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Analytica Chimica Acta XX (2018) XX-XX

https://doi.org/10.1016/j.aca.2018.11.012

Effects of extraction method and storage of dry tissue on marine lipids and fatty acids

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Keywords

Degradation
Freeze-drying
Lyophilization
Lipid class
Marine animal
Protocol

Abstract

Various protocols are currently used to study marine lipids, but there is a growing interest in working on dry samples that are easier to transport. However, reference protocols are still lacking for dry samples. In order to make recommendations on this use, lipid classes and fatty acids (FA) obtained from six analytical protocols using two different tissue states (dry *vs* wet) and three extraction methods (automat *vs* manual potter *vs* leaving the solvent to work on tissue) were compared. Three dry storage modes of tissue (freezer *vs* gas nitrogen *vs* dry room) during one and three months were also compared. These comparisons were made on seven marine species with different lipid profiles, including fishes, crustaceans and mollusks. Lipid classes and FA obtained from wet and dry tissues were similar, but they were affected by the extraction methods. Regardless of tissue state, "Leave to work" methods obtained the highest lipid quantities, followed by manual potter and automat methods (ca. 90% and 80% of "Leave to work" methods, respectively). Linear relationships allowed correction for lipid classes and FA concentrations obtained from different protocols. The repeatability of all protocols still needs to be improved, especially for fish species. Increasing the replicate number for each sample might be an indirect way to improve lipid quantification. Our results show that storing dry tissues in the freezer for more than one month was associated with a decrease in lipids, which is also observed for other storage methods. For qualitative studies of FA (expressed in %), a three-month storage of dry tissue in freezer did not affect the relative composition of species/tissues with a lipid content below 20% of dry weight.

1. INTRODUCTION

Lipids are extensively studied in marine ecology for three main reasons: (i) they constitute the main energy storage form and are associated with energy allocation strategies [1,2]; (ii) they act as ecological tracers as some fatty acids (FA) are conserved during trophic transfers [3,4]; (iii) they are the main component of the cell membrane and involved in physiological processes such as the homeostasis, the immune response, and the hormone biosynthesis [5,6]. Lipids are grouped into classes with FA as building blocks for most complex lipids. FA are carbon chains differing in length and double bond number and position: from zero (saturated FA; SFA) to several double bonds (polyunsaturated FA; PUFA). Among lipid classes, TriAcylGlycerols (TAG) consist of three FA esterified to a glycerol backbone. Animals store TAG when dietary lipids and energy intake exceed demands. Phospholipids (PL) consist of one or two FA esterified to a phosphoric acid and constitute cell membranes. Free sterols (ST) contain no FA, but play an important role for the cell membrane, as well as for lipoproteins and hormone biosynthesis. Beyond these general aspects, the lipid metabolism of animals is linked to taxonomy. For instance, crustaceans are incapable of *de novo* ST synthesis and depend on dietary ST sources [7]. Bivalve mollusks synthesize unusual 'non-methylene interrupted' (NMI) FA which can be used as tracers for bivalves in food chains [8]. Tunas are among the richest species in PUFA docosahexaenoic acid content (DHA; 22:6n-3) [1].

Marine lipids are commonly extracted with the solvent mixture of Folch et al. [9] (chloroform:methanol; 2:1, v/v) using different methods. The most popular methods use automats such as Soxhlet and Accelerated Solvent Extraction (e.g., [10–12]), potter homogenizers (e.g., [13–15]) or leave the solvent to work on ground tissue (e.g., [16–18]). Each extraction method has its own advantages and limitations: automats generally improve repeatability [19,20], manual devices such as potter homogenizers are cheaper than automats, and leaving solvent to work reduces handling steps that generate variability [21].

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Marine ecologists also deal with the constraint of storing tissue when lipids cannot be chemically-extracted immediately after sampling. Tissues can be stored frozen without degradation of marine lipids for several months to several years at -80°C (deep-frozen) [16,22] and at -20°C for some species [20,23,24]. However, the recommended deep-frozen storage is constraining over the long term as it requires suitable infrastructure and logistics; it also proves problematic when transportation is needed from remote areas, as maintaining samples at such temperature is complex. Storage into lipid-adapted solvent is not suitable for aircraft transport, neither for samples intended to be used for various types of complementary analyses such as stable isotopes [25,26] and DNA [27]. Freeze-drying (or lyophilization; a low temperature dehydration process) is a good alternative for marine ecologists as it is compatible with analyses of stable isotopes [28], metallic and organic contaminants [29] and metabolomic analyses such as Nuclear Magnetic Resonance [30]. Few studies have however looked into the effect of drying tissue on subsequent lipid analyses. Dunstan et al. [31] and Murphy et al. [32] conducted tests on the oyster *Crassostera gigas* and the green-lipped mussel *Perna canaliculus*, respectively, and found no significant influence of freeze-drying on lipid composition compared to frozen tissue. Caution seems required for the long-term storage of dry tissues as lipid degradation might have occurred (see [33] on rat liver after nine-month storage). For most marine taxa, the lack of comparison to suitable reference points (e.g., before/after drying and before/after dry storage) lead labs to work on frozen (e.g., [14,15]) as well as on freeze-dried tissues (e.g., [34–36]).

In this study, lipid class and FA compositions obtained from six extraction protocols were compared on a quantitative (concentration) and qualitative basis (percentage). These protocols differed in term of tissue state (wet *vs* dry) and extraction method (automat *vs* manual potter *vs* leave solvent to work). Statistical differences, reproducibility and repeatability were the main criteria used to compare the six protocols, all tested in the same laboratory. Reproducibility was defined as the agreement between test results obtained with different protocols and repeatability as the agreement of replicate tests carried out for each protocol. For dry samples, the effects of storage mode (freezer *vs* gas nitrogen *vs* dry room) and duration (t_0 reference *vs* $t_{+1\ month}$ *vs* $t_{+3\ months}$) were tested. The loss in lipid classes and FA was used as the criteria to assess storage modes, regardless of the lipid degradation products. All protocols for marine lipid extraction and tissue storage were tested on seven species from different phylum and biomes, including temperate, tropical and cultured fish, crustacean, cephalopod, and shellfish, to account for diverse marine lipid profiles.

2. MATERIAL AND METHODS

2.1. Tissue homogenates

Around 40 g of tissue were sampled from each of the seven selected marine species. Tissue samples were collected from (i) a single individual for the emperor red snapper *Lutjanus sebae* (dorsal muscle), the common octopus *Octopus vulgaris* (muscle), and the painted spiny lobster *Panulirus versicolor* (tail muscle), three tropical species from coastal waters of Mahé Island (Seychelles, Indian Ocean); (ii) a pool of individuals for the blue mussel *Mytilus edulis* (n=40; mantle), the European pilchard *Sardina pilchardus* (n=15; dorsal and ventral muscle), the cultured gilthead sea bream *Sparus aurata* (n=4; dorsal muscle), and the Atlantic bluefin tuna *Thunnus thynnus* (n=10; pectoral white muscle). Blue mussels were obtained from coastal waters of western Brittany (France, Atlantic Ocean), European pilchard and Atlantic bluefin tuna were collected in the Gulf of Lions (Mediterranean Sea), and cultured gilthead sea bream were obtained from the Ifremer marine station of Palavas, France. Wild species were collected during research programs, in collaboration with local fishermen. For each species, deep-frozen tissues were homogenized, i.e. minced in small pieces of ca. 3-5 mg over ice packs covered with a sheet of foil to avoid defrost and contamination. Homogenates were not pulverized into a thinner powder so that the grinding performance of the potter homogenizer could be assessed afterwards. Finally, the seven species homogenates were stored in polyethylene bottles at -80°C until further analyses.

2.2. Freeze-drying and water content

Thirty-six sub-samples were freeze-dried from each of the seven species homogenates previously minced (12 and 24 sub-samples for lipid extraction and storage comparison, respectively). For each sub-sample, about 1 g of frozen homogenate was weighted in a cryotube to the nearest 0.1 mg on an Adventurer pro balance (OHAUS, Nänikon, Swiss) and freeze-dried for 48 hours in the dark using an Alpha 1-4 freeze-dryer (Christ, Osterode am Harz, Germany). For each species, 12 dry sub-samples were weighted to gravimetrically measure the water content and lipids were chemically extracted in less than 24 hours after freeze-drying (see 2.3. Lipid extraction protocols). The 24 remaining dry sub-samples were stored using different methods (see 2.4. Storage of dry tissues).

2.3. Lipid extraction protocols

For each specie homogenate, six protocols for lipid extraction (numbered from A to F) were tested over four replicates. Tissues used for lipid extraction were either frozen at -80°C [protocols A, C, E] or freeze-dried [protocols B, D, F] (Fig. 1). Lipids were extracted from tissues with different processing: a pressurized automat [protocols A and B], a Dounce potter homogenizer [protocols C and D], or a 'leave-to-work' period of 24 hours [protocols E and F] (Fig. 1). Before extraction, all tissues were weighted to the nearest 0.1 µg on a XP6 analytical balance (Metler-Toledo, Viroflay, France). Lipids were extracted using a modified solvent mixture of Folch et al. [9] (dichloromethane:methanol; CH_2Cl_2:MeOH; 2:1, v/v) [37,38] with butylated hydroxytoluene as an antioxidant (0.01%; w/w). Tissue and solvent quantities were adapted to each method in such a way that the ratio of modified Folch mixture to sample was 50:1 (w/w) to insure a complete lipid extraction [39].

2.3.1 Protocols A and B [ASE]

An automated pressurized liquid extraction technique of trade name ASE 200 for Accelerated Solvent Extractor (Dionex, Voisins de Bretonneux, France) was used. The ASE program was set to a cycle that includes 5 min preheat and 10 min of static phase heat at 100 °C and pressurized to 130 psi. This program consumes 16-17 mL of the modified Folch mixture. ASE cells contained ca. 300 mg (frozen) or 100 mg (dry) of tissue homogenate mixed with glass beads. For protocol A, ASE cells were assembled and placed into the rack progressively to avoid tissue defrost before extraction. Extracts were flushed with nitrogen and stored 2 to 4 hours at -20°C as they arrived to the end of the ASE cycle.

2.3.2 Protocols C and D [Dounce]

A 15-cm^3 Dounce potter homogenizer (glass/Teflon; Fischer Scientific, Illkirch, France) was used. Lipids were extracted from ca. 120 mg (frozen) or 80 mg (dry) of tissue homogenate with 6 mL of the modified Folch mixture (3 rinses with 2 mL) and transferred into glass vials. Extracts were flushed with nitrogen, vortexed, sonicated for 20 minutes (ultrasound bath in +20 °C-water), and stored 2 to 4 hours at -20°C.

2.3.3 Protocols E and F [Ltw]

The solvent was left to work on the tissue homogenate for 24 hours. Lipids were extracted from ca. 120 mg (frozen) or 80 mg (dry) of tissue with 6 mL of the modified Folch mixture directly added into glass vial. Extracts were flushed with nitrogen, vortexed, sonicated for 20 minutes (ultrasound bath in +20 °C-water), and stored 24 hours at -20°C. This protocol applied on dry samples (protocol-F) was set by Cruz et al. [40] as a reference for lipid extraction from *O. vulgaris* and *S. pilchardus*, and it provided similar results to the AOAC Official Method 996.06 on flaxseed [41]. For the present study, Protocol-F was therefore used as the reference protocol.

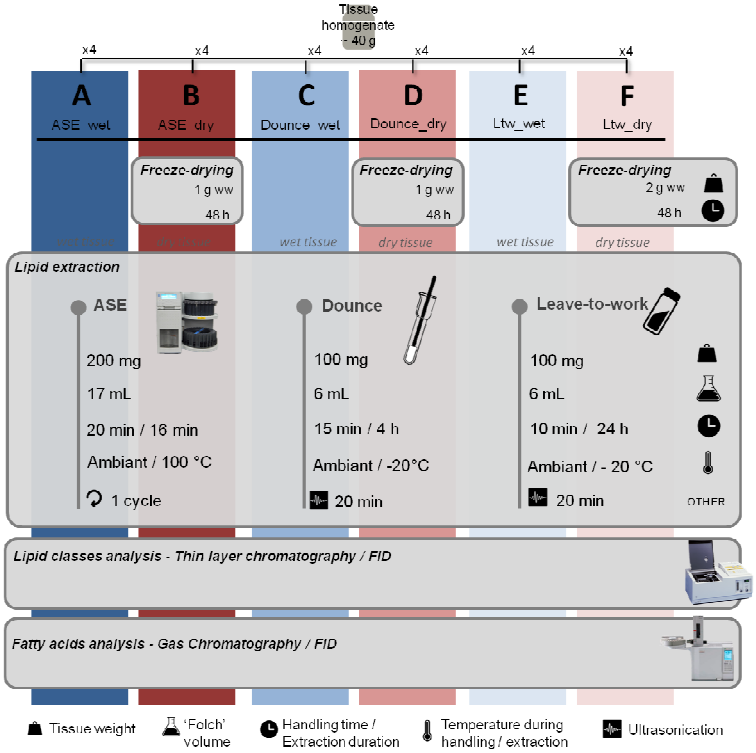


Fig. 1. Outline of the six protocols used for lipid extraction (A to F) compared across seven marine species (see Material & Methods for details). Comparisons are based upon lipid classes and fatty acid analyses. Methods using deep-frozen wet tissues (-80°C) are in blue, those using freeze-dried tissues are in red. Weights, volumes and handling time are given for one sample. Logos legend is at the bottom.

Extracts were brought back to room temperature, mixed with aqueous potassium chloride (0.88%; m/v) to obtain the final ratio of 8:4:3 CH_2Cl_2:MeOH:Water (v/v/v) [9], and were then centrifuged for 8 min at 1000 rpm and 15°C. The lower organic layers containing dissolved lipids were transferred into glass vials using Pasteur pipette and evaporated to dryness with nitrogen using an N-Evap 111 extractor (OA-SYS, Berlin, USA). Lipid extracts were then stored in CH_2Cl_2 and nitrogen at -20°C for one to five days before analyses.

2.4. Storage of dry tissues

For each species, the 24 freeze-dried sub-samples were assigned to three storage modes and two durations, in four replicates. Immediately after freeze-drying, cryotubes containing dry tissues were stored: (i) in a -20°C-freezer; (ii) in dry room (28°C and 30% air humidity), sealed with paraffin and nitrogen (inert gas) or; (iii) straight in dry room (28°C and 30% air humidity). These samples were stored for either one or three months. Protocol-F ('Leave-to-work') was used for lipid extraction. For comparisons with t_0 (reference), lipid classes were analyzed after both storage durations ($t_{+1month}$ and $t_{+3months}$) and fatty acids were analyzed at $t_{+3months}$ because of time and financial constraints. Seven of the 168 replicates were lost during preparation (glass vial broken).

2.5. Lipid class quantification

Lipid extracts in CH_2Cl_2 were spotted on quartz chromarods S5 (i.e. rods covered with silica; Bionis, St Georges Motel, France) using a 5 µL airtight glass syringe. They were separated into lipid classes in a two-phase development system: (i) 40 minutes in 80:20:1 hexane/diethyl-ether/formic acid; and (ii) 15 minutes in pure acetone, followed by two times 10 minutes in 5:4:1 chloroform/methanol/water [42]. Lipid classes were quantified after each separation phase using an Iatroscan MK-6s (Iatron Laboratories, Mitsubishi Chemical Medience, Tokyo, Japan) thin-layer chromatography − flame ionization detector analyzer (TLC−FID) with hydrogen flow set to 160-170 $mL.min^{-1}$. The signal was detected in millivolts and quantified using lipid standards (Cholesteryl palmitate, glyceryl tripalmitate, cholesterol, oleic acid, Diglyceryl palmitate, DL-palmitine and phosphatidil choline; Sigma-Aldrich, St Quentin Fallavier, France) with PeakSimple 3.93 software (SRI Instruments, Earl St. Torrance, USA). The minimum peak area considered was 0.1 $mV.mm^{-1}$. Concentrations in six lipid classes were determined (from least to most polar lipid class): triacylglycerols (TAG), free fatty acid (FFA), sterols (ST), diacylglycerols (DAG), acetone mobile polar lipids (AMPL, including monoacylglycerol, pigments, and glycolipids) and phospholipids (PL). The mean analytical variability for this quantification method was 17% (see section 3.2 for details on each lipid classes) based on 20 measurements of laboratory standards achieved over different days.

2.6. Fatty acid analysis

For FA quantification, tricosanoic acid (23:0; 2.3 µg) was added as internal standard to 250 µl of lipid extract. Lipids were transesterified with 800 µL of H_2SO_4 (3.8 % in MeOH) at 100 °C for 10 min [43] and washed three times with 1.5 mL of hexane-saturated distilled water. Fatty acid methyl esters (FAME) were separated and quantified by gas chromatography coupled with a FID (Varian CP8400 gas chromatograph; Agilent, Les Ulis, France) at the LEMAR Lipidocean facility (Brest, France). Samples (2 µL) were injected in splitless mode at an oven temperature of 60 °C and carried by helium gas simultaneously in two columns to improve FAME identification (polar Zebron ZB-WAX and apolar ZB-5HT columns, both 30 m in length, 0.25 mm internal diameter, 0.25 µm film thickness; Phenomenex, Le Pecq, France). The oven temperature was raised to 150 °C at 50 $°C.min^{-1}$, to 170 °C at 3.5 $°C.min^{-1}$, to 185 °C at 1.5 $°C.min^{-1}$, to 225 °C at 2.4 $°C.min^{-1}$and then to 250 °C at 5.5 $°C.min^{-1}$. FAME were identified by comparing sample retention times to those of commercial standard mixture (37-components FAME Mix; Sigma-Aldrich) using Galaxie 1.9.3.2 software (Agilent). FAME peak area was converted into µg of FA based on the standard peak area. The mean analytical variability for FA quantification was 8.1% based on five measurements of the standard mixture achieved on different days. Co-elution occurs between 20:1n-7 and 22:2i and between 21:5n-3 and 22:3nmi in mussel samples. Thirty-one FA > 0.8% of total FA in at least one sample were kept for data analysis.

2.7. Data analysis

In the subsequent sections, "wet tissue" refers to frozen wet tissue and dry tissue to freeze-dried tissue. All results are expressed in $µg.mg^{-1}$ on a dry weight basis (dw) for comparison across protocols and are presented as mean ± SD. For protocols based on wet tissues (A, C and E), the water content measured before/after freeze-drying was used to convert tissue wet mass into

tissue dry mass. Species were grouped into "Lean" and "Fat" species for visualization purpose, the threshold was arbitrary set at 120 µg.mg^{-1} dw (Lean species: *L. sebae*, *M. edulis*, *O. vulgaris* and *P. versicolor*, and fat species: *S. pilchardus*, *S. aurata* and *T. thynnus*).

Lipid class and FA concentrations (log and square root transformed to achieved normality of residuals in the analysis, respectively) were compared among lipid extraction protocols (three factors: tissue state, extraction method and interaction) and among dry storage (three factors: storage mode, duration and interaction) using MANOVAs (multivariate analyses of variance; F-test). The higher the F value, the stronger the influence of the factor. Normality of residuals was tested with the univariate Shapiro-Wilk test. When residuals were not normally distributed, a non-parametric Scheirer-Ray-Hare test (H-test) was used instead of a MANOVA. Post-hoc tests (parametric TukeyHSD or non-parametric Dunn Holm-adjusted test) were applied to refine differences among the factors' modalities of the lipid extraction protocols and the storage modes factors. Principal Component Analyses (PCA) and PERMANOVAs (multivariate analyses of variance with 999 permutations based on Euclidian distance matrix; Pseudo-F test) were applied on square root transformed % FA to compare FA profiles among lipid extraction protocols and dry storages. PERMANOVA is an analogous to non-parametric MANOVA: it partitions sums of squares of a multivariate dataset among factors and uses a permutation test.

Reproducibility and repeatability were assessed for each species homogenate. Reproducibility was defined for major lipid classes and FAs as the ratio between the quantity obtained with a given protocol to the quantity obtained with the reference protocol-F [40]. The reproducibility of each protocol was considered acceptable when comprised between 90-110%. Repeatability was assessed for each compound with replicate samples through the coefficient of variation (CV), defined as the ratio of SD to the mean. The higher the CV, the lower the repeatability. Repeatability was considered acceptable when CV was below 10%. All statistical analyses were performed using R 3.5.0 software [44], 'MVN', 'vegan' and 'dunn.test' packages.

3. **RESULTS**

3.1. Influence of lipid extraction protocols

The tissue state had little impact on lipid classes and FA concentrations, aside from AMPL that were higher when extracted from wet tissue for all species (Fig. 2; Table S1). However, some species-specific differences were observed. For *L. sebae*, the tissue state only affected lipid classes, especially the minor ones such as DAG (0.8±0.6 and 2.0±1.3 µg.mg^{-1} in dry and wet tissues, respectively; p<0.001) and ST (1.6±0.3 and 2.0±0.3 µg.mg^{-1}; p<0.01, in dry and wet tissues, respectively). For *M. edulis*, dry tissue was associated with higher TAG (20.4±6.3 and 9.6±7.7 µg.mg^{-1} in dry and wet tissues, respectively; p<0.001) and ST contents (4.9±1.3 and 3.7±1.1 µg.mg^{-1} in dry and wet tissues, respectively; p<0.01), but had no influence on FA concentrations (quantitative) (Table 1). For *O. vulgaris*, no impact of the tissue state was observed, excepted for DAG (0.2±0.4 and 2.6±2.2 µg.mg^{-1} in dry and wet tissues, respectively; p<0.001). For *P. versicolor*, while most lipid classes and FA concentrations were affected by tissue state (MANOVAs, Table 1) post-hoc tests detected no difference between dry and wet tissues (DAG: p=0.13; ST: p=0.08; PL: p=0.22; TLC: p=0.28). For *S. pilchardus*, no effect of tissue state was detected, with the exception of some MUFA concentrations being higher in wet tissues, such as cetoleic acid 22:1n-11 (1.8±0.6 and 3.2±1.5 µg.mg^{-1} in dry and wet tissues, respectively; p<0.01). The tissue state had no impact on FA concentrations and lipid classes of *T. thynnus* and only affected ST in *S. aurata* (2.2±0.4 and 2.9±0.7 µg.mg^{-1} in dry and wet tissues, respectively; p<0.01).

The extraction method had a greater influence than tissue state on the quantity of extracted lipids, with higher levels obtained with the Ltw methods, as revealed by the MANOVAs F-values (data not shown). Among lean species, this tendency was particularly evident for *M. edulis*, with TAG ranking 8.8±9.6 < 15.1±4.7 < 21.2±7.3 µg.mg^{-1} and PL ranking 32.0±10.7 < 43.2±4.6 < 49.4±11.2 µg.mg^{-1} with ASE, Dounce and Ltw methods, respectively. For the three other lean species, ASE provided a good extraction for PL, but results were highly variable due to an interaction with the tissue state (see last paragraph below) and FA concentrations were not affected by this pattern, most FA being higher when extracted with the Ltw methods (Fig. 3). For fat species, the extraction method influenced differently lipid classes and FA concentrations. For instance, the extraction method had no effect on the most important lipid class of *S. aurata* (TAG: 205±49.5 µg.mg^{-1}; F=3.0, p=0.07) but most of FA concentrations were higher with Ltw extraction methods such as PUFA (PUFA=32.2±3.6 µg.mg^{-1} with ASE and Dounce extraction methods and 38.1±4.6 µg.mg^{-1} with Ltw methods). Only FA concentrations from *S. pilchardus* and *T. thynnus* were unaffected by the extraction method, despite the higher TAG concentrations obtained with the ASE and Ltw extraction methods, respectively (Table 1).

The interaction between tissue state and extraction method (i.e. the six protocols) affected the lipid classes of most species and the FA of *M. edulis*, *O. vulgaris*, and *P. versicolor*. Regarding lipid classes, PL contents were higher by 52-57% for *L. sebae*, *O. vulgaris*, and *P. versicolor* using protocol-B and TAG contents were higher by 116-138% for *S. pilchardus* and *S. aurata* using protocol-A rather than the five other protocols (Table S1). Regarding FA, protocol-A lead to the lowest concentrations for the lean species whereas no difference was observed across the six protocols for the three fat species (Fig. 3). Regardless of species, significant and strong linear relationships (r^2 around 0.9) between extraction protocols would permit a correction for lipid classes and FA concentrations (Fig. S2). The qualitative FA profiles, expressed in percent, were however similar between the six protocols for each species (Fig. 4a), with no effect of tissue state (Pseudo-F=0.1, p=0.9), extraction method (Pseudo-F=0.1, p=0.9) and their interaction (Pseudo-F=0.1, p=0.9).

3.2. Reproducibility & repeatability of extraction protocols

The reproducibility of the five tested protocols compared to the reference protocol-F varied across species: it was particularly low for *L. sebae* (less than 70% reproducibility for FA, except for protocol-E) and good for *S. pilchardus* (87-120% for FA concentrations across all protocols) (Table 2a). Protocol-D provided similar results to protocol-F with most lipid contents comprised within 90-110% of those obtained with protocol-F, except for *L. sebae* (Table 2a). Protocol-E was also in good agreement with protocol-F for most species, but led to higher lipid classes and FA quantities in lean species. For instance, the maximal overestimation yielded by protocol-E was obtained for 18:1n-9 from *M. edulis* (161% higher, with 0.9±0.2 and 0.6±0.2 µg.mg^{-1} obtained with protocol-E and -F, respectively). In contrast, protocol-A was the most dissimilar to protocol-F due to a lower amount of lipid classes and FA extracted, with an average reproducibility of 86% (min-max=44-198%) for lipid classes and of 78% (min-max=39-155%) for FA across all species. Protocol-B and protocol-C also showed poor reproducibility compared to protocol-F, with lower lipid classes and FA quantities obtained for *L. sebae*, *P. versicolor*, *S. aurata* and *T. thynnus* (Table 2a).

Overall, the repeatability did not fall within the range of the analytical variability (TAG: 21%; FFA: 26%; ST: 8%; DAG: 9%; AMPL: 29%; PL: 10%, and FA: 8%) and was attributable to the extraction protocols (Table 4b). The repeatability also varied across species: *T. thynnus* showed the lowest repeatability among the six protocols (mean CV=39%; min-max=19-67%) and *S. aurata* and *P. versicolor* the best one (CV<10% in most protocols; Table 2b). For lipid classes, the best repeatability was obtained with protocol-E (mean CV=18%; min-max CV=6-36%) and the lowest with protocol-F (mean CV=26%; min-max CV=10-117%). For FA concentrations, the mean repeatability ranked protocol-B (mean CV=11%; min-max CV=1-44%) > protocol-D (mean CV=14%; min-max=1-45%) > protocol-E (mean CV=18%, min-max=3-42%) > protocol-F (mean CV=20%; min-max CV=2-68%) > protocol-A (mean CV=23%; min-max CV=2-45%) and protocol-C (mean CV=23%; min-max CV=1-67%). However, the repeatability of each protocol was also variable across species. For example, protocol-B provided an acceptable repeatability for FA of *M. edulis* (mean CV=4%; min-max=1-9%) but an unacceptable one for *T. thynnus* (mean CV=36%; min-max=24-44 %). No general difference between lean and fat species was noticed for reproducibility and repeatability.

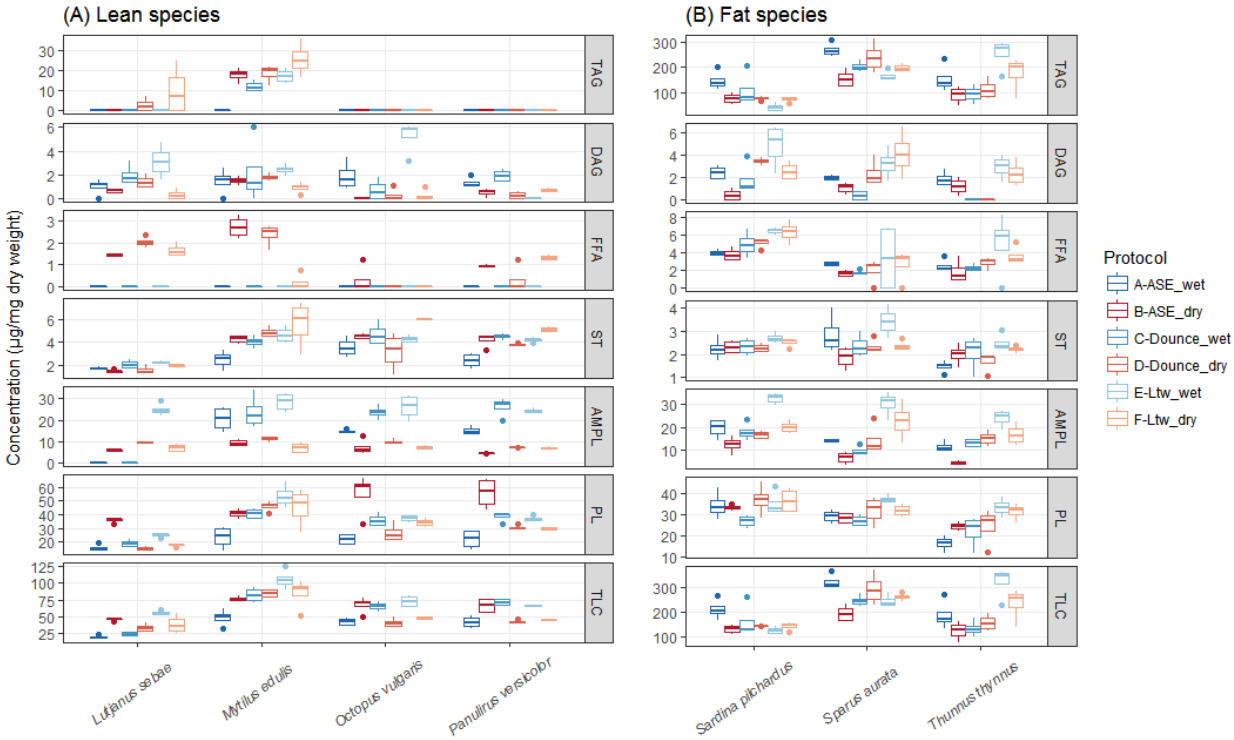
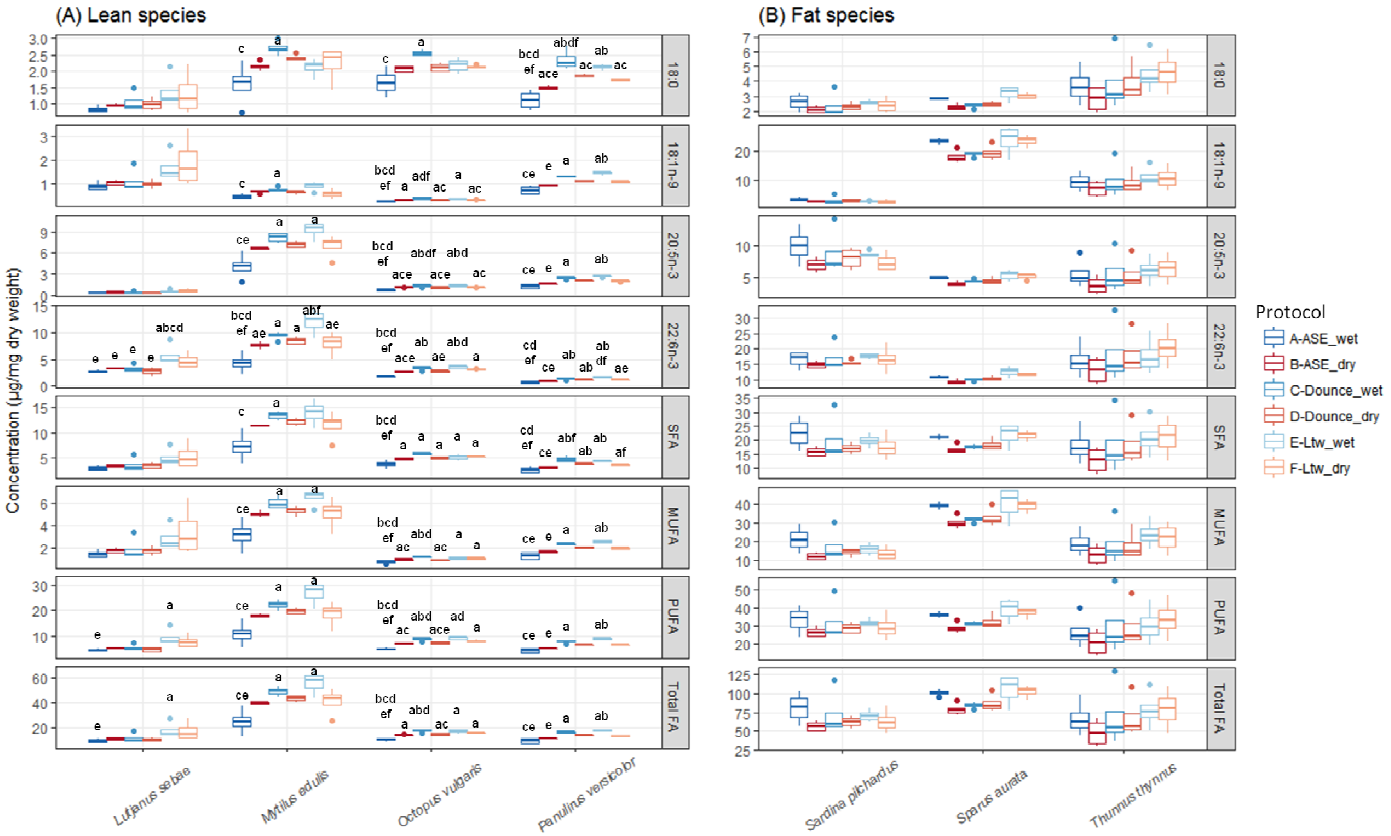


Fig 2. Distribution of lipid classes (triacylglycerols (TAG), diacylglycerols (DAG), free fatty acids (FFA), sterols (ST), acetone mobile polar lipids (AMPL) and phospholipids (PL)) and the total lipid content (TLC) of (A) lean species (TLC < 120 µg.mg^{-1} dry weight) and (B) fat species according to six protocols for lipid extraction (A to F, blue and red based colors for wet and dry tissue, respectively see Material and Methods for details). Thick bar is the median value, points are outliers of four replicates, and the box contains 50% of the data.

Table 1. Probabilities from MANOVA (F-test) or Scheirer-Ray-Hare test (H-test; grey lines) testing the effects of tissue state (dry and wet), method (ASE, Dounce potter and Ltw) and their interaction on lipid class (TAG: triacylglycerols, DAG: diacylglycerols, FFA: free fatty acids, ST: sterols, AMPL: acetone mobile polar lipids, PL: phospholipids) and total lipid content (TLC) and fatty acid concentrations (SFA=Saturated FA; MUFA=Monounsaturated FA; PUFA=Polyunsaturated FA) determined in seven marine species. Codes: ns not significant, * p<0.05, ** p<0.01, *** p<0.001. _ denotes FA <0.8% of total FA.

	Lutjanus sebae			*Mytilus edulis*			*Octopus vulgaris*			*Panulirus versicolor*			*Sardina pilchardus*			*Sparus aurata* (cultured)			*Thunnus thynnus*		
	Tissue state	Method	Tissue state × Method	Tissue state	Method	Tissue state × Method	Tissue state	Method	Tissue state × Method	Tissue state	Method	Tissue state × Method	Tissue state	Method	Tissue state × Method	Tissue state	Method	Tissue state × Method	Tissue state	Method	Tissue state × Method
Lipid classes																					
TAG	ns	ns	ns	***	***	***	ns	ns	ns	_	_	_	ns	**	**	ns	ns	***	ns	**	ns
DAG	***	*	**	ns	ns	***	***	ns	ns	**	***	***	*	***	***	ns	***	**	ns	***	ns
FFA	***	ns	ns	***	ns	ns	ns	ns	ns	ns	***	ns	ns	***	ns	ns	ns	*	ns	**	ns
ST	**	**	ns	**	**	ns	ns	ns	*	**	***	***	ns	ns	ns	**	ns	ns	ns	*	ns
AMPL	***	***	***	***	ns	*	***	**	*	***	*	ns	***	***	ns	*	***	**	***	***	***
PL	*	***	***	ns	***	*	ns	ns	***	*	ns	***	ns	ns	ns	ns	*	ns	ns	***	ns
Total lipid	*	***	***	ns	**	*	*	ns	***	**	ns	***	ns	ns	***	ns	ns	***	*	***	ns
Fatty acids																					
14:0	ns	ns	ns	ns	**	**	_	_	_	_	_	_	*	ns	ns	ns	ns	ns	ns	ns	ns
15:0	_	_	_	ns	***	**	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
16:0	ns	***	ns	ns	***	*	*	***	***	*	***	**	*	ns	ns	ns	**	ns	ns	ns	ns
17:0	ns	**	ns	ns	***	ns	ns	***	*	ns	***	ns	ns	ns	ns	_	_	_	ns	ns	ns
18:0	ns	ns	ns	ns	***	ns	ns	**	**	ns	***	***	ns	ns	ns	ns	***	ns	ns	ns	ns
16:1n-7	ns	ns	ns	ns	*	*	ns	***	***	ns	***	***	*	ns	ns	ns	*	ns	ns	ns	ns
18:1n-9	ns	**	ns	ns	*	**	ns	***	***	*	***	*	ns	ns	ns	ns	*	ns	ns	ns	ns
18:1n-7	ns	***	ns	ns	***	ns	ns	***	**	ns	***	***	ns	ns	ns	*	**	ns	ns	ns	ns
20:1n-9	_	_	_	ns	***	*	_	_	_	_	_	_	**	ns	ns	ns	*	*	ns	ns	ns
20:1n-7	_	_	_	ns	***	**	ns	*	ns	_	_	_	_	_	_	_	_	_	_	_	_
22:1n-11	_	_	_	_	_	_	_	_	_	_	_	_	**	ns	ns	ns	*	*	ns	*	ns
24:1n-9	ns	***	ns	_	_	_	_	_	_	_	_	_	ns	ns	ns	_	_	_	ns	ns	ns
16:4n-3	ns	***	ns	ns	***	ns	_	_	_	_	_	_	*	ns	ns	_	_	_	_	_	_
18:2n-6	ns	**	ns	ns	**	**	_	_	_	*	***	*	ns	ns	ns	ns	*	ns	ns	ns	ns
18:3n-3	_	_	_	ns	**	***	_	_	_	*	***	ns	ns	ns	ns	ns	*	*	ns	ns	ns
18:4n-3	_	_	_	_	_	_	_	_	_	*	**	***	**	ns	ns	ns	ns	*	ns	***	ns
20:2i	_	_	_	ns	**	**	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
20:2j	_	_	_	ns	**	*	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
20:4n-6	ns	***	**	ns	***	*	ns	***	***	*	***	*	ns	ns	ns	_	_	_	_	_	_
20:4n-3	_	_	_	_	_	_	_	_	_	_	_	_	ns	ns	ns	ns	*	ns	ns	ns	ns
20:5n-3	ns	***	ns	ns	***	*	ns	***	***	*	***	*	ns	ns	ns	ns	**	ns	ns	ns	ns
22:3nmi	_	_	_	ns	***	*	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
22:4n-6	ns	***	ns	_	_	_	ns	***	***	**	***	***	_	_	_	ns	***	ns	ns	ns	ns
22:5n-6	ns	***	ns	_	_	_	ns	***	***	*	***	ns	ns	ns	ns	_	_	_	ns	ns	ns
22:5n-3	ns	*	ns	ns	***	*	*	***	***	*	***	***	ns	ns	ns	ns	**	ns	ns	ns	ns
22:6n-3	ns	***	ns	ns	***	***	ns	***	***	*	***	***	ns	ns	ns	ns	***	ns	ns	ns	ns
18:0DMA	_	_	_	ns	***	*	ns	***	***	ns	***	*	_	_	_	_	_	_	_	_	_
n-3 PUFA	ns	***	ns	ns	***	**	ns	***	***	*	***	*	ns	ns	ns	ns	**	ns	ns	ns	ns
n-6 PUFA	ns	***	ns	ns	***	*	ns	***	***	*	***	*	ns	ns	ns	ns	*	ns	ns	ns	ns
SFA	ns	***	ns	ns	***	*	ns	***	***	ns	***	***	*	ns	ns	ns	**	ns	ns	ns	ns
MUFA	ns	*	ns	ns	***	*	ns	***	***	*	***	*	*	ns	ns	ns	*	ns	ns	ns	ns
PUFA	ns	***	ns	ns	***	*	ns	***	***	*	***	*	ns	ns	ns	ns	**	ns	ns	ns	ns
Total FA	ns	***	ns	ns	***	*	ns	***	***	*	***	*	*	ns	ns	ns	*	ns	ns	ns	ns

Fig 3. Distribution of four specific fatty acids (Stearic acid 18:0; Oleic acid 18:1n-9; Eicosapentaenoic acid (EPA) 20:5n-3; Docosahexaenoic (DHA) 22:6n-3) and FA families (SFA=Saturated FA; MUFA=Monounsaturated FA; PUFA=Polyunsaturated FA) of (A) lean species and (B) fat species according to six protocols (A to F, blue and red based colors for wet and dry tissue, respectively; see Material and Methods for details). Thick bar is the median value, points are outliers of four replicates, and the box contains 50% of the data. Letters indicate significant difference among methods at p<0.05 (i.e. a=different from protocol A, b=different from protocol B...).



3.3. Influence of dry storage

The storage of dry tissue had contrasted effects on marine lipid compounds and among species (Table 3). One month of freezer storage had no effect on lipid classes, aside from ST in *O. vulgaris* and *P. versicolor*. The two other storage modes led to lower values of lipid classes, especially for *M. edulis*. For instance, the TAG content of *M. edulis* was 25.4±8.1 µg.mg^{-1} (reference) and decreased to 2.0±0.4 and 2.3±0.7 µg.mg^{-1} after one-month storage in dry room and with nitrogen, respectively (Fig. 5a). No decrease in lipid class concentrations was however observed after one-month storage in dry room or after nitrogen flushing for *T. thynnus* (Fig. 5b).

After three months, a species-specific decrease in lipid class and FA concentrations was observed regardless of the storage mode. *T. thynnus* was the less affected species for both lipid classes and FA concentrations after three months of freezer storage, *P. versicolor* was the most affected for lipid class concentrations and *S. aurata* the most affected for FA concentrations. For instance, the 18:1n-9 in *S. aurata* ranked 23.6±2.1 (reference) > 17.1±2.1 (dry room) > 16.2±2.8 (nitrogen) > 14.0±2.2 µg.mg^{-1} (freezer) (Fig. 6; Table S2f). For the other species, after three months most FA concentrations ranked as: reference ~ freezer > nitrogen > dry room. Some differences between reference and freezer storage were however observed, mostly for *O. vulgaris*, but they corresponded to minor losses: e.g. 22:6n-3 was the most important FA of *O. vulgaris* and it ranked 3.1±0.1 (reference) > 2.9±0.3 (freezer) > 2.9±0.4 (dry room) > 2.8±0.0 (nitrogen) (Table S2c). Finally, although the concentrations decreased for all FA (including SFA, MUFA and PUFA), the FA profiles expressed in percent were the same between reference and freezer storage (Pseudo-F=0.1, p=1.0), and between dry room and nitrogen storages (Pseudo-F=0.3, p=0.05) (Fig. 4b and Table S2). FA profiles were different between reference and dry room storage (Pseudo-F=4.7, p<0.01), and reference and nitrogen storage (Pseudo-F=3.0, p<0.01).

4. DISCUSSION

Six lipid extraction protocols, including different tissue states and extraction methods, and three storage modes of dry tissues, were compared to make recommendations for protocols of lipid analysis in marine animals. For lipid extraction, the tissue state (frozen or freeze-dried) did not affect the results, but the extraction method did. Linear corrections permitted to correct this effect. For the storage of dry tissues, one-month storage in a -20°C-freezer was found acceptable for quantitative studies, while longer storage or other storage modes led to lipid loss. Three-month storage in a -20°C-freezer gave satisfactory results for qualitative studies of FA.

4.1. How to extract lipids from marine tissues?

Except for *P. versicolor*, no difference was observed between dry and wet tissues while the extraction method affected the lipid composition of all studied marine species. Dounce (potter homogenizer) and Leave-to-work methods provided more reproducible results than the ASE ones. Regarding ASE methods, an increase in the extraction temperature up to 120°C might be a solution to extract all lipids and improve the reproducibility [19]. The highest lipid class and FA concentrations were obtained with the two “Leave-to-work” protocols (protocol-E and -F). These two protocols had however a low repeatability, especially protocol-F (chosen as reference protocol), which has been detrimental to the assessment of reproducibility. The difference between protocols is however predictable and the reproducibility might be improved by applying a correction on concentration results. The repeatability of the six protocols was low but of the same order of magnitude as the repeatability obtained with other protocols on frozen fish species (13-18%) [20]. Quadruplicating the samples was probably not enough to assess the extraction variability and could explain this low repeatability. Considering the high variability of the method used for lipid class quantification (mean

CV=17%), 10 replicates would have provided a better overview of the repeatability of each protocol. The mincing of the initial homogenates was ruled out as being responsible for the low repeatability because (i) protocols C and -D included a fine grinding and were also associated with low repeatability, and (ii) some homogenates were obtained from a single individual and a single tissue type without any improvement in repeatability (*L. sebae*, *O. vulgaris* and *P. versicolor*).

Leave the solvent to work on tissue (regardless of tissue state) was the simplest protocol to implement, requiring little handling and preparation (ca. 10 min per sample). In this study, tissue homogenates were minced in small pieces (ca. 3-5 mg). Such a preparation was required for protocols –E and -F to work properly. Several teams already used a similar protocol after ball grinding frozen tissues (e.g. [18]) but it involves handling liquid nitrogen with care (-195°C) and to carefully clean equipment between samples to avoid cross-contamination. A safer and faster processing as the protocol-E might be sufficient to extract lipids but its repeatability should be improved for some species, especially *L. sebae*, *M. edulis* and *T. thynnus*. A continuous agitation during the extraction and a temperature increase (e.g. ambient temperature instead of -20°C) might be beneficial [45]. For the oyster *C. gigas*, Dunstan et al. [31] increased the lipid recovery by rehydrating dry tissue, but this result was not confirmed [46]. When lipid analyses are cheap such as TLC-FID, a compromise could be to use the simplest and fastest extraction protocol (e.g. protocol-E) but to extract lipids in duplicate or triplicate to overcome the low repeatability and the tissue heterogeneity. Such an approach is already used for some contaminant analysis when precision and accuracy tolerances are difficult to achieve (e.g. three replicates of each sample tissue were analyzed for a precise quantification of polycyclic aromatic hydrocarbon and total mercury in fish [47,48]).

Finally, the “Bligh and Dyer” extraction method [49], mainly consisting in a reduction of the solvent to sample ratio from the Folch method, would probably not be affected by the tissue state either. However, this method might have a reduced extraction efficiency for fat dry tissues, as observed on wet tissues [50].

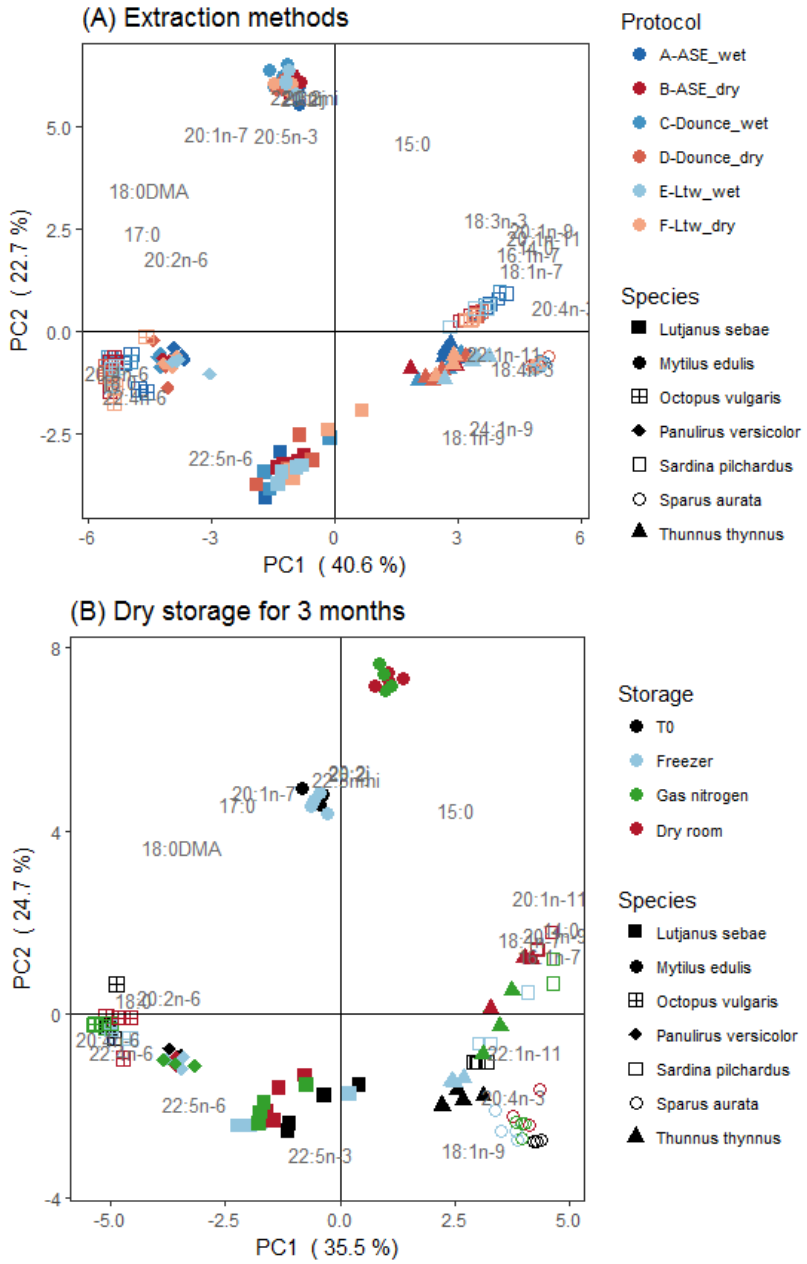


Fig 4. Principal component analyses (PCA) of total fatty acid (FA) percentage composition for seven marine species: (A) according to six lipid extraction protocols (A to F) and (B) three storage modes of dry tissues. Thirty-one FA >0.8% are considered for the PCA but only FA with $\cos^2 > 0.35$ were represented to improve readability.

Table 2a. Percentage of reproducibility for the lipid extraction protocols A to E compared to protocol-F (used as reference) estimated from the results of lipid class (TAG: triacylglycerols, ST: sterols, PL: phospholipids) and total lipid content (TLC) and fatty acid concentrations (SFA=Saturated FA; MUFA=Monounsaturated FA; PUFA=Polyunsaturated FA). Green intensity denotes acceptability (90-110%).

(A) Reproducibility of protocol F (%)

A-ASE_wet

	L. sebae	*M. edulis*	*O. vulgaris*	*P. versicolor*	*S. pilchardus*	*S. aurata*	*T. thynnus*
Lipid class							
TAG	_	_	_	_	198	136	88
ST	88	44	59	46	89	122	66
PL	88	52	65	74	94	92	52
TLC	48	57	89	59	147	122	81
Fatty acids							
16:0	55	61	64	70	123	98	87
18:0	62	71	78	64	110	93	80
18:1n-9	47	79	73	65	128	99	87
18:1n-7	39	62	66	65	127	103	87
20:4n-6	69	56	61	67	111	92	72
20:5n-3	50	58	62	64	135	97	87
22:5n-3	49	55	56	62	128	98	86
22:6n-3	58	56	56	62	100	93	79
n-3	57	58	59	64	114	96	80
n-6	61	58	61	66	113	98	78
SFA	55	63	71	68	127	97	85
MUFA	42	62	65	64	155	100	87
PUFA	58	58	60	65	114	97	79
Total FA	53	60	63	66	128	98	83

B-ASE_dry

	L. sebae	*M. edulis*	*O. vulgaris*	*P. versicolor*	*S. pilchardus*	*S. aurata*	*T. thynnus*
Lipid class							
TAG	_	69	_	_	105	79	51
ST	75	76	75	81	92	79	90
PL	203	89	163	187	91	89	79
TLC	119	90	143	150	91	75	53
Fatty acids							
16:0	63	99	88	83	89	77	63
18:0	72	97	96	86	88	75	61
18:1n-9	54	117	97	82	96	76	65
18:1n-7	45	97	90	82	92	79	58
20:4n-6	83	92	90	85	90	75	66
20:5n-3	62	96	95	80	94	77	58
22:5n-3	54	91	83	79	88	78	57
22:6n-3	69	96	85	78	89	79	64
n-3	67	97	87	79	90	78	62
n-6	72	97	88	84	87	77	63
SFA	63	99	92	85	89	77	60
MUFA	50	99	88	82	88	77	59
PUFA	68	97	88	81	90	77	62
Total FA	62	97	90	82	89	77	61

C-Dounce_wet

	L. sebae	*M. edulis*	*O. vulgaris*	*P. versicolor*	*S. pilchardus*	*S. aurata*	*T. thynnus*
Lipid class							
TAG	_	47	_	_	147	103	54
ST	106	72	78	87	92	101	94
PL	107	89	106	129	73	85	70
TLC	60	97	138	103	114	93	57
Fatty acids							
16:0	66	116	104	125	113	82	89
18:0	79	122	120	135	98	78	84
18:1n-9	57	135	126	119	112	80	90
18:1n-7	50	118	113	123	113	83	88
20:4n-6	86	124	113	127	108	83	88
20:5n-3	65	117	120	124	120	86	88
22:5n-3	63	114	100	117	111	84	87
22:6n-3	70	119	105	119	102	84	89
n-3	68	119	108	121	110	84	89
n-6	76	123	110	126	110	83	88
SFA	67	117	112	132	114	81	86
MUFA	54	118	110	119	130	81	85
PUFA	70	119	109	123	110	84	89
Total FA	65	119	111	123	116	82	87

D-Dounce_dry

	L. sebae	*M. edulis*	*O. vulgaris*	*P. versicolor*	*S. pilchardus*	*S. aurata*	*T. thynnus*
Lipid class							
TAG	27	72	_	_	105	121	64
ST	79	86	53	73	90	99	75
PL	87	101	79	102	101	100	78
TLC	85	100	85	96	100	112	67
Fatty acids							
16:0	64	105	91	101	96	85	88
18:0	75	108	99	107	96	82	85
18:1n-9	52	114	92	102	110	82	85
18:1n-7	46	102	90	101	107	86	85
20:4n-6	69	100	93	107	99	86	87
20:5n-3	53	103	96	104	108	86	88
22:5n-3	61	102	90	102	105	84	81
22:6n-3	59	107	89	101	93	88	88
n-3	57	106	90	103	98	87	88
n-6	64	105	92	106	96	85	89
SFA	65	106	95	106	97	85	85
MUFA	50	105	85	103	105	83	82
PUFA	59	105	91	104	98	86	88
Total FA	59	105	92	104	99	85	85

E-Ltw_wet

	L. sebae	*M. edulis*	*O. vulgaris*	*P. versicolor*	*S. pilchardus*	*S. aurata*	*T. thynnus*
Lipid class							
TAG	_	67	_	_	59	84	143
ST	115	82	71	82	107	145	112
PL	141	118	111	124	96	117	107
TLC	145	125	155	149	88	93	138
Fatty acids							
16:0	98	130	96	124	112	101	99
18:0	106	95	103	123	108	105	97
18:1n-9	88	161	109	132	103	101	106
18:1n-7	76	124	101	128	110	108	103
20:4n-6	145	128	113	141	109	108	80
20:5n-3	104	135	119	138	117	103	97
22:5n-3	93	140	108	139	107	105	103
22:6n-3	121	154	109	140	106	106	87
n-3	120	145	111	139	110	105	91
n-6	129	146	114	140	108	103	87
SFA	96	122	99	121	112	101	99
MUFA	82	129	99	131	118	102	109
PUFA	122	146	113	140	110	104	90
Total FA	104	137	109	133	112	103	98

Legend	< 80	90	100	110	>120

Table 2b. Percentage of repeatability for the six lipid extraction protocols (A to F) corresponding to the coefficient of variation (CV) lipid class (TAG: triacylglycerols, ST: sterols, PL: phospholipids) and total lipid content (TLC) and fatty acid concentrations (SFA=Saturated FA; MUFA=Monounsaturated FA; PUFA=Polyunsaturated FA) estimated from four replicates of each marine species. Green intensity indicates different levels of CV (see legend). Values in bold did not fall within the range of analytical variability (TAG: 21%, ST: 8%, PL: 10%, individual FA: 8%). Note the analytical variability was not assessed for TLC and FA families.

(B) Repeatability (%)

A-ASE_wet

	L. sebae	*M. edulis*	*O. vulgaris*	*P. versicolor*	*S. pilchardus*	*S. aurata*	*T. thynnus*
Lipid class							
TAG	_	_	_	_	18	14	23
ST	10	36	23	27	13	34	11
PL	8	43	17	29	14	14	24
TLC	8	25	10	27	12	14	19
Fatty acids							
16:0	16	39	14	25	23	3	33
18:0	13	41	25	27	22	2	33
18:1n-9	19	25	13	27	25	5	32
18:1n-7	23	44	13	28	26	5	40
20:4n-6	9	44	12	30	19	4	34
20:5n-3	14	44	13	32	28	4	42
22:5n-3	20	44	10	28	26	4	45
22:6n-3	10	41	11	32	17	4	34
n-3	10	42	11	31	22	4	35
n-6	11	42	11	30	22	4	34
SFA	16	40	19	26	25	4	35
MUFA	22	42	16	28	32	5	34
PUFA	11	42	11	31	22	4	35
Total FA	14	42	12	29	25	4	34

B-ASE_dry

	L. sebae	*M. edulis*	*O. vulgaris*	*P. versicolor*	*S. pilchardus*	*S. aurata*	*T. thynnus*
Lipid class							
TAG	_	38	_	_	31	12	47
ST	18	25	14	15	7	27	35
PL	13	26	26	21	20	20	24
TLC	11	25	17	19	23	12	41
Fatty acids							
16:0	8	1	5	4	10	10	36
18:0	3	7	6	4	10	10	31
18:1n-9	8	9	5	4	14	12	41
18:1n-7	11	3	4	5	16	13	39
20:4n-6	2	3	3	4	10	12	24
20:5n-3	7	2	3	5	16	10	38
22:5n-3	11	6	2	5	16	12	44
22:6n-3	3	7	3	4	8	8	33
n-3	3	5	3	4	11	9	35
n-6	3	4	3	4	10	11	28
SFA	7	2	5	4	11	11	37
MUFA	11	6	9	4	16	12	42
PUFA	3	4	3	4	11	10	34
Total FA	6	2	3	4	12	11	37

C-Dounce_wet

	L. sebae	*M. edulis*	*O. vulgaris*	*P. versicolor*	*S. pilchardus*	*S. aurata*	*T. thynnus*
Lipid class							
TAG	_	23	_	_	51	21	40
ST	4	33	23	25	11	14	37
PL	5	32	17	24	8	21	40
TLC	4	19	12	23	33	20	32
Fatty acids							
16:0	44	7	5	10	39	5	59
18:0	29	8	4	13	36	6	52
18:1n-9	46	14	12	4	53	4	67
18:1n-7	57	10	6	1	42	5	61
20:4n-6	17	10	9	8	34	5	37
20:5n-3	40	9	8	8	41	6	58
22:5n-3	51	9	10	8	34	4	65
22:6n-3	21	8	10	9	27	3	55
n-3	24	8	10	8	34	4	57
n-6	22	8	9	7	43	4	47
SFA	41	6	4	12	41	5	60
MUFA	54	10	6	3	48	4	64
PUFA	23	8	10	8	35	4	56
Total FA	35	7	8	4	41	4	60

D-Dounce_dry

	L. sebae	*M. edulis*	*O. vulgaris*	*P. versicolor*	*S. pilchardus*	*S. aurata*	*T. thynnus*
Lipid class							
TAG	129	17	_	_	21	30	40
ST	21	14	54	6	5	19	37
PL	8	15	10	9	13	21	50
TLC	16	8	3	9	11	27	29
Fatty acids							
16:0	18	7	6	3	9	11	41
18:0	18	4	6	2	13	6	30
18:1n-9	18	12	5	3	21	14	41
18:1n-7	20	8	6	3	22	12	42
20:4n-6	25	1	6	3	11	6	20
20:5n-3	23	7	7	3	21	12	45
22:5n-3	26	7	5	3	19	13	44
22:6n-3	29	7	5	3	7	8	37
n-3	26	7	6	4	11	11	41
n-6	19	6	6	3	8	14	31
SFA	19	6	6	4	11	12	41
MUFA	20	8	4	3	16	15	42
PUFA	24	7	6	3	11	12	40
Total FA	16	6	6	2	12	13	41

E-Ltw_wet

	L. sebae	*M. edulis*	*O. vulgaris*	*P. versicolor*	*S. pilchardus*	*S. aurata*	*T. thynnus*
Lipid class							
TAG	_	25	_	_	36	22	24
ST	10	21	23	8	18	11	15
PL	9	18	19	10	26	23	12
TLC	7	15	13	6	13	18	19
Fatty acids							
16:0	38	20	14	6	11	19	33
18:0	36	13	12	4	5	16	30
18:1n-9	38	20	10	6	7	21	29
18:1n-7	42	12	12	7	6	20	37
20:4n-6	35	14	11	6	7	11	27
20:5n-3	38	15	11	3	7	16	35
22:5n-3	40	17	10	6	9	18	38
22:6n-3	36	20	10	7	5	13	32
n-3	36	18	10	5	6	15	33
n-6	35	19	11	6	9	19	30
SFA	37	18	13	4	12	19	33
MUFA	39	12	8	6	20	23	30
PUFA	36	18	10	5	6	16	33
Total FA	37	17	11	5	11	20	32

F-Ltw_dry

	L. sebae	*M. edulis*	*O. vulgaris*	*P. versicolor*	*S. pilchardus*	*S. aurata*	*T. thynnus*
Lipid class							
TAG	117	52	_	_	19	26	51
ST	17	20	14	12	16	15	24
PL	21	18	22	12	20	12	23
TLC	31	27	18	10	16	24	45
Fatty acids							
16:0	47	26	2	4	26	7	32
18:0	51	25	3	2	20	7	27
18:1n-9	56	34	7	3	25	9	39
18:1n-7	68	25	4	5	18	8	35
20:4n-6	25	29	3	2	20	6	19
20:5n-3	50	24	4	2	20	9	35
22:5n-3	58	27	2	4	18	8	39
22:6n-3	31	28	3	3	26	4	30
n-3	33	26	4	2	24	7	32
n-6	33	29	3	3	26	9	24
SFA	51	26	2	3	25	8	32
MUFA	63	28	6	4	25	8	37
PUFA	33	26	3	2	24	7	31
Total FA	46	27	3	3	25	8	33

Legend	1-10	11-15	16-20	>20

Table 3. Probabilities from MANOVA (F-test) or Scheirer-Ray-Hare test (H-test; grey lines) testing the effects storage duration after freeze-drying (t_0, $t_{+1month}$, $t_{+3months}$) and storage mode (freezer, nitrogen, dry room) and their interaction on lipid class concentrations (TAG: triacylglycerols, DAG: diacylglycerols, FFA: free fatty acids, ST: sterols, AMPL: acetone mobile polar lipids, PL: phospholipids) and total lipid content (TLC) determined in seven marine species. Codes: ns not significant, * p<0.05, ** p<0.01, *** p<0.001.

	Lutjanus sebae			*Mytilus edulis*			*Octopus vulgaris*			*Panulirus versicolor*			*Sardina pilchardus*			*Sparus aurata* (cultured)			*Thunnus thynnus*		
	Storage mode	Duration	Storage mode × Duration	Storage mode	Duration	Storage mode × Duration	Storage mode	Duration	Storage mode × Duration	Storage mode	Duration	Storage mode × Duration	Storage mode	Duration	Storage mode × Duration	Storage mode	Duration	Storage mode × Duration	Storage mode	Duration	Storage mode × Duration
Lipid class concentration																					
TAG	**	**	*	***	***	***	ns	ns	ns	_	_	_	***	**	ns	ns	**	ns	***	*	ns
DAG	ns	ns	ns	***	ns	ns	ns	*	ns	***	**	ns	***	*	ns	***	ns	**	***	ns	ns
FFA	*	ns	*	***	ns	ns	*	*	*	***	ns	ns	***	***	ns	***	***	**	***	***	ns
ST	***	***	ns	***	***	ns	***	***	***	***	***	**	**	***	ns	ns	***	ns	**	ns	ns
AMPL	ns	*	ns	**	**	*	*	**	ns	*	**	ns	ns	**	ns	***	**	ns	**	***	ns
PL	**	***	***	**	***	ns	*	**	ns	***	***	***	ns	***	***	***	***	ns	ns	**	*
Total lipid	**	ns	***	***	***	ns	ns	***	***	***	***	***	***	***	ns	*	**	ns	***	**	ns

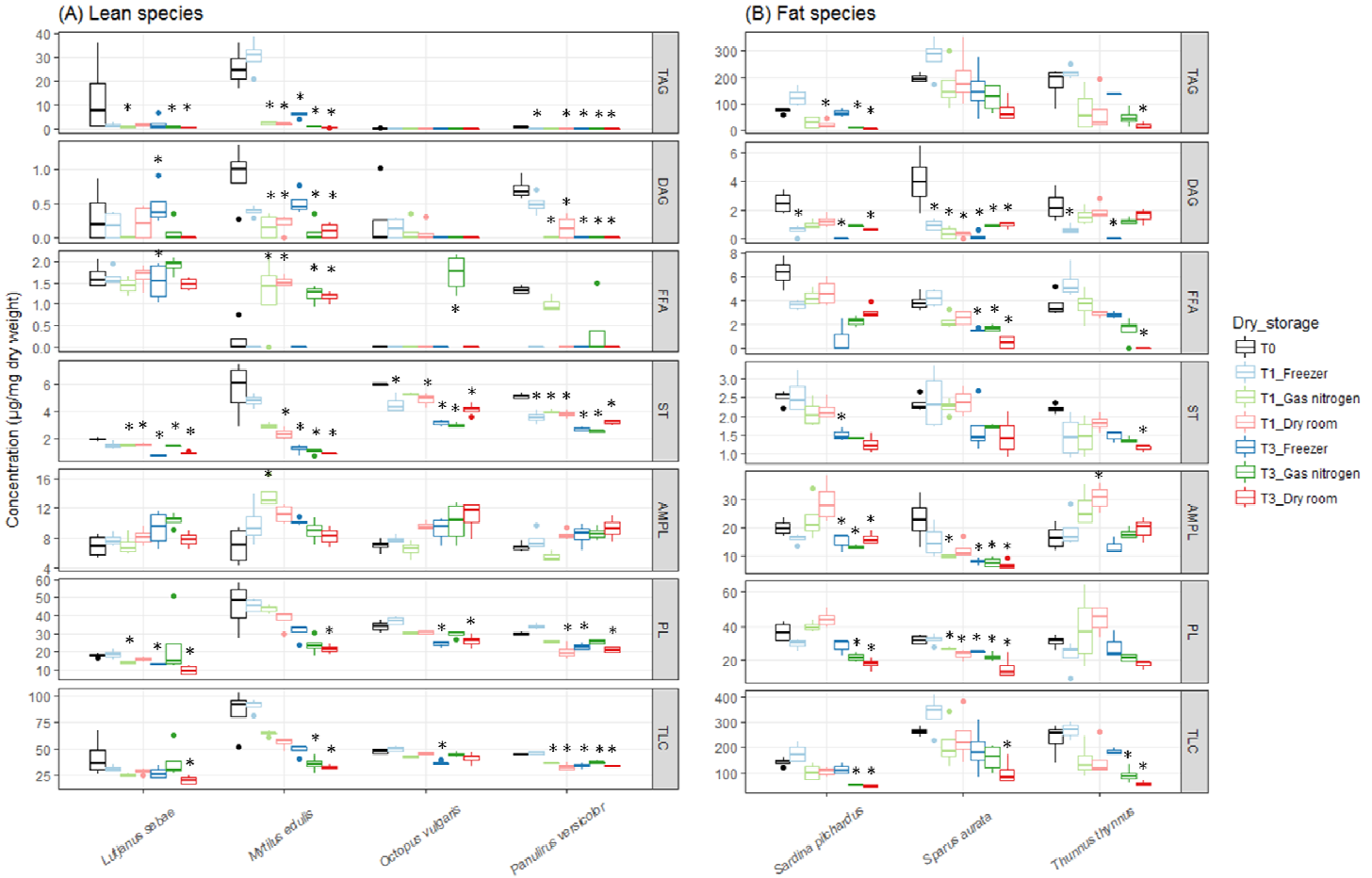


Fig 5. Distribution of lipid classes (triacylglycerols (TAG), diacylglycerols (DAG), free fatty acid (FFA), sterols (ST), acetone mobile polar lipids (AMPL) and phospholipids (PL)) and total lipid content (TLC) in dry tissues of (A) lean species and (B) fat species according to storage duration after freeze-drying ($t_{+1month}$, $t_{+3months}$) and storage mode (freezer, nitrogen, dry room). Reference (t_0) is in black. The thick bar represents the median value and the points are outliers. * denotes difference from t_0 at p<0.05.

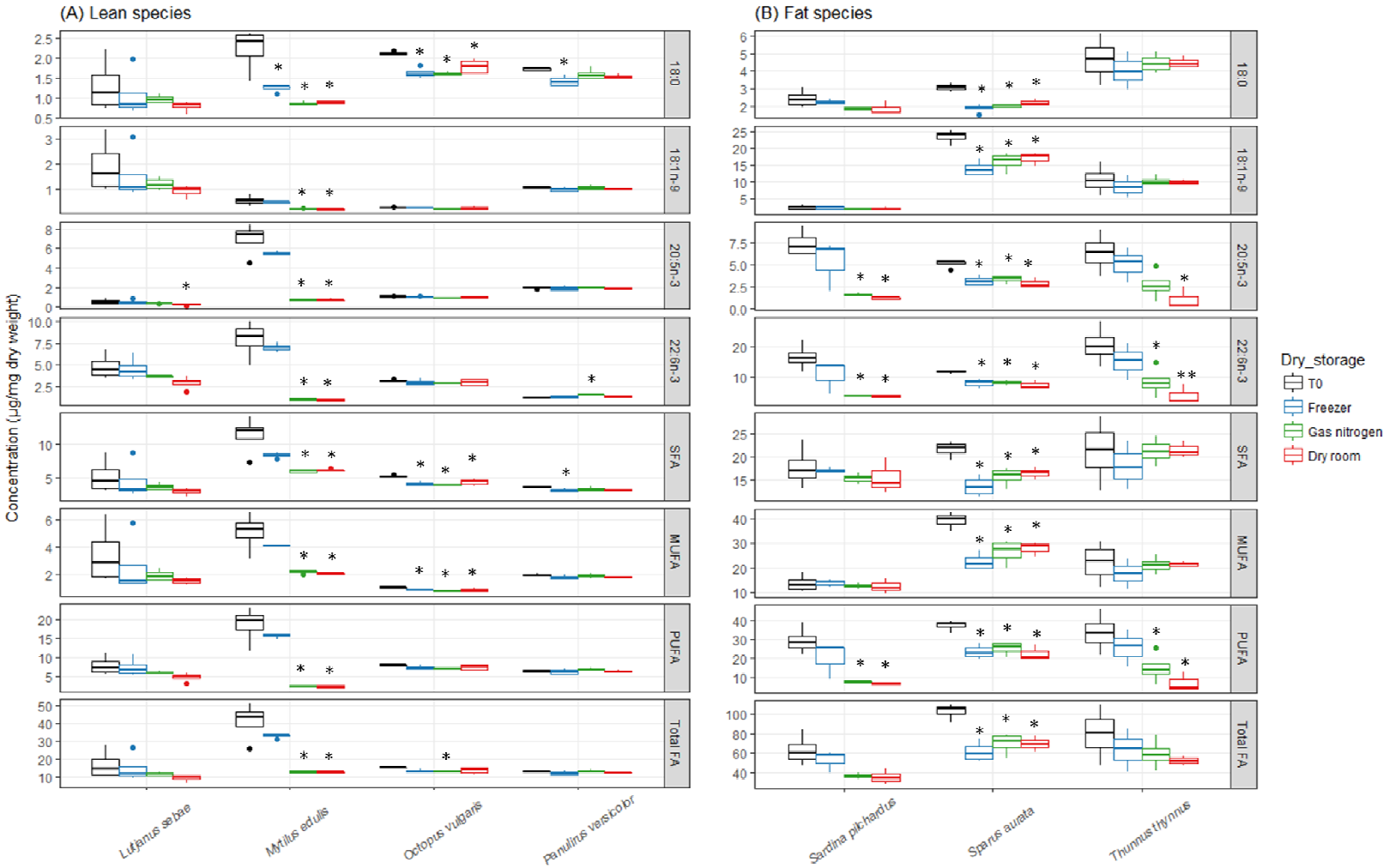


Fig. 6. Distribution of four specific fatty acids (Stearic acid 18:0; Oleic acid 18:1n-9; Eicosapentaenoic acid (EPA) 20:5n-3; Docosahexaenoic (DHA) 22:6n-3) and FA families (SFA=Saturated FA; MUFA=Monounsaturated FA; PUFA=Polyunsaturated FA) in dry tissues of (A) lean species and (B) fat species, after three-month storage in three modes (freezer, nitrogen, dry room). Reference (t_0) is in black. The thick bar represents the median value and the points are outliers. * denotes difference from t_0 at $p<0.05$ (see Table S2 for results on all FA).

4.2. How to store dry tissues?

No loss after one-month storage in -20°C-freezer was found for the different lipid classes. The other storage modes (dry room and nitrogen) were ineffective to prevent lipid class degradation. After a three-month storage, FA were more resistant to degradation than lipid classes in lean species such as *L. sebae* and *P. versicolor*, as little degradation was observed on their dry tissue in freezer. The freezer storage of dry tissue was however ineffective for the cultured *S. aurata*, probably because of its high lipid and FA contents sensitive to oxidation (> 200 $\mu g.mg^{-1}$ dw). Sensitivity to oxidation varied with FA in this species, e.g. 22:6n-3 losses were lower than for 18:1n-9 (the main FA with 24 $\mu g.mg^{-1}$ dw). As for lipid classes, storage modes other than freezer were not efficient to prevent FA oxidation.

Although they did not contain water, dry tissues were better preserved at low temperature (except for FA from *S. aurata*). Considering the two main pathways of lipid oxidation (i.e. enzyme initiated or reaction with oxygen [51]), this result suggested that lipase enzymes might remain active without water, or that lipid reaction with oxygen was slowed down at low temperature (through reduction of molecular excitation).

Lipid degradation also occurred before storage: Rudy et al. [22] found FA degradation for fat species holding on ice before frozen storage. Consequently, a careful attention should be given to tissue conservation from sampling to storage, especially for fat tissues. The best practices remained to collect samples on fresh individuals and to quickly cool them (ideally in liquid nitrogen) before a deep-frozen storage (-80°C) [22]. The removal of the outermost edge of sample before lipid extraction might also reduce the oxidation due to oxygen contact. When samples cannot be kept frozen, because of transport for example, they might be freeze-dried. If they are transported at ambient temperature, dry samples should be immersed into solvent directly on arrival: the total duration between the freeze-drying end and the solvent immersion should not exceed four days at +20°C [24]. If dry tissues can be transported at -20°C (e.g. few hours with ice packs inside an insulated box before being stored again in freezer), the total duration between the freeze-drying and the immersion should not exceed one month. FA profile express in percentage (qualitative) did not differ before/after the freezer storage of dry tissue, except for cultured *S. aurata* (Fig. 4b), suggesting a short-term storage (< three months) of dry tissue is suitable for trophic ecology or qualitative studies of FA in species with lipid content below 20% dw. FA profiles can be compared between wet frozen tissues and three-month dry frozen tissues.

4.3. Checking for lipid oxidation

DAG and FFA are commonly used as a degradation marker for lipid classes [52,14]. Here, except for FFA of *M. edulis*, the DAG and FFA contents did not increase during storage when TAG or PL contents decreased. Meyer et al. [24] obtained similar result on shark lean tissues. This suggests that TAG and PL oxidation did not only lead to DAG or FFA formation but to other compounds undetected here (e.g. malondialdehyde), and DAG and FFA oxidation markers might not be relevant for all marine species.

FA quantities tended to decrease after three-month storage in poor conditions (with gas nitrogen and in dry room) including the SFA which are little subjected to oxidation in comparison to PUFA [53]. SFA loss was also observed in poor condition storage of frozen fishes [22,24]. However, for species with total FA content > 20 $\mu g.mg^{-1}$ (fat species and *M. edulis* in our study), PUFA were oxidized faster than SFA in poor storage conditions of dry tissues. Consequently, the FA ratio 22:6n-3/16:0 could be used to assess the extent of FA degradation from a reference point as suggested by Young et al. [17] on swordfish preys. For instance, in our study, 22:6n-3/16:0 ratios lower than 1.0±0.1, 1.4±0.1, 0.8±0.1 and 1.5±0.1 indicated lipid oxidation for *M. edulis*, *S. pilchardus*, cultured *S. aurata* and *T. thynnus*, respectively.

Conclusions

Lipid compositions were not affected by freeze-drying but the extraction method did influence the results for lipid classes. The highest quantity of lipids extracted was obtained from the homogenates of wet tissues left into solvent mixture for 24 hours. Extractions with manual potter homogenizer led to a lower but reproducible lipid content for most species (ca. 90% reproducibility) while extractions with ASE would require more protocol adjustments. Increasing the number of replicates might help to improve the repeatability of each protocol. Differences among the six protocols were however predictable, allowing to correct concentration results for comparisons among studies using different extraction protocols. One-month storage in freezer might be acceptable for dry tissue (no significant decrease in lipid quantities) whereas storage into a dry room or with gas nitrogen did not prevent lipid degradation. For qualitative studies of FA (in %), a three-month storage in the freezer did not alter the FA profile for species with total lipid <20% dw. The fast oxidation of some FA however requires caution for longer storage durations.

Acknowledgements

We are grateful to Margaux Mathieu-Resuge (LEMAR, Brest, France) for help in fatty acid determination and to Pierre-Yves Carval (Ecloserie du Tinduff, Plougastel, France) for assistance in mussel sampling. We thank our colleagues from Ifremer/REM/RDT and RBE, CNRS, UM and IRD who provided insight and expertise that greatly assisted the research. We warmly thank two anonymous reviewers for their suggestions that greatly improved the manuscript. This research was part of the MERLIN-POPSTAR project funded by Ifremer, France.

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SUPPLEMENTARY MATERIAL

Effects of extraction method and storage of dry tissue on marine lipids and fatty acids

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Key words: Degradation, Freeze-drying, Lipid class, Marine animal, Protocol

Fig S1. Principal component analyses (PCA) of lipid class percentage (root square transformed) for seven marine species (A) according to six lipid extraction protocols (A to F) and (B) three storage modes of dry tissues for one and three months.

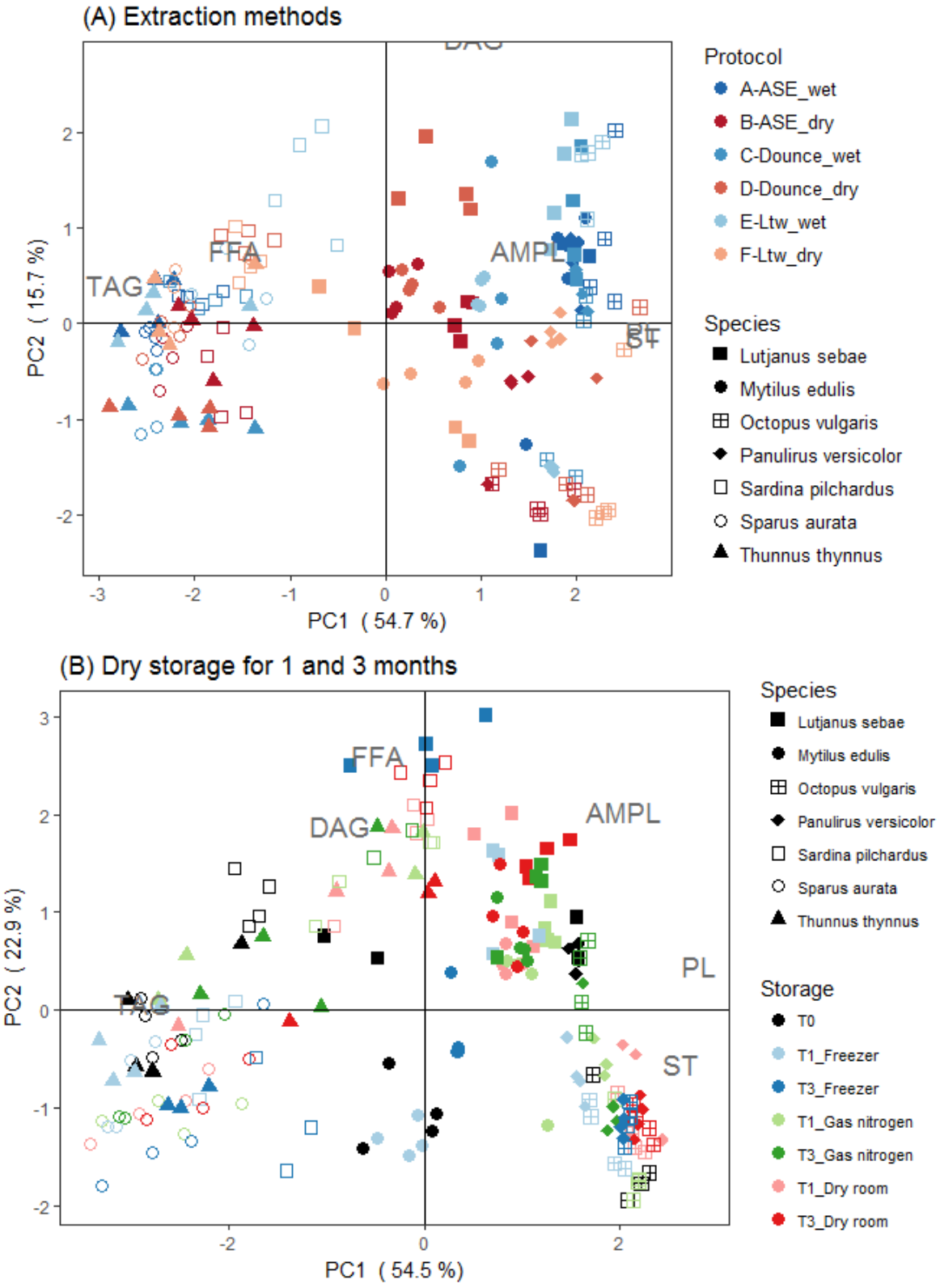

Fig. S2. Scatterplot matrixes between extraction protocols (A to F) of (a) 41 lipid classes and (b) 192 fatty acids from the seven studied species, expressed in µg.mg^{-1} dw. Upper panels: Each point is the mean of four replicates. Dashed line is the linear regression model obtained between two protocols. Green line is the 1:1 line, i.e. no difference between the protocols. Lower panels: the linear regression equation associated to the dashed line from the upper panel, and the adjusted coefficient of determination (r^2).

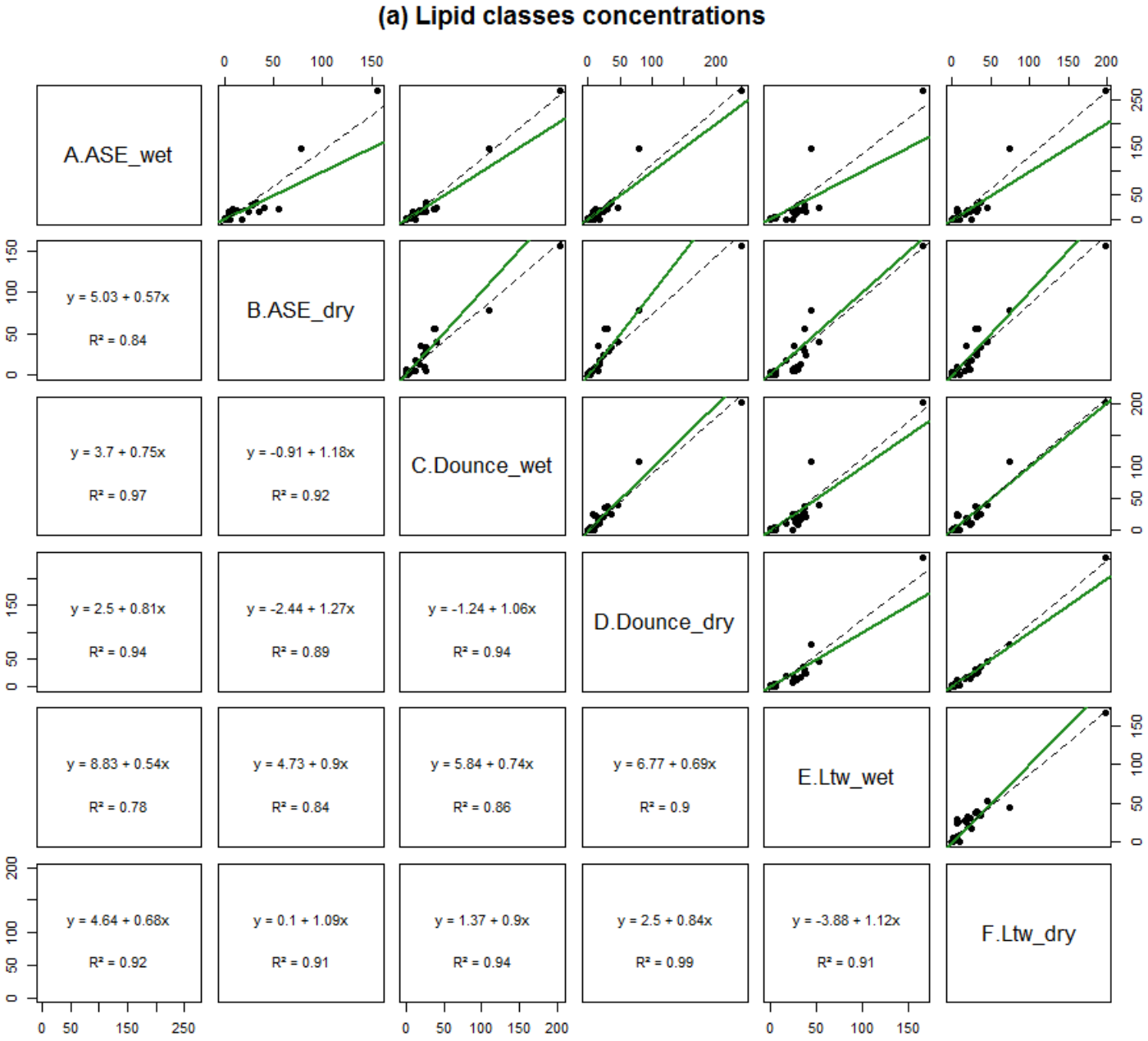

Fig S2b. continued

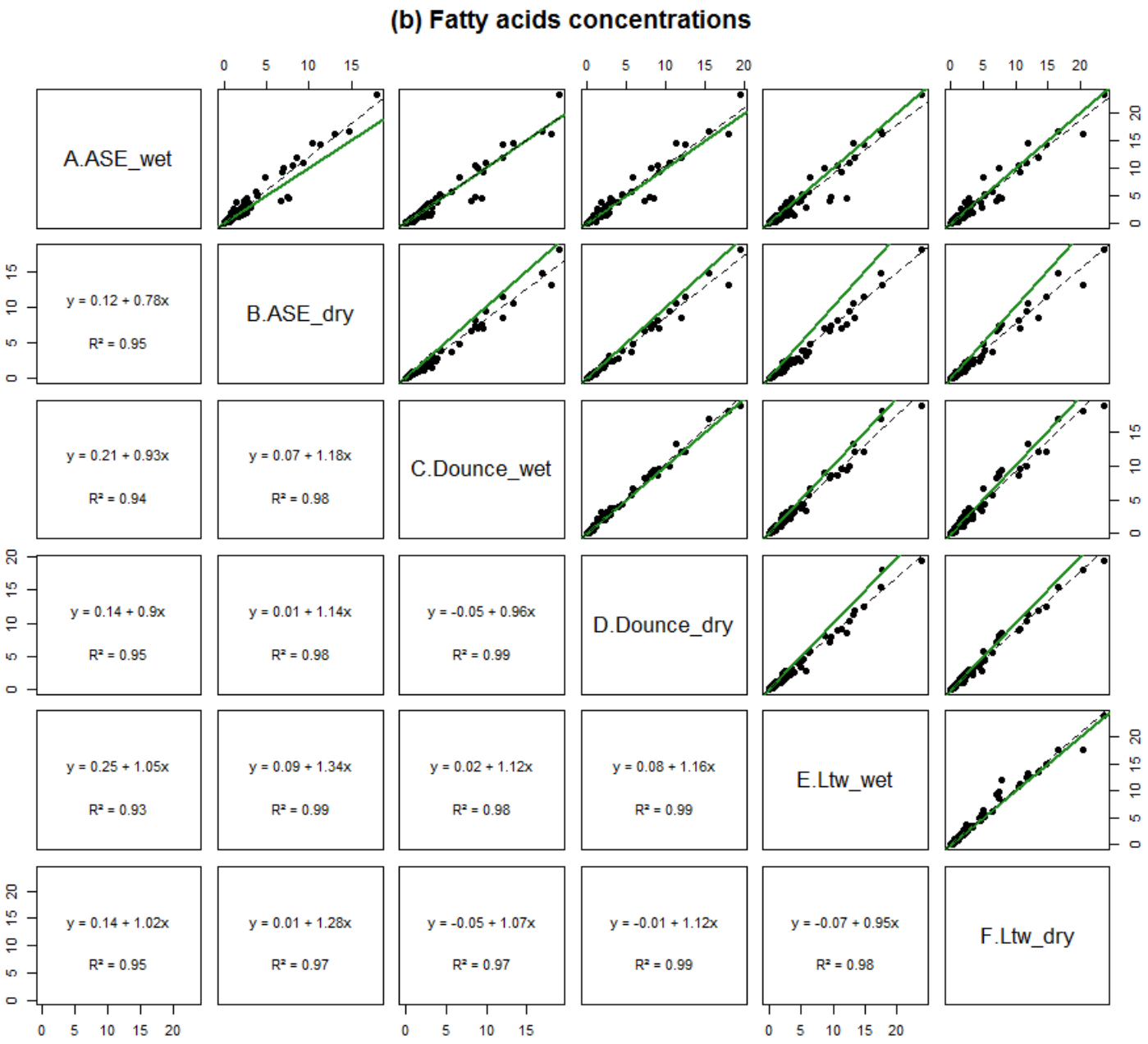

Table S1. Lipid classes (TAG: triacylglycerols, DAG: diacylglycerols, FFA: free fatty acids, ST: sterols, AMPL: acetone mobile polar lipids, PL: phospholipids), total lipid content (TLC), and fatty acids (FA) composition (in µg.mg^{-1} dry weight; mean ± 1 SD of four replicates) of seven marine species according to six lipid extraction methods (A to F; see Material and methods for details). Only FA >0.8% of total FA are given. 'Wet' referred to frozen wet tissue and 'dry' to freeze-dried tissue. SFA=Saturated FA; MUFA=Monounsaturated FA; PUFA=Polyunsaturated FA.

(a) *Lutjanus sebae*

	A-ASE_wet	B-ASE_dry	C-Dounce_wet	D-Dounce_dry	E-Ltw_wet	F-Ltw_dry
(a) Lutjanus sebae						
TAG	0.4 ± 0.7	1.5 ± 0.7	1.1 ± 2.1	3.1 ± 2.8	2.4 ± 0.7	12.9 ± 16.2
FFA	0.7 ± 1.4	1.4 ± 0.1	0.0 ± 0.0	2.0 ± 0.2	4.4 ± 0.3	1.6 ± 0.3
ST	1.7 ± 0.2	1.4 ± 0.2	2.0 ± 0.4	1.5 ± 0.4	2.2 ± 0.1	1.9 ± 0.1
DAG	1.1 ± 0.5	0.7 ± 0.2	2.0 ± 0.8	1.4 ± 0.5	3.1 ± 1.3	0.3 ± 0.4
AMPL	17.4 ± 1.3	5.7 ± 0.5	25.3 ± 4.4	9.5 ± 0.5	24.8 ± 2.9	6.9 ± 1.6
PL	15.6 ± 2.4	35.9 ± 1.7	19.0 ± 2.5	15.5 ± 1.3	25.0 ± 1.3	17.7 ± 1.0
TLC	36.9 ± 4.3	46.6 ± 1.1	49.3 ± 9.1	33.0 ± 5.1	61.9 ± 4.3	41.5 ± 18. 1
14:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.3
16:0	1.8 ± 0.3	2.1 ± 0.2	2.2 ± 1.0	2.1 ± 0.4	3.2 ± 1.2	3.3 ± 1.6
17:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.1
18:0	0.8 ± 0.1	0.9 ± 0.0	1.0 ± 0.3	1.0 ± 0.2	1.4 ± 0.5	1.3 ± 0.7
16:1n-7	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.3	0.3 ± 0.1	0.4 ± 0.2	0.8 ± 0.7
18:1n-9	0.9 ± 0.2	1.0 ± 0.1	1.1 ± 0.5	1.0 ± 0.2	1.7 ± 0.6	1.9 ± 1.1
18:1n-7	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.3
24:1n-9	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.1
16:4n-3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.6 ± 0.2	0.3 ± 0.0
18:2n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1
20:4n-6	0.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	1.1 ± 0.4	0.7 ± 0.2
20:5n-3	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	0.5 ± 0.3
22:4n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.1
22:5n-6	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	0.5 ± 0.1
22:5n-3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.2
22:6n-3	2.7 ± 0.3	3.2 ± 0.1	3.3 ± 0.7	2.8 ± 0.8	5.7 ± 2.0	4.7 ± 1.4
n-3	3.4 ± 0.4	4.0 ± 0.1	4.1 ± 1.0	3.5 ± 0.9	7.3 ± 2.6	6.1 ± 2.0
n-6	1.0 ± 0.1	1.2 ± 0.0	1.3 ± 0.3	1.0 ± 0.2	2.1 ± 0.7	1.6 ± 0.5
SFA	2.8 ± 0.5	3.3 ± 0.2	3.5 ± 1.4	3.4 ± 0.6	5.0 ± 1.9	5.2 ± 2.7
MUFA	1.4 ± 0.3	1.7 ± 0.2	1.9 ± 1.0	1.7 ± 0.4	2.8 ± 1.1	3.4 ± 2.2
PUFA	4.4 ± 0.5	5.2 ± 0.2	5.4 ± 1.3	4.5 ± 1.1	9.4 ± 3.3	7.7 ± 2.5
Total FA	8.9 ± 1.3	10.5 ± 0.6	11.0 ± 3.9	9.9 ± 1.6	17.5 ± 6.4	16.8 ± 7.8

(b) *Mytilus edulis*

	A-ASE_wet	B-ASE_dry	C-Dounce_wet	D-Dounce_dry	E-Ltw_wet	F-Ltw_dry
(b) Mytilus edulis						
TAG	5.2 ± 1.7	17.6 ± 3.4	11.5 ± 2.9	18.2 ± 4.2	16.9 ± 3.3	25.4 ± 8.1
FFA	0.0 ± 0.0	2.7 ± 0.5	0.0 ± 0.0	2.4 ± 0.5	0.0 ± 0.0	0.2 ± 0.4
ST	2.5 ± 0.8	4.3 ± 0.3	4.0 ± 0.5	4.9 ± 0.4	4.6 ± 0.7	5.6 ± 2.1
DAG	1.4 ± 1.1	1.5 ± 0.3	2.4 ± 2.5	1.8 ± 0.3	2.4 ± 0.4	0.9 ± 0.5
AMPL	20.5 ± 5.7	9.1 ± 1.7	23.5 ± 7.4	11.2 ± 1.1	28.5 ± 4.4	7.0 ± 2.6
PL	23.4 ± 7.9	40.5 ± 3.3	40.4 ± 3.8	46.0 ± 3.8	53.4 ± 8.0	45.4 ± 13.7
TLC	53.0 ± 13.4	75.8 ± 3.5	81.8 ± 11.1	84.5 ± 6.1	106.4 ± 14.4	84.6 ± 22.5
14:0	0.5 ± 0.2	0.8 ± 0.0	0.8 ± 0.1	0.9 ± 0.1	1.1 ± 0.2	0.8 ± 0.2
15:0	0.2 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	0.3 ± 0.1
16:0	4.6 ± 1.8	7.5 ± 0.1	8.7 ± 0.6	7.9 ± 0.6	9.7 ± 1.9	7.5 ± 2.0
17:0	0.6 ± 0.3	0.9 ± 0.0	1.1 ± 0.1	1.0 ± 0.0	1.0 ± 0.2	0.9 ± 0.2
18:0	1.6 ± 0.6	2.2 ± 0.1	2.7 ± 0.2	2.4 ± 0.1	2.1 ± 0.3	2.2 ± 0.6
16:1n-7	1.1 ± 0.5	1.8 ± 0.2	2.0 ± 0.3	1.8 ± 0.2	2.1 ± 0.2	1.8 ± 0.5
18:1n-9	0.4 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.9 ± 0.2	0.6 ± 0.2
18:1n-7	0.6 ± 0.3	1.0 ± 0.0	1.2 ± 0.1	1.0 ± 0.1	1.2 ± 0.2	1.0 ± 0.2
20:1n-9	0.4 ± 0.2	0.6 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.9 ± 0.1	0.7 ± 0.2
20:1n-7	0.6 ± 0.3	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.0	1.4 ± 0.2	1.0 ± 0.3
16:4n-3	0.2 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.1
18:2n-6	0.4 ± 0.2	0.7 ± 0.0	0.8 ± 0.1	0.7 ± 0.1	1.0 ± 0.2	0.7 ± 0.2
18:3n-3	0.3 ± 0.1	0.5 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.8 ± 0.2	0.5 ± 0.2
20:2i	0.6 ± 0.2	0.9 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.4 ± 0.3	1.0 ± 0.3
20:2j	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.1
20:4n-6	0.6 ± 0.3	0.9 ± 0.0	1.3 ± 0.1	1.0 ± 0.0	1.3 ± 0.2	1.0 ± 0.3
20:5n-3	4.1 ± 1.8	6.7 ± 0.1	8.2 ± 0.7	7.2 ± 0.5	9.4 ± 1.4	7.0 ± 1.7
22:3nmi	0.2 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.1
22:5n-3	0.3 ± 0.1	0.5 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	0.7 ± 0.1	0.5 ± 0.1
22:6n-3	4.4 ± 1.8	7.6 ± 0.6	9.4 ± 0.7	8.5 ± 0.6	12.1 ± 2.4	7.9 ± 2.2
18:0DMA	1.4 ± 0.6	2.1 ± 0.1	3.1 ± 0.4	2.5 ± 0.1	3.8 ± 0.6	2.5 ± 0.7
n-3	9.4 ± 4.0	15.7 ± 0.7	19.4 ± 1.5	17.2 ± 1.2	23.7 ± 4.3	16.3 ± 4.2
n-6	1.3 ± 0.6	2.2 ± 0.1	2.8 ± 0.2	2.4 ± 0.1	3.3 ± 0.6	2.3 ± 0.7
SFA	7.3 ± 2.9	11.3 ± 0.2	13.4 ± 0.8	12.1 ± 0.7	14.0 ± 2.5	11.5 ± 2.9
MUFA	3.1 ± 1.3	5.0 ± 0.3	6.0 ± 0.6	5.3 ± 0.4	6.6 ± 0.8	5.1 ± 1.4
PUFA	10.8 ± 4.5	17.9 ± 0.8	22.1 ± 1.8	19.6 ± 1.4	27.0 ± 5.0	18.6 ± 4. 9
Total FA	24.5 ± 10.2	39.5 ± 0.8	48.6 ± 3.6	42.9 ± 2.6	56.0 ± 9.7	40 .8 ± 10.8

(c) *Octopus vulgaris*

	A-ASE_wet	B-ASE_dry	C-Dounce_wet	D-Dounce_dry	E-Ltw_wet	F-Ltw_dry
(c) Octopus vulgaris						
TAG	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
FFA	0.0 ± 0.0	0.3 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
ST	3.5 ± 0.8	4.5 ± 0.2	4.7 ± 1.0	3.2 ± 1.6	4.3 ± 0.3	6.0 ± 0.1
DAG	1.9 ± 1.1	0.0 ± 0.0	1.0 ± 0.8	0.3 ± 0.5	5.2 ± 1.4	0.3 ± 0.5
AMPL	14.6 ± 0.8	7.3 ± 3.5	23.6 ± 3.1	9.8 ± 1.2	26.2 ± 5.7	7.0 ± 0.9
PL	21.8 ± 4.3	55.1 ± 14.9	35.8 ± 5.1	26.6 ± 6.7	37.3 ± 2.2	33.8 ± 3.0
TLC	41.9 ± 5.3	67.2 ± 12.1	65.1 ± 6.8	39.8 ± 6.6	73.0 ± 8.4	47.2 ± 2. 7
16:0	1.7 ± 0.2	2.3 ± 0.1	2.8 ± 0.1	2.4 ± 0.2	2.6 ± 0.4	2.7 ± 0.1
17:0	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
18:0	1.6 ± 0.4	2.0 ± 0.1	2.5 ± 0.1	2.1 ± 0.1	2.2 ± 0.3	2.1 ± 0.1
16:1n-7	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
18:1n-9	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
18:1n-7	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
20:1n-7	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:2n-6	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:4n-6	1.7 ± 0.2	2.5 ± 0.1	3.1 ± 0.3	2.5 ± 0.1	3.1 ± 0.3	2.7 ± 0.1
20:5n-3	0.7 ± 0.1	1.0 ± 0.0	1.3 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.0
22:4n-6	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	0.3 ± 0.0
22:5n-6	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
22:5n-3	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
22:6n-3	1.8 ± 0.2	2.7 ± 0.1	3.3 ± 0.3	2.8 ± 0.1	3.4 ± 0.3	3.1 ± 0.1
18:0DMA	0.4 ± 0.2	0.8 ± 0.1	1.0 ± 0.2	0.8 ± 0.1	1.1 ± 0.2	0.8 ± 0.1
n-3	2.6 ± 0.3	3.9 ± 0.1	4.9 ± 0.5	4.1 ± 0.2	5.0 ± 0.5	4.5 ± 0.2
n-6	2.1 ± 0.2	3.1 ± 0.1	3.9 ± 0.4	3.3 ± 0.2	4.0 ± 0.4	3.5 ± 0.1
SFA	3.6 ± 0.7	4.7 ± 0.2	5.7 ± 0.2	4.9 ± 0.3	5.1 ± 0.7	5.1 ± 0.1
MUFA	0.7 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	0.9 ± 0.0	1.0 ± 0.1	1.0 ± 0.1
PUFA	4.8 ± 0.5	7.0 ± 0.2	8.8 ± 0.8	7.3 ± 0.4	9.0 ± 0.9	8.0 ± 0.3
Total FA	9.7 ± 1.1	13.8 ± 0.5	17.0 ± 1.3	14.3 ± 0.8	16.7 ± 1.9	15.4 ± 0.4

(d) *Panulirus versicolor*

	A-ASE_wet	B-ASE_dry	C-Dounce_wet	D-Dounce_dry	E-Ltw_wet	F-Ltw_dry
(d) Panulirus versicolor						
TAG	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
FFA	0.0 ± 0.0	0.9 ± 0.1	0.0 ± 0.0	0.3 ± 0.6	4.2 ± 0.1	1.3 ± 0.1
ST	2.4 ± 0.6	4.2 ± 0.6	4.5 ± 0.3	3.8 ± 0.1	4.2 ± 0.2	5.1 ± 0.2
DAG	1.3 ± 0.5	0.5 ± 0.3	1.9 ± 0.5	0.3 ± 0.3	0.0 ± 0.0	0.7 ± 0.2
AMPL	15.0 ± 1.7	4.5 ± 0.1	26.1 ± 4.4	7.3 ± 0.1	24.3 ± 1.1	6.8 ± 0.6
PL	22.2 ± 7.0	55.9 ± 11.3	38.6 ± 3.6	30.5 ± 2.0	37.0 ± 2.2	29.8 ± 1.0
TLC	40.8 ± 8.9	66.2 ± 11.8	71.1 ± 5.5	42.3 ± 2.8	70.3 ± 2.5	44.4 ± 0. 7
16:0	1.0 ± 0.3	1.2 ± 0.1	1.8 ± 0.2	1.5 ± 0.0	1.8 ± 0.1	1.4 ± 0.1
17:0	0.2 ± 0.1	0.2 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.0
18:0	1.1 ± 0.3	1.5 ± 0.1	2.3 ± 0.3	1.8 ± 0.0	2.1 ± 0.1	1.7 ± 0.0
20:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
16:1n-7	0.2 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0
18:1n-9	0.7 ± 0.2	0.9 ± 0.0	1.3 ± 0.0	1.1 ± 0.0	1.4 ± 0.1	1.1 ± 0.0
18:1n-7	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
16:2n-4	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
18:2n-6	0.2 ± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
20:2n-6	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
20:4n-6	1.4 ± 0.4	1.8 ± 0.1	2.7 ± 0.2	2.3 ± 0.1	3.0 ± 0.2	2.1 ± 0.1
20:5n-3	1.2 ± 0.4	1.5 ± 0.1	2.4 ± 0.2	2.0 ± 0.1	2.7 ± 0.1	1.9 ± 0.0
22:4n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
22:5n-6	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
22:5n-3	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
22:6n-3	0.7 ± 0.2	0.9 ± 0.0	1.4 ± 0.1	1.2 ± 0.0	1.6 ± 0.1	1.1 ± 0.0
18:0DMA	0.4 ± 0.1	0.5 ± 0.0	0.6 ± 0.3	0.5 ± 0.2	0.9 ± 0.0	0.6 ± 0.0
n-3	2.2 ± 0.7	2.7 ± 0.1	4.2 ± 0.3	3.5 ± 0.1	4.8 ± 0.2	3.4 ± 0.1
n-6	1.9 ± 0.6	2.4 ± 0.1	3.5 ± 0.2	3.0 ± 0.1	3.9 ± 0.2	2.8 ± 0.1
SFA	2.4 ± 0.6	3.0 ± 0.1	4.6 ± 0.6	3.7 ± 0.2	4.3 ± 0.2	3.5 ± 0.1
MUFA	1.3 ± 0.4	1.6 ± 0.1	2.3 ± 0.1	2.0 ± 0.1	2.6 ± 0.2	2.0 ± 0.1
PUFA	4.1 ± 1.2	5.1 ± 0.2	7.7 ± 0.6	6.5 ± 0.2	8.7 ± 0.4	6.2 ± 0.1
Total FA	8.5 ± 2.4	10.6 ± 0.4	15.9 ± 0.6	13.4 ± 0.3	17.2 ± 0.8	12.9 ± 0.3

(e) *Sardina pilchardus*

	A-ASE_wet	B-ASE_dry	C-Dounce_wet	D-Dounce_dry	E-Ltw_wet	F-Ltw_dry
(e) Sardina pilchardus						
TAG	147.8 ± 37.4	78.2 ± 21.1	109.6 ± 64.3	78.2 ± 8.8	43.7 ± 16.0	74.6 ± 11.3
FFA	4.0 ± 0.3	3.8 ± 0.7	5.0 ± 1.4	5.1 ± 0.5	6.5 ± 0.3	6.3 ± 1.2
ST	2.2 ± 0.5	2.3 ± 0.3	2.3 ± 0.3	2.3 ± 0.2	2.7 ± 0.2	2.5 ± 0.2
DAG	2.4 ± 0.6	0.6 ± 0.4	1.8 ± 1.4	3.4 ± 0.3	4.8 ± 1.9	2.5 ± 0.8
AMPL	19.5 ± 4.1	12.3 ± 3.7	17.9 ± 4.0	16.5 ± 1.6	32.7 ± 2.4	20.0 ± 2.8
PL	34.2 ± 6.4	33.2 ± 1.3	26.5 ± 2.9	36.9 ± 7.0	35.0 ± 5.5	36.4 ± 5.9
TLC	210.4 ± 41.4	130.3 ± 18.2	163.0 ± 65.1	142.5 ± 1.6	126.2 ± 14.0	142.9 ± 17.6
14:0	4.5 ± 1.5	2.6 ± 0.4	3.8 ± 2.0	2.9 ± 0.5	3.4 ± 0.7	2.8 ± 0.7
16:0	14.6 ± 3.4	10.5 ± 1.0	13.3 ± 5.1	11.3 ± 1.0	13.2 ± 1.5	11.8 ± 3.1
17:0	0.8 ± 0.2	0.5 ± 0.1	0.8 ± 0.4	0.6 ± 0.1	0.7 ± 0.2	0.6 ± 0.2
18:0	2.6 ± 0.6	2.1 ± 0.2	2.4 ± 0.8	2.3 ± 0.3	2.6 ± 0.1	2.4 ± 0.5
16:1n-7	8.2 ± 2.8	4.8 ± 1.0	6.7 ± 3.1	5.8 ± 1.5	6.3 ± 0.8	5.0 ± 1.0
18:1n-9	3.2 ± 0.8	2.4 ± 0.3	2.8 ± 1.5	2.7 ± 0.6	2.5 ± 0.2	2.5 ± 0.6
18:1n-7	2.3 ± 0.6	1.7 ± 0.3	2.0 ± 0.8	1.9 ± 0.4	2.0 ± 0.1	1.8 ± 0.3
20:1n-9	2.7 ± 1.0	1.1 ± 0.2	2.2 ± 1.2	1.3 ± 0.3	1.8 ± 0.8	1.5 ± 0.6
22:1n-11	3.8 ± 1.4	1.4 ± 0.3	3.2 ± 1.6	1.8 ± 0.6	2.6 ± 1.3	2.1 ± 0.9
24:1n-9	0.7 ± 0.1	0.5 ± 0.0	0.6 ± 0.2	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.2
16:4n-3	0.6 ± 0.2	0.4 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
18:2n-6	0.9 ± 0.3	0.6 ± 0.1	0.9 ± 0.5	0.7 ± 0.1	0.8 ± 0.2	0.7 ± 0.2
18:3n-3	0.7 ± 0.2	0.4 ± 0.1	0.6 ± 0.4	0.4 ± 0.0	0.5 ± 0.2	0.5 ± 0.2
18:4n-3	1.3 ± 0.4	0.8 ± 0.1	1.2 ± 0.8	0.8 ± 0.1	1.0 ± 0.3	0.9 ± 0.3
20:4n-6	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.0	0.7 ± 0.1
20:4n-3	0.6 ± 0.1	0.4 ± 0.1	0.6 ± 0.3	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
20:5n-3	10.1 ± 2.9	7.0 ± 1.1	8.9 ± 3.6	8.1 ± 1.7	8.7 ± 0.6	7.4 ± 1.5
22:5n-6	0.5 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.1
22:5n-3	1.2 ± 0.3	0.8 ± 0.1	1.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.2
22:6n-3	16.6 ± 2.8	14.8 ± 1.1	16.9 ± 4.6	15.4 ± 1.0	17.5 ± 0.9	16.6 ± 4.2
n-3	31.0 ± 6.8	24.6 ± 2.6	29.8 ± 10.3	26.7 ± 2.9	29.9 ± 1.8	27.2 ± 6.5
n-6	2.4 ± 0.5	1.8 ± 0.2	2.3 ± 1.0	2.1 ± 0.2	2.3 ± 0.2	2.1 ± 0.5
SFA	22.5 ± 5.6	15.8 ± 1.7	20.2 ± 8.4	17.2 ± 1.8	19.9 ± 2.4	17.7 ± 4.5
MUFA	21.1 ± 6.7	12.0 ± 2.0	17.7 ± 8.6	14.2 ± 2.2	16.1 ± 3.2	13.6 ± 3.4
PUFA	33.4 ± 7.3	26.4 ± 2.8	32.1 ± 11.3	28.7 ± 3.0	32.2 ± 2.0	29.3 ± 7.0
Total FA	80.3 ± 20.4	56.2 ± 6.8	73.0 ± 29.7	62.3 ± 7.2	70.8 ± 7.8	62.9 ± 15.5

(f) *Sparus aurata* (cultured)

	A-ASE_wet	B-ASE_dry	C-Dounce_wet	D-Dounce_dry	E-Ltw_wet	F-Ltw_dry
(f) Sparus aurata						
TAG	268.9 ± 27.6	155.7 ± 32.9	203.3 ± 20.4	239.4 ± 59.0	165.8 ± 21.4	197.8 ± 14.5
FFA	2.8 ± 0.2	1.6 ± 0.4	1.7 ± 0.3	2.9 ± 0.5	5.7 ± 1.2	3.9 ± 0.8
ST	2.8 ± 0.8	1.9 ± 0.5	2.4 ± 0.5	2.3 ± 0.3	3.4 ± 0.6	2.3 ± 0.2
DAG	2.0 ± 0.2	1.0 ± 0.5	0.4 ± 0.4	2.3 ± 1.1	3.2 ± 1.3	4.0 ± 2.0
AMPL	14.2 ± 0.6	6.4 ± 2.6	9.4 ± 2.1	14.2 ± 6.4	30.3 ± 5.4	22.7 ± 8.0
PL	29.1 ± 3.0	28.2 ± 2.5	27.0 ± 2.5	31.8 ± 6.7	37.2 ± 1.9	31.8 ± 2.9
TLC	319.9 ± 30.1	194.8 ± 34.1	244.3 ± 21.6	292.8 ± 61.2	246.2 ± 22.4	263.3 ± 16.3
14:0	3.4 ± 0.2	2.6 ± 0.3	2.8 ± 0.2	2.9 ± 0.6	3.3 ± 0.8	3.4 ± 0.4
16:0	14.3 ± 0.5	11.4 ± 1.2	12.0 ± 0.5	12.5 ± 1.4	14.9 ± 2.8	14.7 ± 1.1
18:0	2.8 ± 0.1	2.3 ± 0.2	2.4 ± 0.2	2.5 ± 0.2	3.2 ± 0.5	3.1 ± 0.2
16:1n-7	5.3 ± 0.3	4.0 ± 0.5	4.3 ± 0.3	4.4 ± 0.8	5.2 ± 1.1	5.2 ± 0.6
18:1n-9	23.4 ± 1.1	18.0 ± 2.2	18.9 ± 0.8	19.5 ± 2.7	24.0 ± 5.1	23.6 ± 2.1
18:1n-7	2.8 ± 0.1	2.1 ± 0.3	2.2 ± 0.1	2.3 ± 0.3	2.9 ± 0.6	2.7 ± 0.2
20:1n-9	3.4 ± 0.2	2.6 ± 0.3	2.7 ± 0.2	2.8 ± 0.5	3.5 ± 1.0	3.3 ± 0.3
22:1n-11	3.3 ± 0.2	2.5 ± 0.3	2.5 ± 0.2	2.7 ± 0.6	3.4 ± 1.2	3.1 ± 0.2
18:2n-6	10.4 ± 0.5	8.1 ± 0.9	8.7 ± 0.4	8.9 ± 1.3	10.8 ± 2.1	10.5 ± 1.0
18:3n-3	2.6 ± 0.1	2.0 ± 0.2	2.1 ± 0.1	2.2 ± 0.4	2.6 ± 0.5	2.6 ± 0.3
18:4n-3	1.3 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.3	1.3 ± 0.2	1.3 ± 0.2
20:4n-6	0.8 ± 0.0	0.6 ± 0.1	0.7 ± 0.0	0.7 ± 0.0	0.9 ± 0.1	0.8 ± 0.1
20:4n-3	1.0 ± 0.0	0.8 ± 0.1	0.8 ± 0.0	0.8 ± 0.2	1.0 ± 0.2	1.0 ± 0.1
20:5n-3	5.0 ± 0.2	4.0 ± 0.4	4.4 ± 0.3	4.5 ± 0.6	5.3 ± 0.8	5.2 ± 0.5
22:5n-3	2.8 ± 0.1	2.2 ± 0.3	2.4 ± 0.1	2.4 ± 0.3	3.0 ± 0.5	2.8 ± 0.2
22:6n-3	10.9 ± 0.5	9.3 ± 0.7	10.0 ± 0.3	10.4 ± 0.8	12.5 ± 1.7	11.8 ± 0.5
n-3	23.9 ± 1.0	19.4 ± 1.8	21.0 ± 0.8	21.7 ± 2.5	26.1 ± 4.0	25.0 ± 1.7
n-6	12.2 ± 0.5	9.6 ± 1.1	10.3 ± 0.4	10.6 ± 1.4	12.8 ± 2.4	12.4 ± 1.1
SFA	21.1 ± 0.8	16.7 ± 1.8	17.6 ± 0.8	18.4 ± 2.2	21.9 ± 4.3	21.7 ± 1.7
MUFA	39.4 ± 1.9	30.2 ± 3.6	31.7 ± 1.4	32.7 ± 4.8	40.2 ± 9.1	39.3 ± 3.3
PUFA	36.1 ± 1.5	29.0 ± 2.9	31.3 ± 1.2	32.3 ± 3.9	38.9 ± 6.4	37.4 ± 2.8
Total FA	101.1 ± 4.3	79.2 ± 8.7	84.3 ± 3.5	87.1 ± 11.5	105.7 ± 20.8	102.7 ± 8.1

(*g*) *Thunnus thynnus*

	A-ASE_wet	B-ASE_dry	C-Dounce_wet	D-Dounce_dry	E-Ltw_wet	F-Ltw_dry
(g) Thunnus thynnus						
TAG	155.4 ± 54.9	90.7 ± 33.5	94.8 ± 31.9	112.8 ± 38.8	253.5 ± 61.1	177.1 ± 67.3
FFA	2.5 ± 0.8	1.8 ± 1.3	2.3 ± 0.4	2.8 ± 0.7	5.0 ± 3.5	3.7 ± 1.0
ST	1.5 ± 0.2	2.0 ± 0.4	2.1 ± 0.7	1.7 ± 0.4	2.5 ± 0.4	2.2 ± 0.1
DAG	1.8 ± 0.6	1.2 ± 0.7	0.2 ± 0.3	0.2 ± 0.3	2.9 ± 1.0	2.3 ± 1.1
AMPL	11.4 ± 2.1	4.3 ± 0.6	12.9 ± 1.8	15.0 ± 3.0	24.0 ± 3.8	16.7 ± 4.5
PL	16.2 ± 3.5	24.5 ± 1.9	21.9 ± 7.4	24.3 ± 8.5	33.3 ± 4.1	31.1 ± 3.7
TLC	188.9 ± 58.3	124.6 ± 37.2	134.2 ± 32.6	157.1 ± 30.5	321.2 ± 61.1	233.1 ± 66.0
16:0	11.8 ± 3.9	8.5 ± 3.0	12.1 ± 7.2	12.0 ± 4.9	13.4 ± 4.4	13.6 ± 4.4
17:0	0.8 ± 0.3	0.5 ± 0.2	0.8 ± 0.4	0.8 ± 0.3	0.9 ± 0.3	0.9 ± 0.3
18:0	3.7 ± 1.2	2.8 ± 0.9	3.9 ± 2.0	3.9 ± 1.2	4.5 ± 1.3	4.7 ± 1.2
16:1n-7	4.1 ± 2.0	2.3 ± 1.2	3.7 ± 2.4	3.3 ± 1.6	4.9 ± 1.9	4.4 ± 2.0
18:1n-9	9.4 ± 3.0	6.9 ± 2.8	9.6 ± 6.5	9.1 ± 3.7	11.3 ± 3.3	10.7 ± 4.2
18:1n-7	2.2 ± 0.9	1.5 ± 0.6	2.2 ± 1.4	2.1 ± 0.9	2.6 ± 1.0	2.5 ± 0.9
20:1n-9	1.2 ± 0.3	0.8 ± 0.3	1.2 ± 0.8	1.1 ± 0.5	1.7 ± 0.5	1.5 ± 0.6
22:1n-11	1.1 ± 0.3	0.7 ± 0.3	1.1 ± 0.8	1.0 ± 0.4	1.9 ± 0.9	1.6 ± 0.9
24:1n-9	0.7 ± 0.1	0.5 ± 0.3	0.5 ± 0.1	0.8 ± 0.2	1.1 ± 0.3	0.8 ± 0.4
18:2n-6	0.9 ± 0.3	0.6 ± 0.2	0.9 ± 0.5	0.9 ± 0.4	1.0 ± 0.3	1.0 ± 0.3
18:3n-3	0.7 ± 0.2	0.4 ± 0.2	0.7 ± 0.5	0.6 ± 0.6	0.7 ± 0.3	0.7 ± 0.3
18:4n-3	0.2 ± 0.4	0.6 ± 0.2	1.0 ± 0.7	1.1 ± 0.7	1.1 ± 0.5	1.1 ± 0.4
20:4n-6	0.8 ± 0.3	0.7 ± 0.2	1.0 ± 0.4	1.0 ± 0.2	0.9 ± 0.2	1.1 ± 0.2
20:4n-3	0.5 ± 0.2	0.3 ± 0.1	0.5 ± 0.4	0.6 ± 0.3	0.6 ± 0.2	0.6 ± 0.2
20:5n-3	5.6 ± 2.3	3.7 ± 1.4	5.7 ± 3.3	5.6 ± 2.5	6.2 ± 2.2	6.4 ± 2.2
22:5n-6	0.5 ± 0.2	0.5 ± 0.1	0.6 ± 0.3	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.2
22:5n-3	1.1 ± 0.5	0.7 ± 0.3	1.1 ± 0.7	1.0 ± 0.4	1.3 ± 0.5	1.2 ± 0.5
22:6n-3	16.2 ± 5.5	13.0 ± 4.3	18.1 ± 9.9	18.0 ± 6.7	17.7 ± 5.7	20.4 ± 6.1
n-3	24.4 ± 8.5	18.9 ± 6.6	27.2 ± 15.5	27.0 ± 11.1	27.8 ± 9.3	30.7 ± 9.7
n-6	2.5 ± 0.8	2.0 ± 0.6	2.8 ± 1.3	2.8 ± 0.9	2.7 ± 0.8	3.1 ± 0.7
SFA	18.1 ± 6.3	12.8 ± 4.8	18.3 ± 11.0	18.0 ± 7.4	21.1 ± 6.9	21.2 ± 6.8
MUFA	19.1 ± 6.6	13.0 ± 5.5	18.7 ± 12.0	17.9 ± 7.5	24.0 ± 7.3	22.0 ± 8.2
PUFA	26.9 ± 9.3	20.9 ± 7.1	30.0 ± 16.8	29.8 ± 12.0	30.5 ± 10.1	33.8 ± 10.4
Total FA	66.4 ± 22.9	48.2 ± 17.9	69.2 ± 41.2	68.0 ± 27.9	78.3 ± 25.0	79.6 ± 26.2

Table S2. Fatty acid concentrations (in µg.mg^{-1} dry weight) and percentages (mean ± 1 SD of four replicates) for seven marine species after three-month storage of dry tissue in different conditions: -20°C-freezer, under nitrogen atmosphere, or in dry room. Signs indicate differences from T_0 concentrations (used as reference): ns=not significant; * p<0.05; ** p<0.01; *** p<0.001. Only FA >0.8% of total FA are given. Lines in grey indicate when non-parametric tests were used. SFA=Saturated FA; MUFA=Monounsaturated FA; PUFA=Polyunsaturated FA.

	Concentration (µg.mg^{-1})							Percentage (% of total FA)			
	T0 (reference)	Freezer		Gas nitrogen		Dry room		T0 (reference)	Freezer	Gas nitrogen	Dry room
(a) Lutjanus sebae											
Total FA	16.8 ± 7.8	14.8 ± 7.8	ns	11.7 ± 1.0	ns	9.3 ± 1.9	ns	_	_	_	_
14:0	0.3 ± 0.3	0.2 ± 0.3	ns	0.1 ± 0.1	ns	0.1 ± 0.0	ns	1.4 ± 0.9	1.0 ± 0.8	0.8 ± 0.4	0.6 ± 0.2
16:0	3.3 ± 1.6	3.0 ± 1.9	ns	2.4 ± 0.3	ns	1.9 ± 0.4	ns	19.8 ± 0.5	19.6 ± 1.8	20.2 ± 1.2	20.6 ± 0.7
17:0	0.3 ± 0.1	0.2 ± 0.1	ns	0.1 ± 0.0	ns	0.1 ± 0.0	*	1.5 ± 0.2	1.0 ± 0.3	1.0 ± 0.2	0.9 ± 0.1
18:0	1.3 ± 0.7	1.1 ± 0.6	ns	1.0 ± 0.1	ns	0.8 ± 0.1	ns	7.6 ± 0.5	7.3 ± 0.2	8.3 ± 0.2	8.6 ± 0.4
16:1n-7	0.8 ± 0.7	0.5 ± 0.7	ns	0.3 ± 0.1	ns	0.2 ± 0.1	ns	3.8 ± 1.9	2.6 ± 2.3	2.6 ± 0.8	2.2 ± 0.4
18:1n-9	1.9 ± 1.1	1.5 ± 1.0	ns	1.2 ± 0.2	ns	0.9 ± 0.2	ns	10.9 ± 1.2	9.9 ± 1.2	10.4 ± 1.1	10.0 ± 0.7
18:1n-7	0.4 ± 0.3	0.3 ± 0.3	ns	0.2 ± 0.1	ns	0.2 ± 0.0	ns	2.5 ± 0.6	2.1 ± 0.6	2.0 ± 0.3	1.9 ± 0.2
24:1n-9	0.1 ± 0.1	0.0 ± 0.0	*	0.0 ± 0.0	*	0.0 ± 0.0	*	0.9 ± 0.7	0.2 ± 0.1	0.4 ± 0.2	0.4 ± 0.3
16:4n-3	0.3 ± 0.0	0.4 ± 0.0	ns	0.4 ± 0.0	ns	0.3 ± 0.1	ns	2.3 ± 1.1	3.1 ± 1.1	3.6 ± 0.2	3.6 ± 0.2
18:2n-6	0.2 ± 0.1	0.1 ± 0.1	ns	0.1 ± 0.0	ns	0.1 ± 0.0	*	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.0	0.7 ± 0.1
20:4n-6	0.7 ± 0.2	0.7 ± 0.2	ns	0.7 ± 0.0	ns	0.5 ± 0.1	ns	4.7 ± 0.9	5.5 ± 1.1	6.0 ± 0.7	5.7 ± 0.4
20:5n-3	0.5 ± 0.3	0.5 ± 0.3	ns	0.3 ± 0.0	ns	0.3 ± 0.1	*	3.1 ± 0.2	3.2 ± 0.1	2.9 ± 0.2	2.7 ± 0.3
22:4n-6	0.2 ± 0.1	0.2 ± 0.1	ns	0.2 ± 0.0	ns	0.1 ± 0.0	ns	1.3 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.2 ± 0.2
22:5n-6	0.5 ± 0.1	0.4 ± 0.1	ns	0.4 ± 0.0	ns	0.3 ± 0.1	ns	3.0 ± 0.6	3.2 ± 0.7	3.1 ± 0.3	3.1 ± 0.2
22:5n-3	0.3 ± 0.2	0.3 ± 0.2	ns	0.2 ± 0.0	ns	0.1 ± 0.0	*	1.8 ± 0.2	2.0 ± 0.4	1.4 ± 0.1	1.3 ± 0.2
22:6n-3	4.7 ± 1.4	4.5 ± 1.4	ns	3.6 ± 0.2	ns	2.9 ± 0.8	ns	29.4 ± 4.2	32.3 ± 5.5	31.4 ± 3.0	30.7 ± 2.5
n-3	6.1 ± 2.0	5.8 ± 2.0	ns	4.6 ± 0.2	ns	3.7 ± 0.9	ns	37.5 ± 4.6	41.3 ± 5.8	39.7 ± 3.5	39.3 ± 2.3
n-6	1.6 ± 0.5	1.6 ± 0.5	ns	1.3 ± 0.1	ns	1.0 ± 0.2	ns	10.2 ± 1.3	11.3 ± 1.7	11.4 ± 1.1	11.1 ± 0.6
SFA	5.2 ± 2.7	4.5 ± 2.9	ns	3.6 ± 0.5	ns	2.9 ± 0.5	ns	30.3 ± 1.5	28.9 ± 3.0	30.3 ± 2.0	30.7 ± 1.2
MUFA	3.4 ± 2.2	2.6 ± 2.2	ns	1.9 ± 0.4	ns	1.5 ± 0.3	ns	19.3 ± 3.5	15.7 ± 4.3	16.2 ± 2.3	16.6 ± 1.7
PUFA	7.7 ± 2.5	7.4 ± 2.5	ns	5.9 ± 0.2	ns	4.7 ± 1.1	ns	47.6 ± 5.9	52.5 ± 7.5	51.1 ± 4.6	50.3 ± 2.9
(b) Mytilus edulis											
Total FA	40.8 ± 10.8	32.9 ± 1.2	ns	13.1 ± 0.7	*	12.8 ± 0.5	*	_	_	_	_
14:0	0.8 ± 0.2	0.7 ± 0.0	ns	0.5 ± 0.0	*	0.5 ± 0.0	*	2.1 ± 0.3	2.1 ± 0.2	3.8 ± 0.1	3.8 ± 0.2
15:0	0.3 ± 0.1	0.3 ± 0.0	ns	0.2 ± 0.0	ns	0.2 ± 0.0	ns	0.8 ± 0.0	0.8 ± 0.0	1.6 ± 0.0	1.6 ± 0.1
16:0	7.5 ± 2.0	5.8 ± 0.3	ns	4.1 ± 0.2	*	4.2 ± 0.1	*	18.4 ± 0.6	17.6 ± 0.3	31.4 ± 0.3	32.9 ± 1.0
17:0	0.9 ± 0.2	0.7 ± 0.0	ns	0.5 ± 0.0	*	0.5 ± 0.0	*	2.2 ± 0.0	2.0 ± 0.1	3.7 ± 0.1	4.0 ± 0.1
18:0	2.2 ± 0.6	1.3 ± 0.1	*	0.9 ± 0.0	*	0.9 ± 0.0	*	5.5 ± 0.3	3.8 ± 0.2	6.6 ± 0.2	7.0 ± 0.1
16:1n-7	1.8 ± 0.5	1.4 ± 0.1	ns	0.7 ± 0.1	*	0.7 ± 0.0	*	4.4 ± 0.5	4.3 ± 0.5	5.6 ± 0.4	5.1 ± 0.3
18:1n-9	0.6 ± 0.2	0.5 ± 0.0	ns	0.2 ± 0.0	*	0.2 ± 0.0	*	1.3 ± 0.2	1.5 ± 0.1	1.8 ± 0.2	1.7 ± 0.1
18:1n-7	1.0 ± 0.2	0.8 ± 0.0	ns	0.4 ± 0.0	*	0.4 ± 0.0	*	2.5 ± 0.1	2.3 ± 0.1	2.9 ± 0.1	2.9 ± 0.1
20:1n-9	0.7 ± 0.2	0.5 ± 0.0	ns	0.4 ± 0.0	*	0.4 ± 0.0	*	1.6 ± 0.1	1.6 ± 0.1	2.8 ± 0.0	2.7 ± 0.1
20:1n-7	1.0 ± 0.3	0.8 ± 0.1	ns	0.4 ± 0.0	*	0.4 ± 0.0	*	2.4 ± 0.1	2.4 ± 0.1	3.2 ± 0.1	3.1 ± 0.1
16:4n-3	0.3 ± 0.1	0.2 ± 0.0	ns	0.1 ± 0.0	*	0.1 ± 0.0	*	0.7 ± 0.0	0.6 ± 0.0	1.1 ± 0.0	1.2 ± 0.0
18:2n-6	0.7 ± 0.2	0.6 ± 0.0	ns	0.1 ± 0.0	*	0.1 ± 0.0	*	1.6 ± 0.1	1.7 ± 0.0	0.8 ± 0.1	0.7 ± 0.0
18:3n-3	0.5 ± 0.2	0.4 ± 0.0	ns	0.1 ± 0.0	*	0.1 ± 0.0	*	1.1 ± 0.1	1.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.0
20:2i	1.0 ± 0.3	0.8 ± 0.1	ns	0.5 ± 0.1	*	0.4 ± 0.0	*	2.5 ± 0.2	2.5 ± 0.1	3.5 ± 0.3	3.2 ± 0.1
20:2j	0.3 ± 0.1	0.2 ± 0.0	ns	0.1 ± 0.0	*	0.1 ± 0.0	*	0.7 ± 0.1	0.7 ± 0.0	0.8 ± 0.0	0.8 ± 0.0
20:4n-6	1.0 ± 0.3	0.8 ± 0.0	ns	0.2 ± 0.0	*	0.2 ± 0.0	*	2.5 ± 0.2	2.4 ± 0.1	1.4 ± 0.0	1.3 ± 0.1
20:5n-3	7.0 ± 1.7	5.5 ± 0.2	ns	0.7 ± 0.0	*	0.7 ± 0.1	*	17.2 ± 0.6	16.8 ± 0.5	5.2 ± 0.1	5.2 ± 0.9
22:3nmi	0.3 ± 0.1	0.3 ± 0.0	ns	0.1 ± 0.0	*	0.1 ± 0.0	*	0.8 ± 0.1	0.8 ± 0.0	0.6 ± 0.0	0.6 ± 0.1
22:5n-6	0.2 ± 0.1	0.1 ± 0.0	ns	0.0 ± 0.0	*	0.0 ± 0.0	*	0.5 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
22:6n-3	7.9 ± 2.2	7.0 ± 0.4	ns	0.9 ± 0.0	*	0.9 ± 0.1	*	19.4 ± 0.5	21.2 ± 0.9	7.1 ± 0.6	6.9 ± 0.7
18:0DMA	2.5 ± 0.7	2.2 ± 0.1	ns	1.2 ± 0.1	*	1.1 ± 0.1	*	6.0 ± 0.6	6.7 ± 0.2	9.2 ± 0.3	8.7 ± 0.4
n-3	16.3 ± 4.2	13.7 ± 0.6	ns	1.9 ± 0.1	*	1.8 ± 0.2	*	40.0 ± 0.5	41.7 ± 0.6	14.6 ± 0.5	14.5 ± 1.3
n-6	2.3 ± 0.7	1.9 ± 0.1	ns	0.4 ± 0.0	*	0.3 ± 0.0	*	5.5 ± 0.2	5.8 ± 0.1	3.0 ± 0.1	2.7 ± 0.2
SFA	11.5 ± 2.9	8.4 ± 0.4	ns	6.0 ± 0.3	*	6.1 ± 0.2	*	28.2 ± 0.6	25.5 ± 0.5	45.5 ± 0.1	47.7 ± 1.2
MUFA	5.1 ± 1.4	4.1 ± 0.1	ns	2.2 ± 0.1	*	2.0 ± 0.1	*	12.5 ± 0.6	12.4 ± 0.7	16.7 ± 0.5	16.1 ± 0.4
PUFA	18.6 ± 4.9	15.6 ± 0.6	ns	2.3 ± 0.1	*	2.2 ± 0.2	*	45.6 ± 0.4	47.5 ± 0.6	17.7 ± 0.5	17.2 ± 1.4

Continued (1/2).

	Concentration (µg.mg^{-1})							Percentage (% of total FA)			
	T0 (reference)	Freezer		Gas nitrogen		Dry room		T0 (reference)	Freezer	Gas nitrogen	Dry room
(c) Octopus vulgaris											
Total FA	15.4 ± 0.3	13.4 ± 1.0	**ns**	12.9 ± 0.2	*	13.7 ± 1.9	**ns**	_	_	_	_
16:0	2.7 ± 0.1	2.1 ± 0.1	**	2.0 ± 0.1	***	2.2 ± 0.2	*	17.3 ± 0.2	15.5 ± 0.3	15.6 ± 0.7	16.4 ± 0.6
17:0	0.3 ± 0.1	0.2 ± 0.0	**ns**	0.2 ± 0.0	**ns**	0.2 ± 0.1	**ns**	2.1 ± 0.5	1.5 ± 0.0	1.5 ± 0.0	1.7 ± 0.7
18:0	2.1 ± 0.1	1.6 ± 0.1	***	1.6 ± 0.0	***	1.8 ± 0.2	*	13.8 ± 0.2	12.1 ± 0.1	12.6 ± 0.3	13.2 ± 0.8
16:1n-7	0.1 ± 0.0	0.0 ± 0.0	**ns**	0.0 ± 0.0	**ns**	0.0 ± 0.0	**ns**	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1
18:1n-9	0.3 ± 0.0	0.3 ± 0.0	**ns**	0.2 ± 0.0	**ns**	0.3 ± 0.1	**ns**	1.9 ± 0.1	2.0 ± 0.1	1.8 ± 0.1	2.0 ± 0.2
18:1n-7	0.3 ± 0.0	0.2 ± 0.0	**	0.2 ± 0.0	***	0.2 ± 0.0	**	1.9 ± 0.0	1.8 ± 0.0	1.7 ± 0.0	1.8 ± 0.1
20:1n-7	0.2 ± 0.0	0.1 ± 0.1	*	0.2 ± 0.0	*	0.2 ± 0.0	**ns**	1.5 ± 0.1	1.1 ± 0.7	1.4 ± 0.0	1.6 ± 0.2
20:2n-6	0.2 ± 0.0	0.1 ± 0.0	*	0.1 ± 0.0	*	0.1 ± 0.0	*	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.2
20:4n-6	2.7 ± 0.1	2.4 ± 0.1	*	2.3 ± 0.0	*	2.4 ± 0.3	*	17.8 ± 0.3	17.8 ± 0.5	18.0 ± 0.3	17.6 ± 0.9
20:5n-3	1.0 ± 0.0	1.0 ± 0.1	**ns**	0.9 ± 0.0	**ns**	1.0 ± 0.1	**ns**	6.8 ± 0.2	7.2 ± 0.2	7.1 ± 0.2	7.2 ± 0.2
22:4n-6	0.3 ± 0.0	0.4 ± 0.0	**ns**	0.4 ± 0.0	**ns**	0.3 ± 0.0	**ns**	2.2 ± 0.1	2.8 ± 0.1	2.8 ± 0.2	2.5 ± 0.3
22:5n-6	0.2 ± 0.0	0.2 ± 0.0	**	0.2 ± 0.0	**	0.2 ± 0.0	**	1.5 ± 0.0	1.5 ± 0.1	1.5 ± 0.0	1.4 ± 0.1
22:5n-3	0.2 ± 0.0	0.2 ± 0.0	**ns**	0.2 ± 0.0	**	0.2 ± 0.0	*	1.5 ± 0.0	1.6 ± 0.1	1.5 ± 0.0	1.5 ± 0.1
22:6n-3	3.1 ± 0.1	2.9 ± 0.3	**ns**	2.8 ± 0.0	**ns**	2.9 ± 0.4	**ns**	20.4 ± 0.4	21.8 ± 0.8	21.7 ± 0.5	21.5 ± 1.1
18:0DMA	0.6 ± 0.4	0.9 ± 0.1	*	0.8 ± 0.0	**ns**	0.8 ± 0.5	**ns**	3.9 ± 2.5	6.8 ± 0.1	6.6 ± 0.0	5.4 ± 3.5
n-3	4.5 ± 0.2	4.2 ± 0.4	**ns**	3.9 ± 0.0	**ns**	4.2 ± 0.5	**ns**	29.2 ± 0.6	31.1 ± 0.7	30.7 ± 0.4	30.7 ± 1.3
n-6	3.5 ± 0.1	3.1 ± 0.2	*	3.1 ± 0.0	*	3.1 ± 0.3	*	23.0 ± 0.3	23.5 ± 0.5	23.7 ± 0.4	22.9 ± 1.3
SFA	5.1 ± 0.2	4.0 ± 0.3	***	3.9 ± 0.1	***	4.3 ± 0.5	*	33.5 ± 0.6	29.6 ± 0.4	30.1 ± 0.6	31.6 ± 0.8
MUFA	1.1 ± 0.1	0.8 ± 0.0	*	0.8 ± 0.0	**	0.9 ± 0.1	*	6.9 ± 0.4	6.2 ± 0.3	6.0 ± 0.3	6.3 ± 0.5
PUFA	8.0 ± 0.3	7.3 ± 0.6	**ns**	7.0 ± 0.1	**ns**	7.3 ± 0.8	**ns**	52.2 ± 0.8	54.6 ± 0.3	54.4 ± 0.7	53.7 ± 2.1
(d) Panulirus versicolor											
Total FA	12.9 ± 0.3	12.0 ± 1.2	**ns**	13.2 ± 0.8	**ns**	12.5 ± 0.5	**ns**	_	_	_	_
16:0	1.4 ± 0.1	1.3 ± 0.1	**ns**	1.4 ± 0.1	**ns**	1.3 ± 0.0	**ns**	11.2 ± 0.3	10.4 ± 0.4	10.2 ± 0.5	10.1 ± 0.2
17:0	0.3 ± 0.0	0.2 ± 0.0	**	0.3 ± 0.0	*	0.2 ± 0.0	*	2.3 ± 0.0	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.0
18:0	1.7 ± 0.0	1.4 ± 0.1	**	1.6 ± 0.1	**ns**	1.5 ± 0.1	**ns**	13.4 ± 0.2	11.8 ± 0.2	12.0 ± 0.4	12.3 ± 0.0
20:0	0.1 ± 0.0	0.1 ± 0.0	***	0.1 ± 0.0	***	0.1 ± 0.0	**	0.8 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.7 ± 0.0
16:1n-7	0.4 ± 0.0	0.4 ± 0.0	**ns**	0.4 ± 0.0	**ns**	0.4 ± 0.0	**ns**	3.0 ± 0.0	3.0 ± 0.1	2.9 ± 0.0	2.9 ± 0.1
18:1n-9	1.1 ± 0.0	1.0 ± 0.1	**ns**	1.1 ± 0.1	**ns**	1.0 ± 0.0	**ns**	8.4 ± 0.1	8.4 ± 0.3	8.2 ± 0.1	8.2 ± 0.1
18:1n-7	0.2 ± 0.0	0.2 ± 0.0	**ns**	0.2 ± 0.0	**ns**	0.2 ± 0.0	**ns**	1.9 ± 0.0	1.8 ± 0.1	1.8 ± 0.0	1.8 ± 0.0
16:2n-4	0.1 ± 0.0	0.1 ± 0.0	**ns**	0.2 ± 0.0	**ns**	0.1 ± 0.0	**ns**	1.0 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.2 ± 0.0
18:2n-6	0.3 ± 0.0	0.3 ± 0.0	**ns**	0.3 ± 0.0	**ns**	0.3 ± 0.0	**ns**	2.4 ± 0.0	2.3 ± 0.0	2.3 ± 0.0	2.3 ± 0.0
20:2n-6	0.1 ± 0.0	0.1 ± 0.0	**ns**	0.1 ± 0.0	**ns**	0.1 ± 0.0	**ns**	1.1 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
20:4n-6	2.1 ± 0.1	2.0 ± 0.2	**ns**	2.2 ± 0.1	**ns**	2.1 ± 0.1	**ns**	16.3 ± 0.1	17.0 ± 0.3	16.8 ± 0.3	17.1 ± 0.1
20:5n-3	1.9 ± 0.0	1.9 ± 0.2	**ns**	2.0 ± 0.1	**ns**	1.9 ± 0.1	**ns**	14.9 ± 0.3	15.4 ± 0.6	15.0 ± 0.4	15.2 ± 0.2
22:4n-6	0.1 ± 0.0	0.1 ± 0.0	**ns**	0.1 ± 0.0	**ns**	0.1 ± 0.0	**ns**	0.9 ± 0.0	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.0
22:5n-6	0.1 ± 0.0	0.1 ± 0.0	**ns**	0.1 ± 0.0	**ns**	0.1 ± 0.0	**ns**	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
22:5n-3	0.2 ± 0.0	0.2 ± 0.0	**ns**	0.2 ± 0.0	**ns**	0.2 ± 0.0	**ns**	1.5 ± 0.0	1.6 ± 0.0	1.4 ± 0.1	1.4 ± 0.0
22:6n-3	1.1 ± 0.0	1.2 ± 0.2	**ns**	1.4 ± 0.1	*	1.2 ± 0.1	**ns**	8.8 ± 0.3	9.8 ± 0.4	10.9 ± 1.0	9.8 ± 0.8
18:0DMA	0.6 ± 0.0	0.7 ± 0.1	**ns**	0.7 ± 0.1	*	0.7 ± 0.0	**ns**	4.8 ± 0.1	5.5 ± 0.2	5.6 ± 0.1	5.9 ± 0.1
n-3	3.4 ± 0.1	3.4 ± 0.4	**ns**	3.8 ± 0.1	**ns**	3.5 ± 0.2	**ns**	26.5 ± 0.5	28.2 ± 1.0	28.8 ± 1.1	27.7 ± 0.6
n-6	2.8 ± 0.1	2.7 ± 0.3	**ns**	2.9 ± 0.2	**ns**	2.8 ± 0.1	**ns**	21.7 ± 0.1	22.5 ± 0.3	22.2 ± 0.3	22.5 ± 0.1
SFA	3.5 ± 0.1	3.0 ± 0.3	**ns**	3.3 ± 0.3	**ns**	3.1 ± 0.1	**ns**	27.2 ± 0.4	24.6 ± 0.7	24.6 ± 0.9	24.8 ± 0.2
MUFA	2.0 ± 0.1	1.8 ± 0.1	**ns**	1.9 ± 0.2	**ns**	1.8 ± 0.1	**ns**	15.2 ± 0.2	14.9 ± 0.6	14.6 ± 0.5	14.7 ± 0.2
PUFA	6.2 ± 0.1	6.1 ± 0.7	**ns**	6.7 ± 0.3	**ns**	6.3 ± 0.3	**ns**	48.2 ± 0.6	50.7 ± 1.2	51.0 ± 1.2	50.2 ± 0.4

Continued (2/2).

	Concentration (µg.mg^{-1})							Percentage (% of total FA)			
	T0 (reference)	Freezer		Gas nitrogen		Dry room		T0 (reference)	Freezer	Gas nitrogen	Dry room
(e) Sardina pilchardus											
Total FA	62.9 ± 15.5	53.2 ± 11.6	ns	36.9 ± 4.2	ns	35.7 ± 8.1	ns	_	_	_	_
14:0	2.8 ± 0.7	2.9 ± 0.3	ns	2.9 ± 0.6	ns	3.0 ± 0.9	ns	4.5 ± 0.2	5.6 ± 0.8	7.7 ± 0.8	8.2 ± 0.5
16:0	11.8 ± 3.1	11.5 ± 0.3	ns	10.2 ± 1.0	ns	10.3 ± 2.5	ns	18.7 ± 0.6	22.3 ± 5.1	27.7 ± 0.4	28.8 ± 0.8
17:0	0.6 ± 0.2	0.5 ± 0.1	ns	0.4 ± 0.1	ns	0.4 ± 0.2	ns	1.0 ± 0.1	1.0 ± 0.2	1.2 ± 0.0	1.0 ± 0.2
18:0	2.4 ± 0.5	2.2 ± 0.1	ns	1.8 ± 0.1	ns	1.8 ± 0.4	ns	3.9 ± 0.2	4.4 ± 1.4	5.0 ± 0.4	5.1 ± 0.5
16:1n-7	5.0 ± 1.0	5.1 ± 0.9	ns	5.0 ± 1.0	ns	4.8 ± 1.3	ns	8.0 ± 1.1	9.7 ± 0.5	13.4 ± 1.1	13.3 ± 0.6
18:1n-9	2.5 ± 0.6	2.3 ± 0.5	ns	2.0 ± 0.2	ns	2.1 ± 0.5	ns	3.9 ± 0.3	4.2 ± 0.2	5.3 ± 0.0	6.0 ± 1.0
18:1n-7	1.8 ± 0.3	1.5 ± 0.3	ns	1.5 ± 0.2	ns	1.4 ± 0.3	ns	2.9 ± 0.4	2.9 ± 0.1	4.0 ± 0.1	3.9 ± 0.2
20:1n-9	1.5 ± 0.6	1.8 ± 0.0	ns	1.6 ± 0.1	ns	1.6 ± 0.5	ns	2.3 ± 0.5	3.5 ± 0.9	4.3 ± 0.1	4.3 ± 0.4
22:1n-11	2.1 ± 0.9	2.5 ± 0.1	ns	2.3 ± 0.1	ns	2.2 ± 0.6	ns	3.2 ± 0.8	4.9 ± 1.0	6.2 ± 0.3	6.1 ± 0.6
24:1n-9	0.6 ± 0.2	0.2 ± 0.3	ns	0.1 ± 0.0	ns	0.1 ± 0.0	*	0.9 ± 0.0	0.5 ± 0.7	0.3 ± 0.1	0.3 ± 0.1
16:4n-3	0.4 ± 0.1	0.3 ± 0.2	ns	0.1 ± 0.0	*	0.1 ± 0.0	*	0.6 ± 0.1	0.5 ± 0.2	0.3 ± 0.0	0.4 ± 0.2
18:2n-6	0.7 ± 0.2	0.5 ± 0.2	ns	0.3 ± 0.0	ns	0.3 ± 0.1	ns	1.1 ± 0.1	1.0 ± 0.3	0.9 ± 0.1	0.8 ± 0.1
18:3n-3	0.5 ± 0.2	0.3 ± 0.2	ns	0.1 ± 0.0	ns	0.1 ± 0.0	*	0.8 ± 0.1	0.6 ± 0.3	0.4 ± 0.0	0.3 ± 0.1
18:4n-3	0.9 ± 0.3	0.7 ± 0.3	ns	0.3 ± 0.1	ns	0.0 ± 0.0	*	1.4 ± 0.2	1.2 ± 0.4	0.8 ± 0.3	0.0 ± 0.0
20:4n-6	0.7 ± 0.1	0.5 ± 0.2	ns	0.2 ± 0.0	*	0.2 ± 0.0	**	1.1 ± 0.1	0.9 ± 0.3	0.5 ± 0.0	0.5 ± 0.0
20:4n-3	0.5 ± 0.1	0.4 ± 0.2	ns	0.1 ± 0.0	*	0.1 ± 0.0	*	0.8 ± 0.0	0.6 ± 0.2	0.3 ± 0.0	0.2 ± 0.0
20:5n-3	7.4 ± 1.5	5.3 ± 2.8	ns	1.7 ± 0.2	*	1.3 ± 0.2	**	11.9 ± 1.2	9.5 ± 3.7	4.5 ± 0.0	3.6 ± 0.7
22:5n-6	0.5 ± 0.1	0.3 ± 0.1	ns	0.1 ± 0.0	**	0.1 ± 0.0	**	0.8 ± 0.0	0.5 ± 0.1	0.3 ± 0.0	0.3 ± 0.1
22:5n-3	0.9 ± 0.2	0.7 ± 0.2	ns	0.2 ± 0.0	**	0.1 ± 0.0	**	1.5 ± 0.2	1.2 ± 0.2	0.5 ± 0.0	0.4 ± 0.1
22:6n-3	16.6 ± 4.2	10.7 ± 5.4	ns	4.0 ± 0.3	*	3.8 ± 0.6	**	26.3 ± 1.3	19.2 ± 6.8	10.9 ± 0.3	10.7 ± 1.7
n-3	27.2 ± 6.5	18.4 ± 9.3	ns	6.5 ± 0.5	*	5.5 ± 0.9	**	43.3 ± 0.5	32.9 ± 11.8	17.7 ± 0.7	15.7 ± 2.5
n-6	2.1 ± 0.5	1.6 ± 0.6	ns	0.8 ± 0.0	*	0.7 ± 0.1	*	3.4 ± 0.1	2.9 ± 0.6	2.1 ± 0.1	1.9 ± 0.2
SFA	17.7 ± 4.5	17.1 ± 0.6	ns	15.3 ± 1.8	ns	15.5 ± 3.9	ns	28.1 ± 0.3	33.2 ± 7.4	41.5 ± 0.0	43.1 ± 1.5
MUFA	13.6 ± 3.4	13.8 ± 1.6	ns	12.5 ± 1.8	ns	12.3 ± 3.1	ns	21.6 ± 0.6	26.4 ± 3.3	33.9 ± 0.9	34.4 ± 1.0
PUFA	29.3 ± 7.0	20.0 ± 9.9	ns	7.3 ± 0.6	*	6.2 ± 1.0	**	46.7 ± 0.5	35.8 ± 12.4	19.8 ± 0.8	17.6 ± 2.7
(f) Sparus aurata											
Total FA	102.7 ± 8.1	61.6 ± 9.9	***	69.9 ± 10.5	**	69.5 ± 7.8	**	_	_	_	_
14:0	3.4 ± 0.4	2.0 ± 0.4	**	2.4 ± 0.4	*	2.4 ± 0.2	*	3.3 ± 0.1	3.3 ± 0.3	3.4 ± 0.1	3.5 ± 0.2
16:0	14.7 ± 1.1	9.4 ± 1.6	***	10.9 ± 1.4	**	11.4 ± 0.9	*	14.3 ± 0.1	15.2 ± 0.5	15.6 ± 0.4	16.4 ± 1.3
18:0	3.1 ± 0.2	1.9 ± 0.2	***	2.0 ± 0.1	***	2.2 ± 0.2	***	3.0 ± 0.1	3.1 ± 0.4	2.9 ± 0.4	3.2 ± 0.4
16:1n-7	5.2 ± 0.6	3.1 ± 0.7	**	3.8 ± 0.6	*	3.8 ± 0.4	*	5.1 ± 0.2	5.1 ± 0.3	5.4 ± 0.1	5.5 ± 0.3
18:1n-9	23.6 ± 2.1	14.0 ± 2.2	***	16.2 ± 2.8	**	17.1 ± 2.1	*	23.0 ± 0.2	22.7 ± 0.3	23.1 ± 0.6	24.5 ± 1.1
18:1n-7	2.7 ± 0.2	1.5 ± 0.3	***	1.7 ± 0.2	***	1.7 ± 0.2	***	2.6 ± 0.1	2.4 ± 0.1	2.4 ± 0.1	2.5 ± 0.2
20:1n-9	3.3 ± 0.3	2.0 ± 0.4	**	2.5 ± 0.7	ns	2.6 ± 0.1	ns	3.3 ± 0.1	3.2 ± 0.6	3.5 ± 0.6	3.8 ± 0.4
22:1n-11	3.1 ± 0.2	1.5 ± 0.3	*	2.0 ± 0.5	**	2.3 ± 0.3	ns	3.0 ± 0.2	2.5 ± 0.3	2.8 ± 0.3	3.3 ± 0.1
18:2n-6	10.5 ± 1.0	6.4 ± 1.0	**	7.3 ± 1.3	**	6.7 ± 1.1	**	10.2 ± 0.2	10.4 ± 0.1	10.4 ± 0.3	9.7 ± 0.7
18:3n-3	2.6 ± 0.3	1.5 ± 0.2	***	1.6 ± 0.3	**	1.4 ± 0.3	***	2.5 ± 0.1	2.5 ± 0.1	2.3 ± 0.1	2.1 ± 0.3
18:4n-3	1.3 ± 0.2	0.6 ± 0.4	**	0.8 ± 0.2	ns	0.7 ± 0.2	*	1.3 ± 0.1	0.9 ± 0.6	1.2 ± 0.1	1.0 ± 0.2
20:4n-6	0.8 ± 0.1	0.6 ± 0.1	**	0.6 ± 0.0	**	0.5 ± 0.1	**	0.8 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
20:4n-3	1.0 ± 0.1	0.6 ± 0.1	**	0.6 ± 0.1	**	0.5 ± 0.1	***	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.0	0.8 ± 0.1
20:5n-3	5.2 ± 0.5	3.2 ± 0.5	***	3.4 ± 0.4	**	2.9 ± 0.6	***	5.0 ± 0.2	5.1 ± 0.1	4.9 ± 0.1	4.2 ± 0.6
22:5n-3	2.8 ± 0.2	1.6 ± 0.4	***	1.8 ± 0.3	**	1.5 ± 0.4	***	2.8 ± 0.0	2.6 ± 0.2	2.5 ± 0.0	2.2 ± 0.4
22:6n-3	11.8 ± 0.5	8.1 ± 1.4	**	8.2 ± 0.7	**	7.3 ± 1.4	***	11.5 ± 0.5	13.1 ± 0.9	11.9 ± 0.8	10.5 ± 1.3
n-3	25.0 ± 1.7	15.7 ± 2.5	***	16.7 ± 2.0	**	14.5 ± 3.2	***	24.3 ± 0.4	25.5 ± 0.7	23.9 ± 0.8	20.8 ± 2.9
n-6	12.4 ± 1.1	7.7 ± 1.2	***	8.5 ± 1.4	**	7.9 ± 1.3	**	12.1 ± 0.1	12.5 ± 0.1	12.2 ± 0.2	11.3 ± 0.8
SFA	21.7 ± 1.7	13.5 ± 2.2	***	15.7 ± 2.0	**	16.4 ± 1.4	*	21.1 ± 0.1	22.0 ± 0.5	22.5 ± 0.6	23.7 ± 2.1
MUFA	39.3 ± 3.3	22.4 ± 3.7	***	26.5 ± 4.9	**	27.9 ± 3.0	*	38.2 ± 0.3	36.3 ± 1.3	37.7 ± 1.4	40.2 ± 1.6
PUFA	37.4 ± 2.8	23.4 ± 3.7	***	25.2 ± 3.4	**	22.4 ± 4.4	*	36.4 ± 0.3	38.0 ± 0.7	36.1 ± 0.6	32.2 ± 3.7
(g) Thunnus thynnus											
Total FA	79.6 ± 26.2	63.6 ± 22.2	ns	59.4 ± 14.8	ns	52.2 ± 5.0	ns	_	_	_	_
14:0	2.1 ± 0.9	1.8 ± 0.6	ns	2.2 ± 0.5	ns	2.4 ± 0.1	ns	2.5 ± 0.4	2.9 ± 0.1	3.8 ± 0.3	4.5 ± 0.6
16:0	13.6 ± 4.4	11.5 ± 3.5	ns	13.8 ± 1.7	ns	13.9 ± 1.2	ns	17.1 ± 0.4	18.3 ± 1.3	23.8 ± 3.3	26.8 ± 3.7
17:0	0.9 ± 0.3	0.7 ± 0.3	ns	0.8 ± 0.1	ns	0.8 ± 0.1	ns	1.1 ± 0.0	1.0 ± 0.0	1.4 ± 0.1	1.5 ± 0.1
18:0	4.7 ± 1.2	4.0 ± 1.1	ns	4.4 ± 0.5	ns	4.5 ± 0.3	ns	6.0 ± 0.5	6.5 ± 0.7	7.7 ± 1.1	8.6 ± 1.1
16:1n-7	4.4 ± 2.0	4.1 ± 1.4	ns	4.7 ± 1.1	ns	4.6 ± 0.3	ns	5.4 ± 0.8	6.5 ± 0.1	8.0 ± 0.7	8.9 ± 0.5
18:1n-9	10.7 ± 4.2	8.6 ± 3.3	ns	10.3 ± 1.3	ns	10.0 ± 0.7	ns	13.3 ± 0.9	13.3 ± 0.5	17.7 ± 2.6	19.3 ± 1.8
18:1n-7	2.5 ± 0.9	2.2 ± 0.7	ns	2.5 ± 0.4	ns	2.4 ± 0.2	ns	3.2 ± 0.1	3.5 ± 0.1	4.3 ± 0.4	4.6 ± 0.6
20:1n-9	1.5 ± 0.6	1.1 ± 0.4	ns	1.5 ± 0.3	ns	1.8 ± 0.2	ns	1.9 ± 0.4	1.8 ± 0.0	2.5 ± 0.3	3.4 ± 0.2
22:1n-11	1.6 ± 0.9	1.2 ± 0.4	ns	1.5 ± 0.5	ns	2.0 ± 0.5	ns	1.9 ± 0.7	1.8 ± 0.0	2.5 ± 0.4	3.7 ± 0.6
24:1n-9	0.8 ± 0.4	0.1 ± 0.0	*	0.2 ± 0.1	*	0.2 ± 0.1	*	1.1 ± 0.6	0.2 ± 0.1	0.4 ± 0.2	0.4 ± 0.3
18:2n-6	1.0 ± 0.3	0.8 ± 0.3	ns	0.7 ± 0.3	ns	0.4 ± 0.2	*	1.3 ± 0.0	1.3 ± 0.1	1.2 ± 0.2	0.8 ± 0.2
18:3n-3	0.7 ± 0.3	0.6 ± 0.2	ns	0.4 ± 0.2	ns	0.2 ± 0.1	*	0.9 ± 0.1	0.9 ± 0.1	0.6 ± 0.2	0.3 ± 0.2
18:4n-3	1.1 ± 0.4	0.9 ± 0.3	ns	0.4 ± 0.4	ns	0.1 ± 0.1	*	1.4 ± 0.1	1.4 ± 0.0	0.7 ± 0.5	0.1 ± 0.1
20:4n-6	1.1 ± 0.2	0.8 ± 0.3	ns	0.5 ± 0.2	*	0.3 ± 0.2	**	1.4 ± 0.3	1.3 ± 0.1	0.8 ± 0.2	0.5 ± 0.2
20:4n-3	0.6 ± 0.2	0.4 ± 0.2	ns	0.3 ± 0.2	ns	0.1 ± 0.1	*	0.7 ± 0.0	0.7 ± 0.0	0.4 ± 0.2	0.2 ± 0.2
20:5n-3	6.4 ± 2.2	5.1 ± 2.0	ns	2.8 ± 1.7	ns	1.1 ± 1.2	*	8.0 ± 0.2	7.9 ± 0.5	4.3 ± 1.7	2.0 ± 2.1
22:5n-6	0.7 ± 0.2	0.5 ± 0.2	ns	0.3 ± 0.2	*	0.1 ± 0.1	**	0.9 ± 0.1	0.8 ± 0.0	0.5 ± 0.1	0.3 ± 0.2
22:5n-3	1.2 ± 0.5	1.0 ± 0.4	ns	0.5 ± 0.3	ns	0.3 ± 0.2	*	1.5 ± 0.1	1.5 ± 0.1	0.9 ± 0.3	0.5 ± 0.4
22:6n-3	20.4 ± 6.1	15.1 ± 5.9	ns	8.5 ± 4.8	*	4.0 ± 3.1	**	25.9 ± 1.8	23.5 ± 1.2	13.4 ± 4.6	7.4 ± 5.1
n-3	30.7 ± 9.7	23.2 ± 9.0	ns	12.9 ± 7.6	*	5.8 ± 4.7	*	38.7 ± 1.7	36.1 ± 1.9	20.4 ± 7.5	10.8 ± 7.8
n-6	3.1 ± 0.7	2.5 ± 0.9	ns	1.7 ± 0.7	ns	1.0 ± 0.5	*	4.1 ± 0.5	3.9 ± 0.2	2.8 ± 0.6	1.8 ± 0.7
SFA	21.2 ± 6.8	18.0 ± 5.4	ns	21.3 ± 2.8	ns	21.5 ± 1.7	ns	26.7 ± 0.4	28.8 ± 2.1	36.7 ± 4.6	41.5 ± 5.4
MUFA	22.0 ± 8.2	17.6 ± 6.3	ns	21.1 ± 3.4	ns	21.3 ± 1.1	ns	27.3 ± 1.9	27.6 ± 0.2	36.1 ± 3.2	41.0 ± 2.6
PUFA	33.8 ± 10.4	25.7 ± 9.9	ns	14.6 ± 8.3	*	6.8 ± 5.2	*	42.8 ± 2.1	40.0 ± 2.1	23.2 ± 8.0	12.6 ± 8.5