Evaluation of food grade solvents for lipid extraction and impact of storage temperature on fatty acid composition of edible seaweeds *Laminaria digitata* (Phaeophyceae) and *Palmaria palmata* (Rhodophyta)

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Abstract

This study evaluated the impact of different food- and non-food grade extraction solvents on yield and fatty acid composition of the lipid extracts of two seaweed species (*Palmaria palmata* and *Laminaria digitata*). The application of chloroform/methanol and three different food grade solvents (ethanol, hexane, ethanol/hexane) revealed significant differences in both, extraction yield and fatty acid composition. The extraction efficiency, in terms of yields of total fatty acids (TFA), was in the order: chloroform/methanol > ethanol > hexane > ethanol/hexane for both species. Highest levels of polyunsaturated fatty acids (PUFA) were achieved by the extraction with ethanol. Additionally the effect of storage temperature on the stability of PUFA in ground and freeze-dried seaweed biomass was investigated. Seaweed samples were stored for a total duration of 22 months at three different temperatures (-20 °C,

4°C and 20 °C). Levels of TFA and PUFA were only stable after storage at -20° C for the two seaweed species.

Keywords: Lipid extraction, seaweeds, PUFA, omega-3 fatty acids, storage, lipid oxidation

Introduction

The deficiency of omega-3 fatty acids of the western diet has been identified as one of the main causes of numerous chronic diseases (Simopoulos, 1991). Omega-3 fatty acids play a major role in the prevention of chronic inflammation, which has been identified as a potential headstone to diseases such as Alzheimer's, diabetes, rheumatoid arthritis and inflammatory bowel disease (Simopoulos, 2008). Also, omega-3 polyunsaturated fatty acids (n-3 PUFA) are important compounds for pre- and postal retina and brain development (Simopoulos, 1991). Health studies have advised to improve the dietary intake of omega-3 fatty acids in the western diet as a preventative measure (Simopoulos, 2002). This has made PUFA one of the target compounds of interest for functional food applications.

Structurally PUFA are made up of long hydrocarbon chains which terminate with a hydroxyl group. Depending on the position of their first double bond, they are classified as omega-3 or omega-6 fatty acids. PUFA play a variety of important functional roles in organisms,

including as structural components of the phospholipid bilayer in cell membranes. The fatty acid composition of this bilayer is important for the regulation of the membrane fluidity and the dietary intake can strongly influence the fatty acid composition of cell membranes. Seaweeds have been identified as a valuable source of PUFA-rich fatty acids with potential application in functional foods (Kumari, Kumar, Gupta, Reddy, & Jha, 2010; Schmid, Guihéneuf, & Stengel, 2014). Seaweeds are rich in n-3 PUFA e.g., stearidonic acid (18:4 n-3, SDA), eicosapentaenoic acid (20:5 n-3, EPA) and even small amounts of docosahexaenoic acid (22:6 n-3, DHA) (Kumari, Kumar, Gupta, Reddy, & Jha, 2010). The growing interest in PUFA-rich lipids from seaweeds for incorporation into foods has lead to an increasing demand for novel extraction techniques with high extraction yields; additionally, incorporation of seaweed lipid extracts into food products requires the application of food grade solvents. One of the most commonly used lipid extraction techniques is based on the method established by Bligh and Dyer (1959) which involves a fixed ratio of chloroform (CHCl_3), methanol (MeOH) and water (H_2O). However this method is unsuitable for food applications because of the use of both CHCl_3 and MeOH , solvents which do not comply with food grade standards. Studies on microalgae have identified efficient extraction techniques using ethanol (EtOH), hexane or a combination of both that have lead to good extraction yields (Fajardo, Cerdan, Medina, Fernandez, Moreno, & Grima, 2007; Yang, Xiang, Sun, Wu, Li, & Long, 2014), but there is little information available on the efficiency of these solvents to extract lipids from seaweeds. A further aspect of concern for the commercial utilisation of algal lipid extracts is the stability of PUFA in stored seaweed biomass. The multiple double bonds of PUFA can readily react with oxygen (Choe & Min, 2006) which can negatively affect the amounts of PUFA in seaweed product and decrease their impact as an omega-3 supplement. Stability of

PUFA in stored seaweed needs to be considered but currently stability of PUFA in stored seaweed biomass is largely unknown (Oh, Lee, & Choe, 2014). In this study, alternative extraction solvents were evaluated including the food grade solvents ethanol and hexane, and a combination of ethanol and hexane. The extraction methods were performed in a time-efficient manner and with low solvent usage which are important factors for commercial application. The extraction solvents were tested on biomass from two commercially valuable macroalgal species commonly found on Irish shores (*Palmaria palmata* and *Laminaria digitata*). The extraction solvents were evaluated for extraction yield, quality of the extracts and percentage of omega-3 fatty acids. In order to evaluate the stability of fatty acids in stored seaweed powder, three storage temperatures (-20 °C, 4 °C and 20 °C) were compared. The effects of storage temperature were evaluated for fatty acid content and composition of freeze-dried and ground seaweed biomass after different intervals of storage.

Materials and methods

Sample collection

Different extraction solvents were compared on biomass of the brown kelp species *L. digitata* (Hudson) Lamouroux (Laminariaceae) and the red seaweed *P. palmata* (L.) Weber & Mohr (Palmariaceae), collected from Co. Clare, western Ireland (53°09'24'' N, 9°07'17'') in November 2012. After collection, seaweed material was frozen at -20 °C and dried in a Labconco Freezone® (Kansas City, MO, USA) freeze-drier. Samples were ground to fine a powder and stored at -20 °C until analysis.

Comparison of solvent mixtures for seaweed lipid extraction

97 Four different solvents were evaluated for lipid extraction from seaweeds, using
98 $\text{CHCl}_3/\text{MeOH}$ and food grade solvents such as EtOH, hexane and both EtOH/hexane. For
99 each analysis, 1 g of freeze-dried and ground seaweed was used. All extraction solvents were
100 compared in a time and solvent efficient manner.

101 **Extraction with $\text{CHCl}_3/\text{MeOH}$:** The extraction was obtained using a protocol modified
102 from Bligh and Dyer (1959): total lipids were extracted overnight under constant stirring with
103 21 ml of $\text{CHCl}_3/\text{MeOH}$ (1/2. v/v) and 2 ml NaCl solution (2.8 g l^{-1}). The next day, 7 ml of
104 CHCl_3 and 14 ml of H_2O were added to create phase separation. The samples were
105 centrifuged (4 min at 1000 rpm) and the lower CHCl_3 phase containing lipids was collected.
106 20 ml of CHCl_3 were added to the sample for a second extraction, vortexed and left for 2 h.
107 After extraction, the sample was centrifuged (4 min at 1000 rpm) and the lower phase was
108 added to the previous extract.

109 **Extraction with EtOH:** Total lipids were extracted using 20 ml of EtOH. Samples were left
110 to extract overnight under constant stirring. The next day samples were centrifuged and EtOH
111 was collected in a separate tube, 20 ml of EtOH was added to the pellet for a 2nd extraction.
112 After 2 h of extraction, the sample was centrifuged and both extracts were pooled.

113 **Extraction with hexane:** Total lipids were extracted using 20 ml of hexane. Samples were
114 left to extract overnight under constant stirring. The next day, samples were centrifuged and
115 hexane was collected in a separate tube. 20 ml of hexane was added to the sample for a 2nd
116 extraction. After 2h of extraction, the sample was centrifuged and both extracts were pooled.

117 **Extraction with EtOH and purification with hexane:** After a crude lipid extraction with
118 EtOH, a purification step was applied using hexane in a modified version of Fajardo, Cerdan,
119 Medina, Fernandez, Moreno, and Grima (2007). By addition of water and hexane to the crude
120 EtOH lipid extract, a biphasic system was formed. Hexane was used to extract lipids while

impurities remain in the aqueous ethanoic phase. Total lipids were extracted with EtOH as previously described. Then, 24 ml of H₂O was added with 13 ml of hexane. Samples were vortexed and centrifuged. The hexane phase was collected in a separate tube. 15 ml of hexane was added to the aqueous ethanoic remaining phase for a 2nd extraction, vortexed and centrifuged, and both hexane extracts were pooled.

After extraction and evaporation using a rotavapor, all lipid samples were resuspended in 8 ml of the respecting extraction solvent. 6 ml were collected in pre-weighed 15 ml Pyrex tubes and evaporated under nitrogen flow before gravimetrical determination of the total lipid extract. The remaining 2 ml of extract was evaporated under nitrogen flow and prepared for analysis of fatty acid contents (TFA) and composition by gas-chromatography coupled with flame-ionization-detector (GC-FID). Purity of the lipid extract in terms of percentage of fatty acids was calculated using the results of the gravimetrical determination of the total lipid extract and FA analysis by GC-FID (for details refer to section Total fatty acid analysis).

Effect of storage temperature on fatty acids in seaweeds

Dried and ground powder (1 g) of *L. digitata* and *P. palmata* was stored in the dark in small sampling bags at room temperature (18-20 °C), in the fridge (4 °C), and in a freezer (-20 °C). Fatty acid contents and composition were analysed initially and then again after 1, 3 and 22 months of storage using the direct transmethylation protocol described in Schmid, Guihéneuf, and Stengel (2014). Samples were analysed in three methodological replicates (n=3).

Total fatty acid analysis

The extraction yield of each extraction solvent was calculated by comparison with the results from direct transmethylation of dried seaweed biomass. For direct transmethylation the method described in Schmid, Guihéneuf, and Stengel (2014) was used on 50 mg freeze-dried

and ground biomass. For quantification of TFA in the different extracts the previously collected fraction was used. For TFA analysis, 20 μ l of 15:0 (5 mg ml⁻¹, Pentadecanoic acid, 98%, Alfa Aesar, Heysham, LA3 2XY, UK) was added before transmethylation to the fine seaweed powder or the lipid extract. Fatty acid methyl esters (FAME) were obtained by the addition of 2 ml of dry MeOH containing 2% (v/v) of H₂SO₄. Samples were closed under nitrogen and heated for 1.5 h at 80 °C. The transmethylation reaction was stopped by the addition of 1 ml of H₂O. After cooling of the sample to room temperature, 2 ml of hexane was added to extract the FAME. All chemicals used were analytical grade (Fischer Scientific, Leicestershire, LE11 5RG, UK).

FAME samples were analysed using an Agilent 7890A GC/5975C MSD Series (Agilent Technologies, Santa Clara, CA, USA) with a fused silica capillary column (DB-WAXetr, 0.25 mm $\times$ 30 m $\times$ 0.25 mm, Agilent) and a flame-ionization-detector (FID). GC-FID conditions were identical to those used by Schmid, Guihéneuf, and Stengel (2014).

Commercially available FAME standards (SupelcoTM 37 Component FAME Mix, Supelco, Bellefonte, PA, USA) and FAME of fish oil (Menhaden Oil, Supelco, Bellefonte, PA, USA) were used for identification of fatty acids.

Results are expressed as the mean values of three replicates (n = 3). Agilent MSD Productivity ChemStation Software was used for instrument control, data acquisition and data analysis (integration, retention times and peak areas).

Statistical analysis

Data were analysed with IBM® SPSS® Statistics software version 21. Significant effects ($p < 0.05$) of extraction solvents on contents of TFA, SFA, MUFA and PUFA [% of DW] and [% of TFA] were determined using one-way analysis of variances (one-way-ANOVA). The

Tukey-test was performed to identify *a posteriori* homogeneous sub-groups that differed significantly (level of significance: $p < 0.05$). Data were $\log_{10}$ transformed prior to analysis to meet requirement of homogeneity of variances. For the results of SFA [% of DW] and [% of TFA] and MUFA [% of DW] in *L. digitata* and SFA[% of TFA] and MUFA[% of TFA] in *P. palmata* the requirements of homogeneity of variances for the one-way ANOVA were not met. In these cases the Welch-ANOVA was performed (level of significance: $p < 0.05$). The Games-Howell-test was applied to identify *a posteriori* homogeneous sub-groups that differed significantly (level of significance: $p < 0.05$).

A mixed ANOVA (level of significance: $p < 0.05$) was conducted to analyse the effects of storage temperature (between-subject factor) and time (within-subject factor) on the levels of TFA in the seaweed samples. Tukey-tests were performed to find *a posteriori* homogeneous sub-groups that differed significantly (level of significance: $p < 0.05$). Data were $\log_{10}$ transformed to meet requirements for homogeneity of variances prior to statistical analysis. Individual one-way repeated measures ANOVA were conducted for each storage temperature to see if total fatty acid levels of the biomass changed in comparison to levels in the initial biomass.

Results

Total fatty acid level of the different extracts

The level of TFA of the lipid extracts from *L. digitata* and *P. palmata* are displayed in Table 1 and 2, respectively. In the lipid extract from *L. digitata* biomass, the highest percentages of TFA (20-21%) in the lipid extract were achieved by extraction with CHCl_3 :MeOH and EtOH. The lipid extracts obtained using hexane and EtOH:hexane as solvents reached 10-11 % of TFA.

In *P. palmata* the highest percentage of TFA (21%) was obtained in the extract with CHCl₃:MeOH. The extraction using EtOH reached a TFA level of 19%, while the TFA level of the extract obtained using hexane was lower with 8% of TFA. The extraction with EtOH and subsequent purification with hexane only achieved 5% of TFA.

Fatty acid extraction yield and composition using different extraction solvents

The comparison of the extraction solvents revealed a strong variability in quantities of TFA extracted, as well as in the fatty acid composition of the extracts. The results for *L. digitata* and *P. palmata* are displayed in Figs. 1 and 2, respectively. The extraction efficiency in terms of yields of TFA (Table 1 and 2) for both species was in the following order: CHCl₃:MeOH > EtOH > hexane > EtOH:hexane. Using direct transmethylation of the grounded freeze-dried biomass, the fatty acid content was at 0.95% of DW for *L. digitata* and 1.76% of DW for *P. palmata*. The composition of *L. digitata* contained 28.4% of SFA, 17.2% of MUFA and 49.9% of PUFA. In *P. palmata* the TFA composition consisted of 32.6% of SFA, 3.7% of MUFA and 62.5% of PUFA. These results were used as a reference to calculate the fatty acid extraction yield and compare each lipid extraction solvent.

The extraction yield of the Bligh and Dyer (1959) method was at 77.03% in *L. digitata* and 56.32% for *P. palmata* (Tables 1 and 2). The main discrepancy was due to the significantly ($p<0.001$) lower concentration of extracted PUFA in both species (Fig. 1 and 2). According to direct transmethylation, 0.49% of DW of *L. digitata* was present as PUFA but the lipid extraction method with CHCl₃:MeOH only yielded a significantly lower ($p<0.01$) 0.31% of DW. In *P. palmata*, 1.10% of DW was as PUFA but the CHCl₃:MeOH extraction only yielded a lower ($p<0.001$) 0.46% of DW. In the fatty acid composition of the CHCl₃:MeOH extract of *L. digitata*, there was a higher proportion of SFA (40.57%) ($p<0.01$), and lower levels ($p<0.05$) of PUFA (42.71%). The fatty acid composition of the *P. palmata* extract

contained a higher proportion ($p<0.001$) of SFA (46.36%), and lower levels ($p<0.001$) of PUFA (46.39% of TFA).

Using EtOH, a total of 0.38% of DW of TFA were extracted from *L. digitata* and 0.46% of DW in *P. palmata* resulting in a yield of 40.46% and 25.80%, respectively. The levels of PUFA in percentage of TFA (Table 1) in *L. digitata* were similar ($p=0.80$) to the one obtained from direct transmethylation with 33.09% of SFA, 17.64% of MUFA and 48.04% of PUFA. For *P. palmata* this solvent achieved a lower percentage ($p<0.001$) of PUFA in comparison to the direct transmethylation (Table 2), with an extract composition of 43.70% of SFA, 5.95% of MUFA and 47.91% of PUFA.

Extraction with hexane from *L. digitata* biomass yielded 30.06% of TFA in comparison to direct transmethylation, lower than the EtOH extract. The extract contained a higher proportion ($p<0.05$) of SFA (43.51%), percentages of MUFA was similar to direct transmethylation and EtOH extraction with 16.06 %, and lower levels ($p<0.01$) of PUFA was observed with 39.37% of TFA. For *P. palmata* the extraction with hexane yielded only 9.26% of TFA in comparison to direct transmethylation. The hexane extract from *P. palmata* contained a higher proportion ($p<0.001$) of SFA with 51.16% of TFA, while the proportion of MUFA accounted for 7.74% and PUFA level was lower ($p<0.001$) with 40.85%.

The extraction with EtOH:hexane resulted in the lowest yield in both species. For *L. digitata* the extraction with EtOH:hexane only yielded 11.57% of extraction achieved from direct transmethylation. The TFA were composed of 47.57% of SFA, 15.66% of MUFA and 32.93% of PUFA. In *P. palmata* the extraction yield was even lower, with only around 5.66% of the TFA extracted by direct transmethylation. The TFA composition of the extract contained 56.02% of SFA, 7.89% of MUFA and the lowest levels ($p<0.001$) of PUFA of all extracts (27.34% of TFA).

Effect of storage temperature on fatty acids

The TFA contents and composition of the biomass after storage under different temperatures and times of *L. digitata* are displayed in Table 3. Mixed-design ANOVA revealed significant within-subject effects of storage time ($F(2,12)=74.5, p<0.001$) and interactive effects of storage time x storage temperature ($F(4,12)=63.3, p<0.001$). Between-subject analysis revealed a significant main effect of storage temperature ($F(1, 6)=43.3, p<0.001$) on TFA levels in the samples, and Tukey test indicated that samples stored at room temperature differed significantly from those stored in a fridge ($p<0.01$) and freezer ($p<0.001$). There was no significant difference between storage in a fridge or freezer ($p=0.234$) for *L. digitata*. The individual repeated measures ANOVA revealed that, storage in the fridge and the freezer, had no impact on TFA ($F(3, 6)=0.1, p=0.977$) levels. TFA content was constant around 1% DW, with a composition of 29% of SFA, 17% of MUFA and 51% of PUFA. Similarly, in the samples stored at 4 °C, there was no significant ($F(3, 6)=3.3, p=0.102$) decrease in TFA observed after 3 and 22 months, with around 0.96% of DW after 22 months of storage. By contrast, the samples stored at room temperature exhibited a significant decrease ($F(3, 6)=87.1, p<0.001$) in TFA which was accompanied by changes in fatty acid composition. After 3 months, the TFA content had decreased by 20% and was at 0.83% of DW; after 22 months the TFA content was just over half of the initial value with 0.56% of DW. PUFA levels after 3 months were at 41.72% of TFA. After 22 months the proportion of PUFA only accounted for 18.36% of the TFA. The levels of EPA and ARA were low in the sample stored at room temperature, with 3.19% and 2.69% of TFA respectively.

The TFA contents and composition of the stored samples of *P. palmata* are displayed in Table 4. The mixed-design ANOVA showed an effect of temperature ($F(2, 12)=635.0, p<0.001$) and interactive effects of temperature x storage time ($F(4,12)=200.3, p<0.001$) on TFA levels. Between-samples analysis also revealed a significant main effect of temperature

on TFA levels in the stored samples ($F(1, 6)=5734.1, p<0.001$). The Tukey test revealed that there was a significant difference between samples stored at room temperature in comparison to fridge ($p<0.001$) and freezer ($p<0.001$). There was also a significant difference between samples stored in the fridge and at room temperature ($p<0.001$). Individual repeated measures ANOVA was applied to determine whether TFA levels changed at the different storage temperatures; TFA levels in samples stored at $-20\text{ }^{\circ}\text{C}$ were stable ($F(3, 6)=0.544, p=0.670$) at 1.8% of DW. The fatty acid composition of the biomass remained unchanged, with 32% of SFA, 4% of MUFA and 62% of PUFA. In biomass stored at $4\text{ }^{\circ}\text{C}$, there was a significant ($F(3, 6)=634.9, p<0.001$) decrease after 3 months (1.69% of DW). After 22 months a significant deviation from the initial TFA content was observed, with only 1.15% of DW. The percentage of PUFA was lower with 41.91% mainly due to a lower level of EPA with 33.08% of TFA. TFA in samples stored at room temperature changed over time ($F(3, 6)=625.4, p<0.001$): after 1 month, a decrease in TFA, and lower levels of PUFA were observed. Levels of EPA were at 46.43% of TFA, in comparison to 51.18% in the control (initial) samples. After 3 months, TFA levels were about 15% lower containing only 1.52% of DW. After 22 months, TFA levels were only about half of those detected in the initial biomass. The fatty acid composition consisted of 64.84% of SFA, 6.37% of MUFA and only 22.27% of PUFA after 22 months of storage. Levels of EPA were only at 11.26% of TFA, representing approximately 1/5 of the initial value.

Discussion

Impact of various extraction solvents on seaweed lipid extracts

Extraction yield, TFA content and composition of the extracts obtained from the two seaweed species under investigation strongly depended on the extraction solvent. The highest extraction yield was achieved by the method described by Bligh and Dyer (1959) which is commonly used for the extraction of lipids from different organisms, though initially

291 developed for the extraction of phospholipids from fish muscle. The method uses a polar
292 (MeOH) and non-polar (CHCl₃) solvent mixture. Typically, lipids in seaweeds are present in
293 forms of neutral or polar lipids (Guschina & Harwood, 2009); neutral lipids function as
294 storage compounds and are predominantly present as triacylglycerols (TAG), composed of
295 three fatty acyl chains, which are esterified to a glycerol backbone. On the other hand, polar
296 lipids play an important role as structural components of the cellular membranes and are
297 mainly present as phospho- and glycolipids (Gurr, Harwood, & Frayn, 2002; Ohlrogge &
298 Browse, 1995). A polar solvent, e.g. MeOH, can thus more easily penetrate the cell wall and
299 then extract the polar lipid fraction. In addition, this can support an enhanced extraction of
300 neutral lipids by CHCl₃ resulting in an overall good extraction yield of the method (Li,
301 Naghdi, Garg, Adarme-Vega, Thurecht, Ghafor, et al., 2014).

302 In comparison to the direct transmethylation, a loss in TFA, and particularly of PUFA, was
303 observed in all other lipid extraction solvents. All extraction methods were conducted in a
304 simple two-step extraction in order to keep solvent usage low and conduct the study with
305 greatest economical effectiveness. Longer extraction times, higher solvent to biomass ratios
306 and multiple repeated extractions are all factors that can improve lipid yield (Fajardo, Cerdan,
307 Medina, Fernandez, Moreno, & Grima, 2007; Suganya & Renganathan, 2012). Also, in
308 comparison to the direct transmethylation, larger amount of dried seaweed biomass was used
309 for the lipid extraction techniques, but the ratio of solvent to biomass was similar to that in
310 commonly used extraction techniques (Yang, Xiang, Sun, Wu, Li, & Long, 2014).

311 Ryckebosch, Muylaert, and Foubert (2012) have shown that the application of the Bligh and
312 Dyer (1959) method can lead to an underestimation of the total lipid content in microalgae
313 with high percentages of lipids of more than 10% of DW, which are much higher than the
314 levels typically present in seaweeds. Similarly, Lewis, Nichols, and McMeekin (2000)
315 highlighted that the total lipid extraction and subsequent determination of TFA lead to an

underestimation in comparison to direct transesterification method. This should be considered when the Bligh and Dyer (1959) method is applied solely for the evaluation of TFA contents and composition in seaweed; direct transmethylation or transesterification methods should be considered instead.

All lipid extraction methods involved longer processing times and more analytical steps which could potentially have lead to lipid oxidation causing lower levels of PUFA in the lipid extracts. The addition of anti-oxidative compounds thus has been suggested as a preventive measure for PUFA oxidation (Christie, 1982).

Of the food grade solvents, extraction with EtOH achieved the best results in *L. digitata* and *P. palmata* regarding total lipid yield and TFA content. The efficiency of the solvents strongly depends on the partitioning of lipids into polar and neutral lipids (Li, et al., 2014).

The polar solvent EtOH extracted more fatty acids than the non-polar solvent hexane; this could indicate a higher amounts of fatty acids partitioned into polar lipids in both species but further data on lipid class composition would be needed to confirm this. Previous studies on *P. palmata* and some kelp species (*L. bongardiana*, *L. yezoensis*, *L. longipes*) suggest that a large proportion of their fatty acids is partitioned into polar lipids, particularly in *P. palmata* (Gerasimenko, Martyyas, & Busarova, 2012; Mishra, Temelli, Oraikul, Shacklock, & Craigie, 1993).

Extraction with EtOH yielded the highest levels of PUFA. In Yang, Xiang, Sun, Wu, Li, and Long (2014), an extraction with EtOH achieved results similar to an extraction with CHCl₃:MeOH. This may be explained by the bio-characteristics of the algae with easily broken cell walls, which allowed EtOH to enter the cells and extract high levels of lipids. The modified Bligh and Dyer (1959) and the EtOH methods achieved the highest contents of PUFA in terms of proportion of fatty acids in *L. digitata*, with slightly lower levels of PUFA for the EtOH extract in *P. palmata*. The percentage of fatty acids in the EtOH extract was at

341 around 20%, similar to that in the CHCl₃:MeOH extract. The remaining 80% of a lipid
342 extract may be made up of the glycerol backbone and the polar heads of the lipids and other
343 potentially soluble compounds such as pigments, proteins binding to lipids and
344 carbohydrates (Ohlrogge & Browse, 1995).

345 An extraction with the non-polar solvent hexane achieved a better TFA extraction from *L.*
346 *digitata* in comparison to *P. palmata*. Fleurence, Gutbier, Mabeau, and Leray (1994);
347 Gerasimenko, Martyyas, and Busarova (2012) have shown that Laminariales can have 30-
348 60% of the total pool of lipids partitioned into neutral lipids (Fleurence, Gutbier, Mabeau, &
349 Leray, 1994; Gerasimenko, Martyyas, & Busarova, 2012), higher than in *P. palmata* (Mishra,
350 Temelli, Ooraikul, Shacklock, & Craigie, 1993). A higher level of neutral lipids could have
351 potentially promoted the more effective extraction performance of hexane in *L. digitata* in
352 comparison to *P. palmata*; but an improved understanding of lipid class composition of the
353 samples are required to support this conclusion. Ambrozova, Misurcova, Vicha, Machu,
354 Samek, Baron, et al. (2014) reached higher lipid yields by hexane extraction in different
355 seaweeds, including *P. palmata*, but only with a much higher solvent biomass ratio and a
356 biomass containing low levels of EPA.

357 The extraction using EtOH:hexane modified from Fajardo, Cerdan, Medina, Fernandez,
358 Moreno, and Grima (2007) uses EtOH and then, in a second step, hexane to extract lipids,
359 while most of the impurities remain in the hydro-alcoholic phase. Hexane favours neutral
360 lipids which are usually less rich in PUFA; this could explain lower levels of PUFA in these
361 extracts in comparison to the crude EtOH extracts. The method achieved a yield of 80% in
362 the diatom *Phaeodactylum tricornutum* (Fajardo, Cerdan, Medina, Fernandez, Moreno, &
363 Grima, 2007), but produced only low yields and did not improve the level of TFA of the
364 extracts for the two macroalgae investigated in this study. The high quantities of TAG

accumulated by *P. tricornutum* may be responsible for the more effective extraction (Alonso, Belarbi, Fernandez-Sevilla, Rodriguez-Ruiz, & Grima, 2000).

Implications of solvent choice for potential applications

The partitioning of algal fatty acids into different lipid classes also affects their bioavailability for humans (Michalski, Genot, Gayet, Lopez, Fine, Joffre, et al., 2013). According to solvent polarity, specific lipids from different lipid classes may be favoured during the extraction process; EtOH produces food grade extracts rich in polar lipids and PUFA (Li, et al., 2014), hexane food grade extracts rich in neutral lipids (Ambrozova, et al., 2014), and EtOH:hexane food grade extracts enriched in TAG with fewer impurities and less polar lipids (Fajardo, Cerdan, Medina, Fernandez, Moreno, & Grima, 2007). For the absorption in the human body and their biological function, the structural difference of lipids is considered very important, as reviewed by Michalski, et al. (2013). Depending on application, the intake of PUFA in polar form or TAG may be more favourable. For example, for the reduction of glucose in hypercholesterolemic patients, the consumption of polar rich krill oil was more effective than TAG-rich fish oil (Bunea, El Farrah, & Deutsch, 2004). In contrary, the intake of DHA (20:6 n-3) by pigs was more efficient in form of TAG from unicellular algae than in the form of polar lipids (Mathews, Oliver, Phillips, Odle, Diersen-Schade, & Harrell, 2002).

Effects of storage temperature on TFA content and composition in seaweeds

Degradation of lipids by oxidation is a major concern during storage of dried foods. Oxidative deterioration can lead to toxic and deteriorative processes and the decline of vitamins, chlorophylls, carotenes, amino acids and proteins, or enzymes and the development of off-flavour in foods (Angelo & Ory, 1975; Gardner, 1979). Due to their chemical

properties, lipids and fatty acids are prone to oxidation by reaction with atmospheric singlet or triplet oxygen. Oxidation of lipids can take place by different mechanisms, i.e. autoxidation, photosensitized or enzymatic oxidation (Choe & Min, 2006). The process of autoxidation is a complicated free radical chain reaction involving initiation, propagation and termination steps. In the initiation step a spontaneous abstraction of hydrogen of the fatty acid by atmospheric oxygen takes place. The hydrogen atom attached to carbon atoms between two double bonds has low dissociation energy and can easily be removed to form fatty acid radicals. This makes particularly lipids with high levels of PUFA susceptible to oxidation (Choe & Min, 2006).

Temperature has been identified as an important factor influencing the degree of lipid oxidation in different types of oils and nuts (Lin, Wu, Zhu, Chen, Huang, Li, et al., 2012; Maskan & Karatas, 1999). The present study has demonstrated, for both algal species, that only biomass ground and stored at -20 °C was stable over 22 months, and storage for extended periods of seaweed biomass can only be recommended under these conditions. The initiation step with spontaneous abstraction of hydrogen atoms from the lipids is an endothermic reaction that requires considerable amounts of activation energy (Choe & Min, 2006), it appeared that the low temperatures (-20 °C) sufficiently protected the fatty acids. Ground biomass of *L. digitata*, but not of *P. palmata*, was stable at 4 °C. The fatty acid composition of the both species differs strongly with the high levels of EPA in *P. palmata* and a more balanced composition with a variety of different PUFA (EPA, ARA (arachidonic acid, 20:4 n-6) and SDA (stearidonic acid, 18:4 n-3)) in *L. digitata*. Nazemroaya, Sahari, and Rezaei (2009) have demonstrated the critical importance of fatty acid composition for lipid oxidation in stored fish, with species with a higher level of PUFA exhibiting a stronger degree of degradation. During the experimental exposure of several months, the biomass

initially containing higher levels of PUFA showed lower level of PUFA after six months of storage (Nazemroaya, Sahari, & Rezaei, 2009).

The presence of compounds with anti-oxidative properties can reduce the level of lipid oxidation in foods (Choe & Min, 2009). Antioxidants act as a hydrogen donor and slow down the process of oxidation by scavenging of free radicals. Both algal species studied here contain a number of compounds with anti-oxidative properties (Connan, Delisle, Deslandes, & Gall, 2006; Yuan, Bone, & Carrington, 2005). Differential antioxidant levels and composition may have influenced the differences in lipid oxidation in the two seaweeds analysed at 4 °C. Also, it has previously been shown that antioxidants can be affected by storage. For example, in dried laver (*Porphyra* sp., Rhodophyta) levels of antioxidants decreased significantly after storage for 14 days under dark conditions (Choe & Oh, 2013).

In the present study, both species displayed a similar degree of degradation at room temperature. Hence the storage of seaweed biomass, especially if ground, at room temperature, even for short periods of time should be avoided due to the strong impact on the levels of PUFA and TFA. Throughout the study the samples were kept in darkness in a dried state; both conditions are factors that can further promote lipid oxidation. For example the exposure to light energy can support the abstraction of hydrogen from lipids (Oh, Lee, & Choe, 2014). An additional factor to consider when storing seaweed biomass for high value applications is moisture as higher moisture content can also accelerate chemical reactions like lipid oxidation (Choe & Oh, 2013; Rockland & Nishi, 1980). In the present study, samples were freeze-dried and stored in small plastic bags to avoid hydration over the duration of storage.

Conclusions

The results of this study have demonstrated a strong influence of solvents on lipid extraction yield, as well as the composition, of the resultant lipid extract. For both seaweed species examined here, EtOH achieved an extract rich in PUFA using food grade solvents. Other extraction techniques, longer extraction times and multiple consecutive extractions may further enhance the yield of the food grade solvents but cost and time effectiveness need to be considered. To gain a more accurate overview of the fatty acid composition in seaweeds as well as to allow comparability of different studies, a method involving direct transmethylation should be considered.

Storage temperature and duration can strongly influence the fatty acid profile and content of seaweeds, particularly for ground biomass conserved as powder. When frozen at -20°C, fatty acid content and composition of the seaweeds biomass was stable while higher temperatures led to degradation of PUFA in both species. Storage at room temperature cannot be recommended for scientific investigation or commercial application due to the strong degradation of PUFA.

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Table 1 Fatty acid composition of the lipid extracts obtained with different extraction solvents from *L. digitata* (Phaeophyceae)

<i>Laminaria digitata</i>	Extraction method				
Fatty acids [% of total fatty acids]	Direct transmethylation	Chloroform/ methanol	Ethanol	Hexane	Ethanol/Hexane
12:0	n.d.	n.d.	n.d.	0.92±0.26	n.d.
14:0	7.92±0.34	7.83±1.18	8.73±0.28	7.41±0.33	7.29±0.82
16:0	19.17±0.55	29.88±0.47	22.81±0.57	29.49±1.98	35.44±4.43
18:0	1.30±0.65	2.85±0.30	1.56±0.27	5.69±1.19	4.85±0.88
Sum SFA	28.40±1.22	40.57±1.40	33.09±0.74	43.51±2.75	47.57±4.50
16:1	4.58±1.40	5.34±0.20	4.77±0.55	5.59±0.89	5.45±0.55
18:1	12.62±0.38	8.97±5.25	12.86±0.39	10.47±0.86	10.22±1.01
Sum MUFA	17.19±1.11	14.31±5.05	17.63±0.18	16.06±1.33	15.66±1.37
18:2 n-6	4.80±0.48	4.98±0.29	5.99±0.10	5.58±0.49	6.35±1.00
18:3 n-6	7.10±0.17	6.08±0.27	6.24±0.26	4.84±0.51	4.63±0.74
18:4 n-3	12.85±1.03	11.82±0.92	14.45±0.29	6.72±1.57	5.76±0.23
20:2 n-3	n.d.	n.d.	n.d.	n.d.	n.d.
20:3 n-6	0.66±0.17	n.d.	n.d.	n.d.	n.d.
20:4 n-6	10.19±0.87	8.11±1.12	6.67±0.46	9.08±1.11	5.08±0.66
20:4 n-3	0.64±0.15	n.d.	n.d.	n.d.	n.d.
20:5 n-3	13.69±0.45	11.72±1.72	14.68±0.13	13.15±2.15	11.13±1.69
22:6 n-3	n.d.	n.d.	n.d.	n.d.	n.d.
Sum PUFA	49.93±3.06	42.71±4.10	48.04±0.88	39.37±2.36	32.93±4.22
Sum n-3	27.18±1.61	23.54±2.49	29.13±0.16	19.87±0.95	16.88±1.85
Sum n-6	22.75±1.60	19.17±1.64	18.90±0.81	19.50±1.43	16.05±2.38
Ratio n-3/n-6	1.20±0.05	1.23±0.04	1.54±0.06	1.02±0.03	1.06±0.05
Other	4.48±1.42	2.42±0.12	1.25±0.08	1.06±0.03	3.83±0.54
Total FA in % of DW	1.03±0.08	0.73±0.04	0.38±0.02	0.29±0.02	0.11±0.01
Yield of FA in % of direct transmeth.	-	77.03±1.81	40.46±4.47	30.06±0.06	11.57±0.25
Crude lipid extract in % DW	-	3.52±0.28	1.98±0.41	2.77±0.65	1.33±0.56
% of FA in crude lipid extract	-	20.97±2.61	20.07±4.80	10.75±2.87	9.54±4.52

Data of individual fatty acids are given as percentage of total FAME; total fatty acids are given in % of DW. Data are presented as mean of three replicate samples (=3) ± SD.

Table 2 Fatty acid composition of the lipid extracts obtained with different extraction solvents from *P. palmata* (Rhodophyta)

<i>Palmaria palmata</i>	Extraction method				
Fatty acids [% of total fatty acids]	Direct transmethylation	Chloroform/ methanol	Ethanol	Hexane	Ethanol/Hexane
12:0	0.42±0.05	0.22±0.04	0.69±0.12	1.38±0.07	0.90±0.40
14:0	7.79±0.34	9.67±0.20	8.72±0.26	8.24±0.29	5.46±1.39
16:0	23.71±0.83	34.45±0.43	32.49±0.88	35.93±0.41	42.32±1.05
18:0	0.64±0.56	2.02±0.09	1.81±0.35	5.60±0.73	7.34±1.11
Sum SFA	32.56±1.67	46.36±0.29	43.70±0.70	51.16±0.67	56.02±2.28
16:1	0.92±0.27	1.65±0.95	1.60±0.25	2.64±0.26	3.02±0.99
18:1	2.76±0.41	3.73±0.06	4.35±0.18	5.10±0.14	4.84±0.66
Sum MUFA	3.68±0.43	5.38±1.01	5.95±0.10	7.74±0.14	7.86±0.56
18:2 n-6	1.89±0.19	1.59±0.02	1.72±0.12	2.79±0.62	2.36±0.92
18:3 n-6	2.35±0.17	2.15±0.04	2.61±0.12	2.55±0.22	3.02±0.22
18:4 n-3	3.31±0.97	3.04±0.07	4.02±0.17	2.29±0.25	3.07±1.28
20:2 n-3	1.47±0.48	n.d.	0.83±0.72	n.d.	n.d.
20:3 n-6	n.d.	n.d.	n.d.	n.d.	n.d.
20:4 n-6	2.26±0.65	1.17±0.07	1.58±0.18	2.02±0.78	2.53±1.20
20:4 n-3	n.d.	n.d.	n.d.	n.d.	n.d.
20:5 n-3	51.18±2.10	37.71±1.52	36.42±0.81	30.15±0.55	16.36±0.86
22:6 n-3	n.d.	0.73±0.11	0.73±0.63	1.05±0.69	n.d.
Sum PUFA	62.47±1.96	46.39±1.33	47.91±0.88	40.85±0.69	27.34±2.60
Sum n-3	54.49±3.04	41.48±1.34	41.17±0.65	33.49±0.87	19.43±2.14
Sum n-6	7.97±1.16	4.90±0.01	6.74±0.55	7.36±0.41	7.91±0.50
Ratio n-3/n-6	6.98±1.47	8.46±0.30	6.13±0.53	4.56±0.33	2.45±0.15
Other	1.30±0.18	1.87±0.23	2.44±0.13	0.25±0.43	8.77±0.21
Total FA in % of DW	1.77±0.03	0.99±0.04	0.46±0.05	0.16±0.01	0.10±0.01
Yield of FA in % of direct transmeth.	-	56.32±2.99	25.8±3.38	9.26±0.54	5.66±0.44
Crude lipid extract in % of DW	-	4.64±0.16	2.45±0.40	2.19±0.18	2.08±0.28
% of FA in crude lipid extract	-	21.44±0.69	18.71±2.13	7.50±0.48	4.86±0.78

Data of individual fatty acids are given as percentage of total FAME; total fatty acids are given in % of DW. Data are presented as mean of three replicate samples (=3) ± SD.

466 **Table 3 Fatty acid contents and composition of ground seaweed biomass of *L. digitata* (Phaeophyceae) after storage at room**
467 **temperature, 4°C and -20°C, for 1, 3 and 22 months**
468

<i>L. digitata</i>	Initial	20°C			4°C			-20°C		
FA [% of TFA]	Storage [mo.]	1	3	22	1	3	22	1	3	22
12:0	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
14:0	7.92±0.34	8.43±0.95	9.88±0.34	15.17±0.97	7.13±0.64	8.56±0.81	8.21±0.19	8.09±0.34	8.04±0.31	8.02±0.79
16:0	19.17±0.55	18.07±1.14	24.23±0.16	35.14±0.06	16.36±0.56	19.73±0.80	18.71±0.15	19.57±0.47	19.47±0.03	19.42±0.73
18:0	1.30±0.65	1.69±0.29	1.22±0.07	2.52±0.32	tr.	0.99±0.17	1.52±0.30	1.33±0.70	1.32±0.03	1.32±0.11
Sum SFA	28.40±1.22	28.19±1.74	35.33±0.47	52.82±0.77	23.49±0.89	29.28±0.26	28.44±0.25	28.99±0.37	28.84±0.32	28.76±0.17
16:1	4.58±1.40	6.26±0.78	5.31±0.01	5.76±0.39	5.18±1.20	4.67±0.06	4.52±0.07	4.67±3.40	4.65±0.16	4.64±0.08
18:1	12.62±0.38	13.40±0.33	15.27±0.12	17.43±0.55	13.19±0.64	12.92±0.69	12.59±0.09	12.88±0.91	12.81±0.30	12.78±0.11
Sum MUFA	17.19±1.11	19.65±1.04	20.59±0.11	23.19±0.89	18.37±0.72	17.58±0.63	17.11±0.02	17.55±2.83	17.46±0.21	17.42±0.18
18:2 n-6	4.80±0.48	4.97±0.31	4.98±0.07	2.95±0.09	5.29±0.78	4.73±0.22	4.76±0.02	4.91±0.56	4.88±0.07	4.87±0.10
18:3 n-6	7.10±0.17	6.95±0.18	6.89±0.21	3.53±0.06	7.39±0.33	7.07±0.42	7.36±0.10	7.25±0.23	7.21±0.15	7.19±0.13
18:4 n-3	12.85±1.03	11.71±0.42	10.90±0.09	4.44±0.010	13.07±1.00	12.27±0.66	13.30±0.08	13.12±1.01	13.05±0.13	13.01±0.31
20:2 n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:3 n-6	0.66±0.17	0.66±0.04	0.44±0.02	1.10±0.20	0.79±0.13	0.83±0.05	0.69±0.02	0.68±0.11	0.67±0.03	0.67±0.01
20:4 n-6	10.19±0.87	10.66±0.51	8.07±0.33	2.69±0.10	12.44±0.52	11.04±0.53	10.32±0.07	10.40±0.87	10.35±0.31	10.32±0.23
20:4 n-3	0.64±0.15	0.57±0.04	0.38±0.04	0.46±0.08	0.62±0.37	0.75±0.06	0.66±0.02	0.65±0.06	0.65±0.02	0.65±0.07
20:5 n-3	13.69±0.45	12.82±0.41	10.05±0.38	3.19±0.2	16.21±0.42	14.23±0.63	14.22±0.16	13.98±1.07	13.91±0.14	13.87±0.06
22:6 n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sum PUFA	49.93±3.06	48.35±0.49	41.72±0.67	18.36±0.55	55.82±1.19	50.91±0.41	51.32±0.36	50.99±3.29	50.71±0.44	50.57±0.22
Sum n-3	27.18±1.61	25.11±0.43	21.34±0.27	8.10±0.21	29.91±1.64	27.24±0.21	28.19±0.27	27.75±1.85	27.60±0.04	27.53±0.41
Sum n-6	22.75±1.60	23.25±0.60	20.38±0.41	10.26±0.38	25.91±0.66	23.67±0.20	23.13±0.12	23.23±1.44	23.11±0.45	23.05±0.21
Ratio n-3/n-6	1.20±0.05	1.08±0.04	1.05±0.01	0.79±0.02	1.16±0.09	1.15±0.00	1.22±0.01	1.19±0.01	1.19±0.02	1.19±0.03
Other	4.48±1.42	3.80±0.59	2.37±1.10	5.62±1.97	2.32±1.41	2.22±0.51	3.14±0.30	2.47±0.92	3.00±0.26	3.25±0.22
Total FA	1.03±0.08	1.15±0.11	0.83±0.01	0.56±0.01	1.03±0.08	0.89±0.03	0.96±0.02	1.01±0.07	1.01±0.04	1.02±0.04

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470 **Data of individual fatty acids are given as percentage of total FAME; total fatty acids are given in % of DW. Data are presented as**
471 **mean of three replicate samples (=3) ± SD.**

Table 4 Fatty acid contents and composition of ground seaweed biomass of *P. palmata* (Rhodophyta) after storage at room temperature, 4°C and -20°C for 1,3 and 22 months

<i>P. palmata</i>	Initial	20°C			4°C			-20°C		
FA [% of TFA]	Storage [mo.]	1	3	22	1	3	22	1	3	22
12:0	0.42±0.05	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
14:0	7.79±0.34	8.89±0.33	10.50±0.49	16.70±0.16	7.93±0.29	8.74±0.35	12.26±0.09	8.05±0.28	8.06±0.43	7.92±0.10
16:0	23.71±0.83	25.46±1.23	26.56±0.89	46.55±0.44	25.69±0.57	23.24±1.33	33.87±0.30	22.96±1.13	22.99±1.16	22.59±0.22
18:0	0.64±0.56	0.50±0.06	0.92±0.13	1.59±0.05	0.62±0.29	1.01±0.32	1.24±0.02	0.86±0.06	0.77±0.07	0.76±0.06
Sum SFA	32.56±1.67	34.85±1.21	37.99±0.49	64.84±0.27	34.24±1.01	33.00±1.30	47.37±0.35	31.87±1.30	31.83±1.06	31.27±0.15
16:1	0.92±0.27	0.65±0.06	1.38±0.16	1.76±0.34	0.26±0.11	0.94±0.55	1.14±0.10	0.70±0.04	0.79±0.52	0.78±0.07
18:1	2.76±0.41	2.93±0.04	3.10±0.04	4.61±0.17	2.86±0.24	2.79±0.13	3.96±0.13	2.68±0.41	2.69±0.16	2.64±0.07
Sum MUFA	3.68±0.43	3.58±0.08	4.48±0.12	6.37±0.42	3.12±0.14	3.73±0.53	5.09±0.20	3.38±0.38	3.48±0.37	3.42±0.07
18:2 n-6	1.89±0.19	2.62±0.21	2.17±0.03	2.47±0.71	2.51±0.29	1.77±0.30	1.73±0.08	1.98±0.23	1.98±0.27	1.94±0.11
18:3 n-6	2.35±0.17	2.50±0.54	2.61±0.24	2.23±0.04	2.49±0.48	2.29±0.14	1.73±0.08	2.72±0.22	2.73±0.12	2.68±0.14
18:4 n-3	3.31±0.97	3.17±0.28	3.51±0.16	2.17±0.02	3.08±0.23	2.82±0.49	1.66±0.06	3.32±0.33	3.33±0.41	3.27±0.17
20:2 n-3	1.47±0.48	1.36±0.51	1.04±1.80	2.78±0.12	1.78±0.10	2.78±0.30	2.33±0.04	1.47±0.49	1.47±0.61	1.45±0.08
20:3 n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:4 n-6	2.26±0.65	2.06±0.50	2.11±0.17	1.36±0.01	1.88±0.43	2.08±0.23	1.38±0.08	2.30±0.17	2.3±0.14	2.26±0.12
20:4 n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:5 n-3	51.18±2.10	46.43±2.44	41.87±1.31	11.26±0.26	47.14±1.47	48.18±1.61	33.08±0.31	49.64±0.10	49.73±1.41	48.85±0.13
22:6 n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sum PUFA	62.47±1.96	58.14±1.93	53.95±0.55	22.27±0.58	58.90±1.88	60.41±0.72	41.91±0.06	61.43±0.76	62.02±0.77	60.45±0.32
Sum n-3	54.49±3.04	49.60±2.38	45.91±1.46	13.42±0.26	50.23±1.60	51.49±1.23	34.74±0.25	52.96±0.32	53.53±1.11	52.11±0.08
Sum n-6	7.97±1.16	8.53±0.72	8.04±1.79	8.85±0.54	8.67±0.28	8.92±0.70	7.17±0.20	8.47±0.50	8.49±0.34	8.33±0.28
Ratio n-3/n-6	6.98±1.47	5.85±0.74	5.90±1.32	1.52±0.10	5.79±0.02	5.80±0.56	4.85±0.17	6.27±0.34	6.32±0.39	6.26±0.21
Other	1.30±0.18	3.42±0.68	3.58±0.71	6.52±0.32	3.74±1.00	2.87±0.57	5.63±0.49	3.32±0.22	2.68±0.64	4.87±0.16
Total FA	1.77±0.03	1.71±0.07	1.52±0.04	0.88±0.01	1.73±0.02	1.69±0.06	1.15±0.02	1.83±0.02	1.82±0.05	1.86±0.05

Data of individual fatty acids are given as percentage of total FAME; total fatty acids are given in % of DW. Data are presented as mean of three replicate samples (=3) ± SD.

Fig 1

Comparison of detected TFA, SFA, MUFA and PUFA after extraction by different methods in comparison to direct transmethylation in biomass from *L. digitata*. A: direct transmethylation, B: CHCl₃:MeOH, C: EtOH, D: hexane, E: EtOH:hexane. Results are displayed as mean of three replicates (n=3) ± SD. Significant effects (p<0.05) of extraction solvents on contents of TFA and PUFA were determined using one-way analyses of variances (one-way-ANOVAs). The Tukey-test was performed to find *a posteriori* homogeneous sub-groups that differed significantly (level of significance: p< 0.05) which are indicated by different letters. For the results of SFA and MUFA the Welsh-ANOVA was performed (level of significance: p< 0.05). The Games-Howell-test was performed to find *a posteriori* homogeneous sub-groups that differed significantly (level of significance: p < 0.05) which are indicated by different letters.

Fig 2

Comparison of detected TFA, SFA, MUFA and PUFA after extraction by different methods in comparison to direct transmethylation in biomass from *P. palmaria*. A: direct transmethylation, B: CHCl₃:MeOH, C: EtOH, D: hexane, E: EtOH:hexane. Results are displayed as mean of three replicates (n=3) ± SD. Significant effects (p<0.05) of extraction solvents on contents of TFA, SFA, MUFA and PUFA were determined using one-way analyses of variances (one-way-ANOVAs). The Tukey-test was performed to find *a posteriori* homogeneous sub-groups that differed significantly (level of significance: p< 0.05) which are indicated by different letters.

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609

Figure 1

Figure 2