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Dichloromethane as a Solvent for Lipid Extraction and Assessment of Lipid Classes and Fatty Acids from Samples of Different Natures

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The usefulness of the solvent mixture dichloromethane/methanol for lipid extraction and the determination of lipid classes and fatty acids in samples of different natures was conducted. Two different extraction methods were compared, one containing chloroform/methanol, another containing dichloromethane/methanol. Total lipid extraction showed some minor differences but no variation in the lipid classes. Regarding the fatty acid profile, in *Echium virescens* seeds, 17 major fatty acids could be identified and quantified, and all were equally extracted when either solvent system was employed. In *Echium acanthocarpum* hairy roots, 17 major fatty acids were quantified, showing some statistical differences for one cell line in favor of chloroform. The data obtained from the liquid nutrient medium were also comparable. The cod roe sample showed 31 major fatty acids, showing no statistical differences between the two solvent systems. Contrarily, the CH_2Cl_2 method was able to extract 31 main fatty acids found in European seabass dorsal muscle more efficiently than the $CHCl_3$ method. The results indicate that, for lipid extraction and fatty acid assessment, dichloromethane/methanol can readily replace the commonly employed chloroform/methanol, thus avoiding the major health, security, and regulatory problems associated with the use of chloroform.

KEYWORDS: Chloroform; cod roe; dichloromethane; *Echium*; fatty acid assessment; fish flesh; lipids; lipid classes

INTRODUCTION

Fatty acids (FAs), either ω-3 or ω-6 long chain highly unsaturated FAs (HUFAs), are renowned compounds due to their health-beneficial properties (*1*). They are known to prevent or relieve myocardial infarction (*2, 3*), participate in early visual and neurological development during pregnancy and early postnatal life (*4, 5*), and play a role in the control of some psychic disorders (*6, 7*) and prevention of metabolic syndrome (*8, 9*), just to name a few. The most important FAs participating in the prevention of these illnesses are mainly the ω-3 FAs, which are mainly supplied through fish or fish oil intake (*10*). Moreover, the ω-6 HUFA, arachidonic acid, is also an important FA acting in the regulation of eicosanoids (*1*). Since mammals have very limited capacity to de novo synthesize these compounds from the two essential fatty acids of plant origin, linoleic acid (18:2$n-6$, LA) and α-linolenic acid (18:3$n-3$, ALA), they must be taken through the diet. There exists therefore a large demand for ω-3 and ω-6 FAs for dietary and medicinal purposes (*11*).

Regarding lipid and fatty acid research, their extraction from either plant or animal sources uses traditional analytical methods. The procedure usually requires a relatively long time and often conventional solvents, normally chloroform ($CHCl_3$) (*12–14*). Other solvents such as hexane have also been employed but with lesser efficacy (*15*). The use of hot hexane extraction provides information on lipid content but hinders determination of lipid classes and FAs. Currently, there are many restrictions for $CHCl_3$ use, and in many cases the solvent is banned. It has been classified as a probable human carcinogenic, although exposure at normal background levels is unlikely to have any adverse health effects (*16*). Furthermore, with respect to European regulations, $CHCl_3$ should be banned in products and processes because of its negative impact on the environment and human health (*17*). Hence, the maximum allowable atmospheric concentration in the workplace is 2 ppm or 10 mg/m^3, and it should not be used in concentrations equal to or higher

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than 0.1% by weight in substances and preparations for human consumption or use. Therefore, a substitute solvent is in demand due to these new and stricter regulations. On the other hand, dichloromethane (CH_2Cl_2) has also been suspected of being toxic for humans and a carcinogen; nevertheless, the threshold limit in the workplace is 50 ppm or 177 mg/m^3, approximately 20−25 times higher than that of $CHCl_3$. Moreover, the use of CH_2Cl_2 involves fewer restrictions and drawbacks compared with $CHCl_3$ and concerning environmental issues is considered slightly greener. In addition, CH_2Cl_2 is not only less hazardous but also 15−18% cheaper than $CHCl_3$, which can also be considered in evaluation of research costs.

Conversely, lipid extraction has also been performed with more state-of-the-art techniques such as supercritical carbon dioxide extraction (*18, 19*), an alternative technology that does not use solvents. It is more rapid and less expensive to run, although it requires sophisticated and expensive equipment that is not readily available in most laboratories.

We compared data on the efficiency of these organic solvents for the extraction of lipids and the determination of FAs from various types of samples. The obtained data validate the use of CH_2Cl_2 as an appropriate extraction solvent for lipid and FA research.

MATERIALS AND METHODS

Animal and Plant Materials. Different plant and animal materials were employed in this study, comprising seeds of *Echium virescens* (Boraginaceae) collected at El Palmar (Buenavista del Norte, Tenerife, Spain) in June 2006, frozen roe of Atlantic cod (*Gadus morhua*) kept at −80 °C until analysis, dorsal muscle from 10 month old cage-cultured European seabass (*Dicentrarchus labrax*) also kept at −80 °C, and hairy roots from two cell lines of established *Echium acanthocarpum* hairy root cultures and liquid culture medium.

Induction and Establishment of *E. acanthocarpum* Hairy Root Cultures. Seeds of *E. acanthocarpum* donated by Jardín Botánico Viera y Clavijo (Gran Canaria, Spain) were surface sterilized by a brief immersion in 70% EtOH, followed by treatment with an aqueous solution of 5% (v/v) commercial bleach submerged for 25 min, and finally washed five times with sterile distilled water.

Subsequently, surface-sterilized seeds were allowed to in vitro germinate on solid B_5 medium (*20*), supplemented with 3% sucrose and 3−4 mg/L GA_3 (gibberelic acid) and solidified with 0.7% agar, with the pH adjusted to 6.0 prior to autoclaving, and cultured in the dark until the beginning of germination.

In vitro germinated plants approximately 50−60 days old were employed for guided infection with *Agrobacterium rhizogenes* strain LBA1334 harboring a pBIN19-*gus* intron plasmid by repeatedly stabbing the internodal stem areas with a fine needle containing bacteria. Treated plants were returned to the same culture vessel, containing the same medium as above but without GA_3 and incubated in light conditions, until hairy roots emerged. Hairy roots ca. 3−4 mm in length that developed after 25−30 days were excised from the stem and transferred to a liquid medium as above without GA_3 but containing the antibiotic cefotaxime (100 mg/L) for several subcultures. Finally, actively growing bacterium-free hairy roots were cut into small segments and cultured in Erlenmeyer flasks (250 mL) containing 30 mL of sterile liquid medium supplemented with 3% sucrose on an orbital shaker at 95 rpm in the dark at 25 ± 2 °C. For fatty acid extraction, 0.35 g (fresh weight) of hairy root fragments were subcultured in each flask and allowed to grow, and six replicate flasks were harvested at days 24−26.

Lipid Extraction, Transesterification, and Analysis of Fatty Acids. Freeze-dried cod roe and European seabass dorsal muscle as well as *E. virescens* seeds were separately powdered using a mortar and pestle with liquid nitrogen. After homogenization, the samples were extracted following two different extraction methods described below, with slight modifications of that reported (*12*). Regarding *E. acanthocarpum* cultures, after separation of the hairy roots from the liquid nutrient medium by vacuum filtration, the former were weighed and freeze-dried at −80 °C for 24 h using a lyophilizer (Christ Alpha 2-4, Osterode, Germany). The total dry weight was recorded, and two equal portions were taken and extracted using the following two extraction methods.

$CHCl_3$ Method. Total lipid of ca. 400 mg of dry matter of different samples was extracted by immersion in 10−12 mL of a mixture of $CHCl_3$/MeOH (2:1, v/v) contained in capped glass test tubes, performing occasional gentle hand agitation for 2 h. The samples were filtered and transferred to a new test tube to which 2.5 mL of an aqueous solution of KCl (0.88%, w/v) was added with strong agitation, followed by centrifugation at 1500 rpm (239*g*) at 4 °C for 5 min. The aqueous upper phase was discarded and the organic phase evaporated under a N_2 stream. The total lipid was weighed, and the samples were resuspended in $CHCl_3$/MeOH (2:1, v/v) with the antioxidant BHT (butylhydroxytoluene; 0.01%, w/v) to obtain a final concentration of 20 mg/mL. This generally used lipid extraction method also extracts other soluble matter; therefore, it may also contain pigments and other compounds.

CH_2Cl_2 Method. This method was the same as the $CHCl_3$ method; however, all extractions used CH_2Cl_2/MeOH (2:1, v/v) instead of $CHCl_3$/MeOH. Analogously, the final sample was resuspended in CH_2Cl_2/MeOH (2:1, v/v) with the antioxidant BHT (0.01%, w/v) to a concentration of 20 mg/mL.

In a parallel procedure, the liquid medium from the hairy root cultures was divided into two equal volumes (ca. 15−18 mL); one was extracted 3× with three equal volumes of a mixture of $CHCl_3$/MeOH (2:1, v/v) and the other sample 3× with three equal volumes of a mixture of CH_2Cl_2/MeOH (2:1, v/v). Due to the formation of an emulsion, the two phases formed were separated after centrifugation. Finally, the organic solvent was evaporated under a stream of N_2 and each sample resuspended in hexane to obtain a final concentration of 5 mg/mL.

Transesterification of Lipids. Total lipid extracts (2 mg) were subjected to acid-catalyzed transesterification by dissolving the sample in 1 mL of toluene (to ensure that the neutral lipids were completely dissolved) plus 2 mL of a mixture of MeOH/1% H_2SO_4 and incubated in a capped glass test tube for 16 h at 50 °C (*13*). Prior to transmethylation, heneicosaenoic acid (21:0) was added to the lipid extracts as an internal standard (50 μg).

Transesterification was followed by the addition of 2 mL of an aqueous solution of K_2CO_3 (2%, w/v) and 5 mL of hexane/diethyl ether (1:1), plus 0.01% BHT (w/v), with strong agitation. The mixture was centrifuged at 1500 rpm (239*g*) at 4 °C for 5 min. The upper phase was kept and the lower phase washed again with 5 mL of hexane/diethyl ether (1:1); the two upper phases were pooled together and evaporated under a stream of N_2. Finally, the resulting fatty acid methyl esters (FAMEs) were dissolved in 100 μL of hexane and stored in sealed glass vials at −20 °C until analysis.

Purification of FAMEs was conducted by preparative thin layer chromatography employing silica gel G-25 glass sheets (Macherey-Nagel, Germany), eluted with a solvent system composed of hexane/ethyl ether/acetic acid, 97.7% (90:10:1), and visualized using iodine. The FAMEs which ran close to the solvent front were scraped off the glass sheet and extracted with 10 mL of hexane/diethyl ether (1:1). Finally, the samples were dissolved in 0.5−1.0 mL of hexane and kept at −20 °C until analysis.

All chemicals and solvents were of analytical grade and supplied by Sigma-Aldrich (Madrid, Spain) and Merck (Darmstadt, Germany).

Gas Chromatography of FAMEs. Analysis and quantification of FAMEs were conducted by GC, employing a Shimadzu GC-14A apparatus (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (250 °C) and a Supelcowax 10 fused silica capillary column (30 m × 0.32 mm i.d.) (Supelco, St. Louis, MO) and helium as the carrier gas. Samples (0.6 μL) were injected into the system by an on-column autoinjector (Shimadzu AOC-17) at 50 °C. For separation of compounds, a temperature program comprising 10 min at 180 °C, followed by an increase of 2.5 °C/min to reach the final temperature of 215 °C, was employed.

Fatty acids were identified according to their t_R compared with standards of commercial FAs (linoleic acid methyl ester, methyl

Table 1. Amount of Lipids Present in Different Samples Extracted Using the $CHCl_3$ Method or CH_2Cl_2 Method[a]

sample	lipid amt (mg/g of DW)	
	$CHCl_3$ method	CH_2Cl_2 method
E. virescens (seeds)	109.870 ± 9.271	107.467 ± 9.036
E. acanthocarpum hairy roots HR E1.5	59.842 ± 17.146	39.387 ± 6.618*
E. acanthocarpum hairy roots HR E1.16	84.958 ± 22.555	51.560 ± 29.259*
liquid medium	342.667 ± 125.932	254.284 ± 38.203
Atlantic cod roe	73.349 ± 3.678	68.091 ± 6.432
dorsal muscle of European seabass	73.323 ± 14.257	110.466 ± 13.414*

[a] Each value is the mean ± standard deviation of six replicates expressed as milligrams per gram of DW, except liquid medium samples, which is expressed as milligrams per liter. When present, an asterisk indicates a statistical difference within rows between the two extraction methods ($P < 0.05$).

γ-linolenate, methyl oleate, stearidonic acid methyl ester, and heneicosanoid acid) (Sigma-Aldrich, Madrid, Spain) and a well-characterized fish oil mix. They were quantified relative to the amount of heneicosanoid acid (50 μg) added as an internal standard prior to transmethylation and compared with a calibration curve employing these standards.

Fractionation of Lipids into Classes. Lipid classes were separated as previously described (*21, 22*) by one-dimensional double-development HP-TLC using ethyl acetate/2-propanol/$CHCl_3$/MeOH/0.25% KCl (w/v) (25:25:25:10:9, by volume) as the polar solvent system and hexane/diethyl ether/glacial acetic acid (80:20:2, by volume) as the neutral solvent system.

Lipid classes were visualized after the samples were sprayed with copper acetate reagent [aqueous solution of 3% cupric acetate (w/v) with 8% orthophosphoric acid (v/v)] and then heated at 160 °C for 10 min, followed by calibrated scanning densitometry using a Shimadzu CS-9001 PC dual-wavelength flying spot scanner (*21*) (Shimadzu, Kyoto, Japan). A well-characterized densitogram from fish roe was used as a standard for the identification of lipid classes.

Statistical Analysis. The means ($n = 6$) and standard deviation of the means were calculated to assess the variation within replicates. Analyses of variance (ANOVA) and Student's *t* tests were conducted to evaluate differences between the means of the different extraction systems. Data presented as percentage values were transformed by an arcsine transformation to angular values prior to statistical analysis. All analyses were performed at a significance level of $P < 0.05$.

RESULTS

Samples of different natures, i.e., cod roe, cage-cultured European seabass dorsal muscle, *E. virescens* seeds, *E. acanthocarpum* hairy roots, and liquid nutrient medium from such cultures, were employed to evaluate the ability of CH_2Cl_2 vs $CHCl_3$ to efficiently extract lipids and for assessment of the resultant lipid classes and FAs.

Total lipids extracted from *E. virescens* seeds gave comparable results using either solvent mixture (**Table 1**). Regarding the FAs, the yield of 17 major FAs was determined (**Figure 1**). The most abundant compounds were α-linolenic acid (18:3*n*−3), followed by γ-linolenic acid (18:3*n*−6), linoleic acid (18:2*n*−6), and then oleic acid (18:1*n*−9), stearidonic acid (18:4*n*−3), and palmitic acid (16:0), irrespective of the extraction method employed. It can be seen that, for most of the FAs evaluated, the CH_2Cl_2 method extracted slightly larger amounts compared to the $CHCl_3$ method, although these differences virtually were not statistically significant ($P < 0.05$) (**Figure 1**).

The application of the two extraction methods was also tested with the novel *E. acanthocarpum* hairy root culture. This is the first account of a Boraginaceae species hairy root establishment and the first report of the induction and establishment of a hairy root for FA production. Two different hairy root cell lines were assessed (HR E1.5 and HR E1.16);in both cases larger amounts of total lipids were extracted when $CHCl_3$ was employed (**Table 1**). Concerning FAs, in the HR E1.5 cell line, the isolated amount of FAs tended to be larger when the CH_2Cl_2 method was employed; however, statistically higher yields were only observed for three FAs, 16:0, 18:1*n*−7, and 18:2*n*−6 ($P < 0.05$). For all other FAs, the results were comparable, with 18:2*n*−6 being the most abundant (**Figure 2**). On the other hand, for the HR E1.16 cell line, the $CHCl_3$ method seemed to give higher FA amounts, with statistical differences for 7 out of 17 FAs ($P < 0.05$). Again 18:2*n*−6 was the most abundant followed by 16:0 (**Figure 3**).

Analogously, the FA content of the liquid nutrient medium of HR E1.5 hairy roots was also evaluated by employing both solvent systems. When the mixture of liquid medium and extraction cocktail was separated, an emulsion formed in the separating funnel, and centrifugation of the lower organic phase

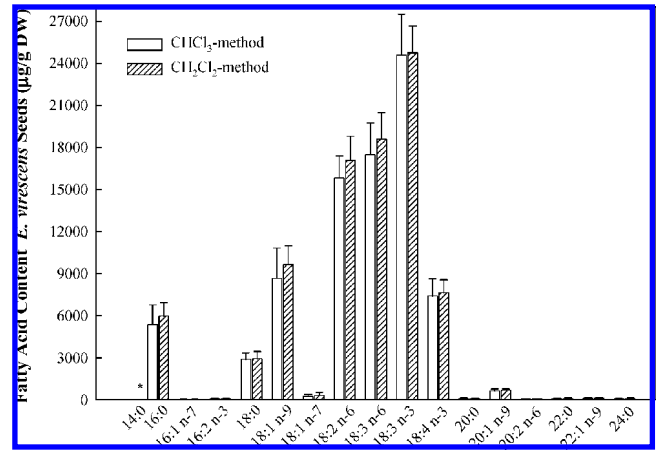

Figure 1. Amounts of different fatty acids obtained from *E. virescens* seeds employing either the $CHCl_3$ method or the CH_2Cl_2 method as the extraction solvent system. Each value is the mean ± standard deviation of six replicates expressed as micrograms per gram of DW. When present, an asterisk indicates a statistical difference for a particular fatty acid between the two extraction methods ($P < 0.05$).

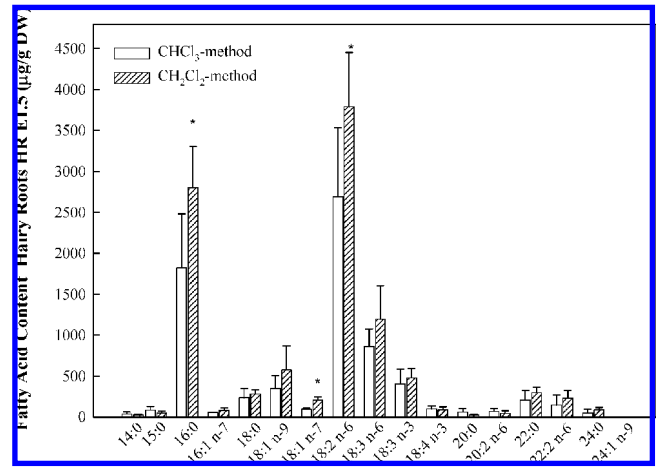

Figure 2. Amounts of different fatty acids obtained from HR E1.5 *E. acanthocarpum* hairy root cultures employing either the $CHCl_3$ method or the CH_2Cl_2 method as the extraction solvent system. Each value is the mean ± standard deviation of six replicates expressed as micrograms per gram of DW. When present, an asterisk indicates a statistical difference for a particular fatty acid between the two extraction methods ($P < 0.05$).

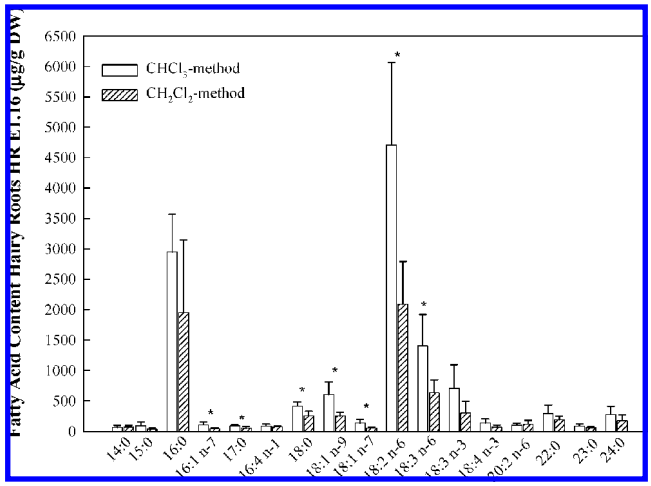

Figure 3. Amounts of different fatty acids obtained from HR E1.16 *E. acanthocarpum* hairy root cultures employing either the $CHCl_3$ method or the CH_2Cl_2 method as the extraction solvent system. Each value is the mean ± standard deviation of six replicates expressed as micrograms per gram of DW. When present, an asterisk indicates a statistical difference between the two extraction methods for a particular fatty acid ($P < 0.05$).

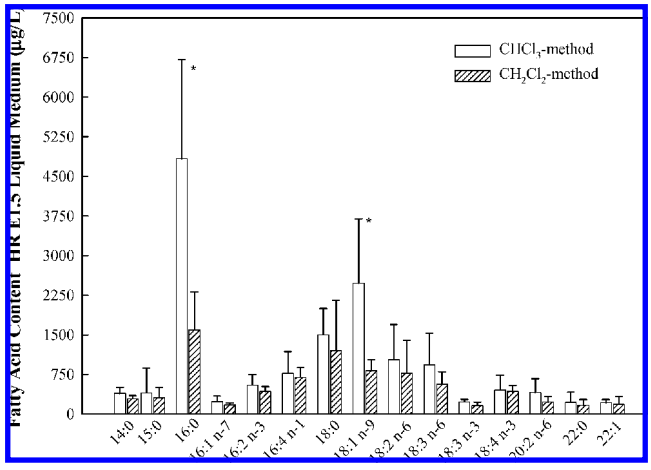

Figure 4. Amounts of different fatty acids obtained from the liquid nutrient medium of *E. acanthocarpum* hairy root cultures HR E1.5 employing either the $CHCl_3$ method or the CH_2Cl_2 method as the extraction solvent system. Each value is the mean ± standard deviation of six replicates expressed as micrograms per liter. When present, an asterisk indicates a statistical difference between the two extraction methods for a particular fatty acid ($P < 0.05$).

containing lipids and FAs was necessary to clearly separate it and harvest the compounds of interest. The data showed that there was no statistical difference between the amounts of total lipid (**Table 1**) and FAs extracted by either method except for 16:0 and 18:1$n-9$, the amounts of which were larger with the $CHCl_3$ method (**Figure 4**).

In a similar fashion, an animal sample, Atlantic cod roe, was also extracted employing both methods. The amounts of lipids extracted (**Table 1**) and the relative amounts of FAs (**Figure 5**) were similar with both methods, highlighting the utility of CH_2Cl_2 with this animal sample. Here, the most abundant fatty acid was DHA (22:6$n-3$), followed by palmitic acid (16:0), oleic acid (18:1$n-9$), and EPA (20:5$n-3$) (**Figure 5**).

Another animal sample, cage-cultured European seabass dorsal muscle, was also evaluated. The amount of lipid extract was statistically lower when $CHCl_3$ was employed (**Table 1**). Similar to Atlantic cod roe, the most abundant highly unsaturated

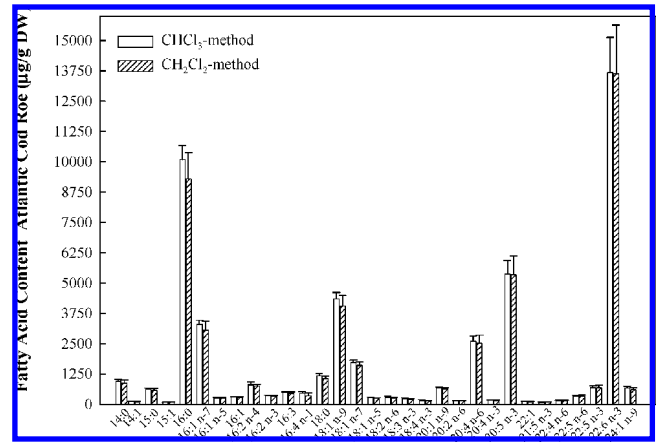

Figure 5. Amounts of different fatty acids obtained from Atlantic cod (*G. morhua*) roe employing either the $CHCl_3$ method or the CH_2Cl_2 method as the extraction solvent system. Each value is the mean ± standard deviation of six replicates expressed as micrograms per gram of DW. When present, an asterisk indicates a statistical difference for a particular fatty acid between the two extraction methods ($P < 0.05$).

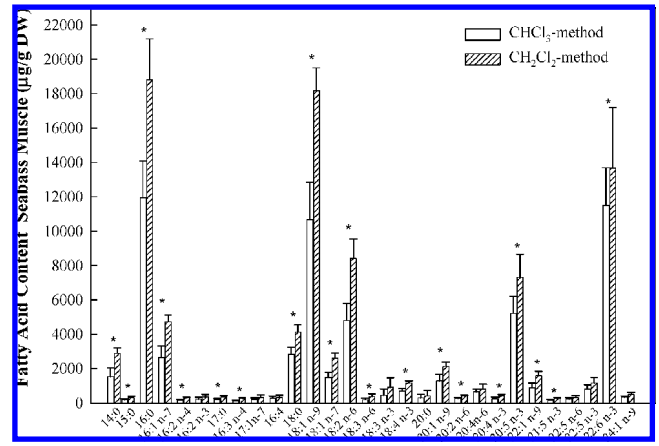

Figure 6. Amounts of different fatty acids obtained from the dorsal muscle of cage-cultured European seabass (*D. labrax*) employing either the $CHCl_3$ method or the CH_2Cl_2 method as the extraction solvent system. Each value is the mean ± standard deviation of six replicates expressed as micrograms per gram of DW. When present, an asterisk indicates a statistical difference for a particular fatty acid between the two extraction methods ($P < 0.05$).

fatty acids were DHA (22:6$n-3$) and EPA (20:5$n-3$), followed by minor amounts of ARA (20:4$n-6$). The most abundant saturated fatty acid was palmitate (16:0), followed by the other unsaturated FAs, e.g., oleic and linoleic acids (**Figure 6**). In this case, extraction using the CH_2Cl_2 method gave statistically higher FA yields compared to the $CHCl_3$ method.

Finally, the different lipid classes present in the samples were also qualitatively evaluated after extraction with the two different organic solvents (**Table 2**). As expected, each sample type showed a particular distribution of polar and neutral lipids. No statistical difference was observed between the two methods for any source. *E. virescens* seeds showed mainly neutral lipids, TAG, while although not all the lipids appearing on the TLC plate could be identified for the hairy root samples, the same number and sequence of the lipid's spots appeared (five polar and seven neutral) regardless of the extraction solvent mixture employed. Finally, the lipid class profiles of the two marine samples were also almost identical (**Table 2**).

Table 2. Main Lipid Classes Analyzed from Different Samples Extracted Using the $CHCl_3$ Method or CH_2Cl_2 Method[a]

sample	lipid[b]	concn (%)	
		$CHCl_3$ method	CH_2Cl_2 method
E. virescens seeds	CHO	9.670 ± 3.402	3.601 ± 0.695
	FFA	2.887 ± 0.073	3.581 ± 0.713
	TAG	75.464 ± 0.149	74.979 ± 0.622
	EST	5.312 ± 1.012	2.675 ± 0.842
	unknown	6.694 ± 2.612	15.163 ± 1.407
E. acanthocarpum hairy root HR E1.5	PC	7.141 ± 0.214	5.245 ± 1.592
	PS	1.969 ± 1.198	3.693 ± 1.101
	PI	2.610 ± 0.181	4.754 ± 0.886
	PE	7.141 ± 1.129	8.192 ± 0.491
	CHO	20.366 ± 6.105	22.023 ± 0.823
	EST	32.010 ± 5.558	19.885 ± 1.412
	unknown	33.050 ± 3.530	40.677 ± 4.513
E. acanthocarpum hairy root HR E1.16	PC	7.606 ± 1.844	8.828 ± 0.126
	PS	3.330 ± 1.445	3.441 ± 1.968
	PI	5.255 ± 2.250	6.958 ± 0.611
	PG	3.459 ± 1.036	1.445 ± 0.611
	PE	5.535 ± 0.854	7.564 ± 0.123
	CHO	17.923 ± 2.599	13.279 ± 0.491
	EST	28.868 ± 3.647	13.789 ± 4.188
	unknown	34.620 ± 13.524	52.744 ± 3.716
Atlantic cod roe	LPC/SM	4.789 ± 1.657	5.164 ± 1.710
	PC	12.479 ± 3.431	11.348 ± 1.178
	PS	0.674 ± 0.104	0.627 ± 0.030
	PI	1.372 ± 0.094	1.350 ± 0.070
	CL/PG	0.813 ± 0.025	0.636 ± 0.119
	PE	5.464 ± 0.216	5.067 ± 0.622
	CHO	15.112 ± 0.335	15.142 ± 0.360
	FFA	33.010 ± 2.484	33.911 ± 1.085
	TAG	10.898 ± 1.252	8.002 ± 3.469
	EST	8.904 ± 0.796	12.325 ± 3.523
	unknown	7.037 ± 1.656	6.325 ± 0.913
dorsal muscle of European seabass	LPC/SM	5.620 ± 1.288	4.387 ± 0.557
	PC	10.037 ± 0.851	8.526 ± 0.356
	PS	2.247 ± 0.140	1.819 ± 0.066
	PI	2.260 ± 0.143	2.015 ± 0.331
	CL/PG	0.432 ± 0.154	0.703 ± 0.151
	PE	5.798 ± 0.464	5.115 ± 0.111
	CHO	9.246 ± 1.215	8.471 ± 0.816
	FFA	11.245 ± 1.954	12.037 ± 0.764
	TAG	48.644 ± 4.740	53.480 ± 3.193
	EST	1.951 ± 0.427	3.038 ± 0.315
	unknown	4.151 ± 1.153	3.404 ± 0.877

[a] Percentage values were transformed by the arcsine transformation prior to statistical analysis. No statistical difference was observed between the two methods for any source. [b] CHO = cholesterol, CL/PG = cardiolipin/phosphatidylglycerol, EST = esters of waxes and cholesterol, FFA = free fatty acid; LPC/SM = lysophosphatidylcholine/sphingomyelin, PC = phosphatidylcholine, PE = phosphatidylethanolamine, PI = phosphatidylinositol, PS = phosphatidylserine, TAG = triacylglycerol.

DISCUSSION

The main objective of our study was to assess the extraction efficiency and if possible validate the use of the less hazardous and less toxic solvent CH_2Cl_2 for the extraction of total lipids and determination of lipid classes and FA profiles. The extraction method design has to take into account the nature of the material to be evaluated, which would normally display different chemical compositions; accordingly, for this study, samples of different natures were assessed, comparing the commonly used lipid extraction solvent $CHCl_3$ against CH_2Cl_2. Furthermore, it is generally agreed that $CHCl_3$/MeOH mixtures are optimal for extracting total lipids from different plant and animal tissues (*12, 13, 23, 24*), and in some instances, this chlorinated solvent has been substituted by CH_2Cl_2, hexane, etc., providing different outcomes when nonpolar or semipolar solvent extractions are compared (*25*). On the other hand, for sphingolipid analyses, $CHCl_3$ (based on ref *23*) has also been used, although the best extraction method for this type of lipid was the mixture of propan-2-ol/water/hexane, which also improves its solubility (*26*).

Here, a comparison of the profiles and amounts of FAs, lipid classes, and lipids obtained was made when using $CHCl_3$ ($CHCl_3$ method) or CH_2Cl_2 (CH_2Cl_2 method) as the extraction solvent. The variety of samples analyzed also allowed a comparison of the extraction performance of these solvent mixtures according to the nature of the sample. Although total lipid extraction may have been more effective for some samples if nonpolar solvents had been employed (such as hot hexane extraction), this would have however prevented us from assessing the resultant FA profiles or lipid classes, the main objective of our research.

Although the total lipid amount was larger for hairy roots extracted with $CHCl_3$, rather than CH_2Cl_2, the reverse was true for European seabass samples. There was no difference for the remaining samples (**Table 1**). Where observed, the difference may be due to the extraction of compounds of nonlipid nature such as pigments and other metabolites, known to occur when the $CHCl_3$/MeOH mixture is employed, mainly for the plant samples, or due to the possible differences in the natures of the samples. Thus, in hairy root plant samples, the lipid content was larger after extraction with the $CHCl_3$ method, which likely also extracted other compounds which accounted for the difference; this could also be due to minor differences in the polarity of the solvents. Moreover, both solvents show similar eluotropic values (solvent strength parameter), 0.40 for $CHCl_3$ and 0.42 for CH_2Cl_2 on silica gel, demonstrating their analogy. Furthermore, the resultant FA profiles with the various samples studied were generally comparable or higher in the seabass muscle sample when CH_2Cl_2 was employed, which further supports the use of CH_2Cl_2 for FA research (**Figures 1–6**).

In this study, the possible differences among the lipid classes obtained following extraction of the different samples using the two solvents were also investigated. It has been shown that for a given sample there was no statistical difference between the proportions of neutral or polar lipids tentatively quantified with either solvent employed (**Table 2**). Thus, as expected, *E. virescens* seeds showed mainly neutral lipids, TAG, since it is in this compartment where oils are commonly stored compared to other plant organs, with comparable results obtained between the two extraction methods. The lack of significant amounts of TAG in *E. acanthocarpum* hairy roots is also consistent with it being mainly a structural organ rich in phospholipids and sterol esters. The results were similar to those reported for oat roots (*27*). On the other hand, the lipid class profiles of the two marine samples were also consistent with data reported for these types of samples (*10*). The cultured fish sample possessed a large proportion of TAG, while the fish roe possessed more polar lipids. These results indicate that the use of CH_2Cl_2 in the extraction mixture generates the same results as compared with $CHCl_3$, and no variation in the lipid classes could be attributed to the use of either solvent.

Similarly, results on the lipid extraction efficiency and FA profiles from soil samples using CH_2Cl_2 or $CHCl_3$ have been reported (*28*). Furthermore, the comparison between CH_2Cl_2 and $CHCl_3$ for lipid studies (cholesterol, triacylglycerides, and phospholipid) from human serum and rat liver tissue was reported, obtaining virtually similar amounts of these lipids when either solvent was utilized (*29*). Similarly, the use of CH_2Cl_2 was also evaluated for tropane alkaloid extraction from plant samples, and it was reported that CH_2Cl_2 showed an extraction efficiency equal to that of $CHCl_3$ (*30*).

Regarding the FA profiles, in the plant samples, many of the results were similar, showing no statistical differences, except for a few FAs from *E. acanthocarpum* HR E1.5 and HR E1.16 hairy roots (**Figures 2** and **3**). For hairy root HR E1.5, CH_2Cl_2 was able to give a higher quantity of certain FAs, while $CHCl_3$ was more efficient for some FAs from HR E1.16 hairy roots. This could be attributed to the different phenotypes of the roots, HR E1.16 being larger with shorter branching and a thicker tissue, while those of HR E1.5 were shorter and very thin with more abundant branching. These samples could also possess different molecular components of nonlipid nature, which would facilitate or hinder the isolation of FAs with either solvent system. Furthermore, the larger variation within some samples, particularly in the root samples and liquid medium, could also be due to the fact that the volume of aqueous wash mixture was not optimized for the CH_2Cl_2-based solvent, since it is known that the volume of aqueous medium needed to produce a clean break in phases with the lipid lying in the halogenated phase is not the same for both solvents.

As expected, the FA profiles obtained from plant samples did not show any FAs of a carbon chain longer than 20 and with the maximum number of unsaturated carbon bonds of 4, e.g., 20:2*n*−6 and 18:4*n*−3, again in agreement with published data (*31*). Contrarily, the fish samples displayed longer carbon chain FAs, also with a larger number of unsaturated bonds, e.g., 24:1*n*−9 and 22:6*n*−3 (*10*). For all these compounds, and others of similar carbon chain length and number of unsaturated bonds, CH_2Cl_2 displayed a very efficient extraction capacity, very comparable to that of $CHCl_3$, suggesting that the degree of unsaturation or the length of the carbon chain, or both, does not affect the extraction efficiency (**Figures 5** and **6**). These results demonstrate that CH_2Cl_2 operates as a satisfactory extraction solvent independently of the nature of the samples. Moreover, with animal samples both solvents gave comparable results, and interestingly, the CH_2Cl_2 method offered larger FA yields for the European seabass muscle sample (**Figure 6**).

The presented data indicate and validate that CH_2Cl_2, a less hazardous solvent with fewer restrictions and drawbacks for its use, is an effective extraction solvent for FA research, irrespective of the type of sample to be analyzed. Therefore, CH_2Cl_2 can readily replace $CHCl_3$ in fatty acid studies.

ABBREVIATIONS USED

$CHCl_3$, chloroform; CH_2Cl_2, dichloromethane; DW, dry weight; FA, fatty acid; FAMEs, fatty acid methyl esters; HUFAs, highly unsaturated fatty acids.

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