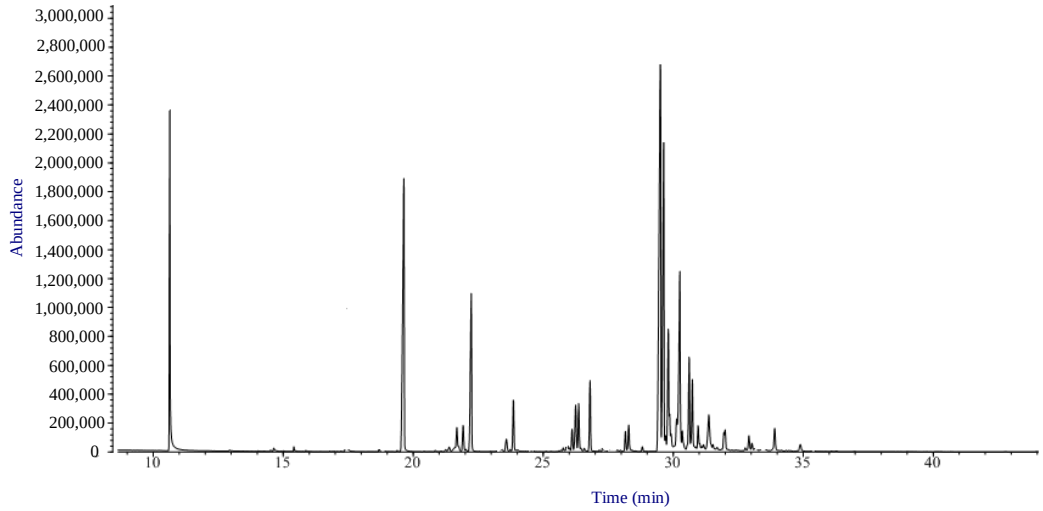
* ME = methylated; EE = ethylated; TMS = trimethylsilylated.



(**A**)



(**B**)



(**C**)

Figure 2. Total ion chromatograms (TICs) acquired from chromatographic fractions of crude extract obtained from *P. lividus* shells: fraction A (**A**); fraction C (**B**), and fraction D (**C**).

Fraction D was acquired after a transesterification process. Although it is feasible that GC-MS measurements for monoglycerides, especially with low molecular weight, are possible without derivatization procedures [25], in order to facilitate the detection and increase sensitivity, the residue was subjected to a common transesterification reaction by methanol (methanolysis) and monoglycerides were converted in their methyl esters. TIC of this fraction (Figure 2C) shows the presence of many 1-monoglycerol esters of fatty acids already identified as ME and the most abundant are 20:4n-6 ME (30.21%), 20:5n-3 ME (32.34%), and 20:1n-9 ME (14.42%) (Table 1).

Using the RSLDE process, 180 mg of extract were obtained from 80 g of shells, which corresponds to a yield of 0.225%.

In fr A, the concentrations of eicosapentanoic acid (EPA) (26.5%) in the injected solution (C_{EPA}) (1 µL) and in the crude extract (C^*_{EPA}) were respectively:

$$C_{EPA} = 153.1\frac{mg}{L}; C^*_{EPA} = 9.9\frac{mg}{g_{extract}}.$$

From the data of the percent concentrations in Table 1, the concentration of any component, x, may be calculated using formulas:

$$C_x = C_{EPA}\frac{\%x}{\%EPA}; C^*_x = C^*_{EPA}\frac{g_{fr}}{g_{extract}}.$$

Analogously for frs C and D, concentrations can be calculated by taking into account that the concentrations of arachidonic acid (ARA) (61.4%) were respectively $C_{ARA} = 142.1\frac{mg}{L}$ and $C^*_{ARA} = 24.6\frac{mg}{g_{extract}}$ and the concentrations for EPA (32.3%) were $C_{ARA} = 138.2\frac{mg}{L}$ and $C^*_{ARA} = 1.8\frac{mg}{g_{extract}}$.

The lipid component in the extract represented 28.27% and the percentage of any class of fatty acids and their esters are summarized in Table 2.

Table 2. Summary of the yield (%) of any class of fatty acids in the extract.

Component	Yield (%)
Fatty acids ME	7.7
Fatty acids EE	4
Free fatty acids	12.1
1-Monoglycerides	4.5
Total	28.3

After developing a strategy and considering the fatty acid yield, the economic profitability should be estimated in order to generate a model to maximize the potential profits from the recovered material. The RSLDE application to the shells of sea urchins has an advantage in this respect, to transform waste material into an economic resource for industry and to reduce the environmental impact derived from the disposal. Apart from the advantages, the possibility of exploiting a by-product should take into account the costs related to the proposed strategy. In this respect, in Table 3 there is an overview of cost for the activities in laboratory of the shell extraction. If an optimization result is required, more detailed data (i.e., cost of chemicals) should be considered.

Table 3. Overview of cost for the rapid solid liquid dynamic extraction (RSLDE) application to the shells of sea urchins.

Cost	Low	Medium	High
Shell preparation	✔		
Chemicals		✔	
Naviglio Extractor	✔		
Energy		✔	
Labor *	✔		

* Naviglio extractor is an automated device and does not require special expertise.

Although this strategy has been developed in a laboratory, the RSLDE could be used in large-scale industrial processes to produce shell extract, making the process cheap, and more efficient in terms of time, as well as ecologically benign.

4. Discussion

In Mediterranean countries there is a long and historical tradition of harvesting and eating sea urchins. In particular, Italy represents the largest market for *Paracentrotus lividus* in Europe, because the annual consumption is about 2000 tons [8]. The edible portion of the sea urchins constitutes approximately 10% of the total weight [26]. Hence, in Italy 1800 tons of sea urchin shells are unwanted material. It would be very important and of interest for a broad audience to reuse these shells as an example of circular economy.

In this study, analysis of *P. lividus* sea urchin shells was performed, and the fatty acid composition was reported for the first time.

Echinoderms, and particularly edible sea urchins, have been widely regarded for the proximate analysis and fatty acid contents of their tissues [27–29]. Investigations were performed on the gonadal tissue of diverse species of sea urchins, due to being of interest as seafood for human consumption [30–36]. The importance of polyunsaturated fatty acids (PUFAs) for human health and nutrition has stimulated chemists, biochemists and biotechnologists to focus their attention on lipids and fatty acids from marine organisms. In fact, marine FAs were extensively reviewed for their occurrence, roles, and for analytical strategies used for their screening [37,38].

The extracts analyzed in the present work were obtained by rapid solid liquid dynamic extraction (RSLDE), a convenient extractive technique which is easy to implement on the base of automated devices and does not require special expertise [12–14]. Contrary to previous studies conducted on red (*Strongylocentrotus franciscanus*) and green (*S. droebachiensis*) sea urchins using different hard strategies to obtain extracts [39,40], RSLDE allowed us to investigate the composition of shells without using a high volume of acidic solvents. In fact, RSLDE was preferred for the extraction and detection of shell components because the solvents, such as water and ethanol, do not alter the natural composition of the shells, which might be affected by hard treatments. The application of RSLDE to the shells of sea urchin, commonly considered to be material with low or no commercial value, permits the extraction of fatty acids of much higher commercial value to be reused in different fields of application, from aquafeed and agri-food industries to cosmeceuticals and nutraceuticals.

The shells RSLDE yield is 0.225% and, according to the Italian sea urchins' consumption [8], the present strategy is capable of providing annually 4.1 tons of shell extract which contain approximately 1.2 tons of fatty acids and their esters.

The main fatty acid component found in shell extract of *P. lividus* was 5,8,11,14,17-eicosapentaenoic acid (EPA) and its esters followed by arachidonic acid (ARA) and its esters in all studied fractions and also many monounsaturated and polyunsaturated fatty acids, in lower percentages.

EPA performs many essential functions in biological membranes and acts as a precursor of a variety of lipid regulators in cell metabolism. These pieces of evidence have led to the flourishing of notable interest in the development of commercial processes to produce EPA. Oils rich in EPA have been marketed for centuries. In addition to their cardiovascular benefits, these oils are generally required for health advantages associated with the nervous system and eyes, as well as general inflammation and multiple inflammatory affections [41]. ARA is becoming increasingly considered as a needed nutritional supplement, especially important for newborns. Due to its importance in development, especially of the central nervous system and retina, the Food and Agricultural Organization (FAO)/World Health Organization (WHO) recommended ARA supplements in infant formula [42]. Interestingly, the 1-monoglycerol ester of arachidonic acid was found in a high percentage in the studied extract. 1-arachidonoylglycerol has antiproliferative effects, as reported by Fowler et al. who studied the activity of this 1-monoglycerol ester in comparison with the endocannabinoid 2-arachidonoylglycerol [43].

5. Conclusions

A circular economy is a system where the value of products is maintained for as long as possible, waste and the use of resources are minimized and these are kept in the economy when a product has reached the end of its life cycle, in order to reuse it several times and create additional value. In this vein, certain research sectors have been focusing on the recovery of food waste for some years now, to solve the problem of waste disposal, transforming the latter into raw materials. Therefore, in this study, the green methodology of extraction, RSLDE, was used for the recovery and enhancement of the shells of sea urchins (*P. lividus*). Subsequently, the obtained extract was characterized from the molecular point of view, in order to find applications for various sectors, such as animal feed, ingredients, nutraceuticals, and cosmetics.

Author Contributions: P.C., A.T., D.N. and A.A. conceived and organized the manuscript; P.C. and A.T. provided the samples; M.M.S. and M.C. performed the experimental part; M.M.S., F.S., M.G. and A.A. wrote, edited and reviewed the manuscript.

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