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Selection of an esterification catalyst for assay of total fatty acid content in cyanobacteria and algae using gas chromatography

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Hydrochloric acid, acetyl chloride and boron trifluoride have been tested as the acid catalysts for direct esterification/transesterification and consequent gas chromatographic determination of the total fatty acid content in cyanobacteria and algae. Acetyl chloride resulted to be the most efficient and was applied for the analysis of freshwater cyanobacteria *Aphanizomenon flos-aquae*, *Planktothrix agardhii* and green algae *Monoraphidium griffithii, Scenedesmus acutus.* All tested species contained significant quantities of palmitic acid and α-linolenic acid. The oleic acid and linoleic acid occurred in the highest amounts in green algae *M. griffithii* and *S. acutus.*

Keywords: cyanobacteria, algae, fatty acids, direct transesterification

INTRODUCTION

Nowadays microalgae are increasingly investigated as possible sources of bioactive compounds that possess antioxidant, antiproliferative, antimutagenic, antidiabetic, anticoagulant, antibacterial and antitumor activities [1–4]. Fatty acids (FA) are valuable chemical compounds provided by the algae. Algal fatty acids can be an important source of food, feed or fuel, omega-3 long chain polyunsaturated fatty acids are considered as a promising alternative of fish oil [5, 6]. Thus, the accurate identification and quantification methods of fatty acids are needed.

Numerous methods are used to assay the lipid content in algae. Most of them use solvent extraction and consequent determination by weighting or chromatography [5, 7]. By weighting only the total lipid content can be evaluated, meanwhile chromatography also allows quantifying individual fatty acids. Gas chromatography (GC) is the most popular technique for fatty acids determination. However, lipids and fatty acids are insufficiently volatile for direct GC analysis and should be derivatized to more volatile compounds.

Different strategies can be used for gas chromatographic algal FA analysis. The first one is to extract the lipids with the proper solvent and then accomplish a transesterification. Using the second approach algae are treated with a strong alkali and fatty acids are subsequently esterified using an acidic catalyst. The third method, direct transesterification, does not require multiple steps as the esterification agent is added directly to the biomass [8, 9]. In this case, the separate lipid extraction step is avoided and fatty acids are simultaneously extracted and transesterified. Direct transesterification results

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in a significant reduction in the length of the total procedure and in the use of a minimal sample amount [9].

Transesterification involves the cleaving of an ester bond by an alcohol and can be catalysed by either a base or an acid. Alkaline catalysts, such as sodium methoxide or sodium or potassium hydroxide in methanol, transesterify complex lipids quickly and at lower temperatures than required by acid catalysts, but they do not esterify free fatty acids [10]. Acid catalysts, such as hydrochloric acid, sulphuric acid or boron trifluoride (BF_3) in methanol, require heating and longer reaction times than basic catalysts, but can transesterify complex lipids as well as esterify free fatty acids [5]. Thus, acid catalysts should be used when the total fatty acid content has to be determined.

Currently, numerous papers on acid catalysts for lipid esterification/transesterification are published, however, there is a little consensus on which gives the most accurate results [8–12].

The aim of this study was to test and to compare the effectiveness of the most popular acid catalysts for the direct esterification/transesterification and consequent gas chromatographic determination of total fatty acid content in cyanobacteria and microalgae.

EXPERIMENTAL

Reagents and solutions

Ethyl acetate (≥99.9%), n-hexane (≥98.0%), boron trifluoride-methanol complex (20% solution in methanol), hydrochloric acid (≥32%) were purchased from Merck (Germany). Methanol (≥99.95%), acetone (≥99.9%), chloroform (≥99.8%) were purchased from Roth (Germany). Acetyl chloride (>99.0%), tricosanoic acid (≥99.0%) were purchased from Sigma-Aldrich (USA). The following fatty acid methyl ester (FAME) standard was used (Larodan, Sweden): Mixture ME 100 (10 mg/ml) solution in dichloromethane (methyl butyrate 4%; methyl hexanoate 4%; methyl octanoate 4%; methyl decanoate 4%; methyl undecanoate 2%; methyl dodecanoate 4%; methyl tridecanoate 2%; methyl tetradecanoate 4%; methyl myristoleate (9c) 2%; methyl pentadecanoate 2%; methyl 10c-pentadecenoate 2%; methyl hexadecanoate 6%; methyl palmitoleate (9c) 2%; methyl heptadecanoate 2%; methyl 10c-heptadecenoate 2%; methyl octadecanoate 4%; methyl oleate (9c) 4%; methyl elaidate (9tr) 2%; methyl linoleate (9c, 12c) 2%; methyl linoelaidate (9tr, 12tr) 2%; methyl linolenate (9c, 12c, 15c) 2%; methyl gamma linolenate (6c, 9c, 12c) 2%; methyl eicosanoate 4%; methyl 11c-eicosenoate 2%; methyl 11c, 14c-eicosadienoate 2%; methyl eicosatrienoate (11c, 14c, 17c) 2%; methyl 8c, 11c, 14c-eicosatrienoate 2%; methyl arachidonate (5c, 8c, 11c, 14c) 2%; methyl eicosapentaenoate (5c, 8c, 11c, 14c, 17c) 2%; methyl heneicosanoate 2%; methyl docosanoate 4%; methyl erucate (13c) 2%; methyl docosadienoate (13c, 16c) 2%; methyl docosahexaenoate (4c, 7c, 10c, 13c, 16c, 19c) 2%; methyl tricosanoate 2%; methyl tetracosanoate 4%; methyl nervonate (15c) 2%.

Cultivation methods of cyanobacteria and microaalgae

The strains of two cyanobacteria species *Aphanizomenon flos-aquae* 2012/KM1/D3 and *Planktothrix agardhii* RU/19/6/F53, and two green algal species *Scenedesmus acutus* 2012/CUL/C3 and *Monoraphidium griffithii* 2013/DVA1/C3 isolated from Lithuanian aquatic ecosystems were used for analyses. The strains were cultured in the MWC medium [13], at 20 °C, 12:12 h day:night cycle. Cultures were harvested by centrifuging at 8000 g for 10 min at 4 °C and pellets were dried in a vacuum freeze drier (ZIRBUS VaCo 2) and stored at –20 °C.

Instrumentation

GC–MS was performed on a PerkinElmer Clarus 580 series gas chromatograph coupled to a PerkinElmer Clarus 560 S mass spectrometer (MS) (PerkinElmer, Shelton, USA). The GC system was equipped with the Elite-5MS capillary column (30 m × 0.25 mm id, 0.25 µm film thickness) coated with methylpolysiloxane (5% phenyl).

GC–FID was performed on a Shimadzu GC-2010 Plus series gas chromatograph with a flame ionisation detector (FID). The GC system was equipped with the Rtx®-2330 capillary column (105 m × 0.25 mm id, 0.2 µm film thickness) coated with 90% biscianopropyl/10% phenylcyanopropyl polysiloxane.

Centrifugation was carried out with a Boeco S-8 centrifuge (Germany).

Gas chromatographic conditions

GC–MS conditions

Helium was employed as a carrier gas with a constant flow of 1 ml min^{-1}. The injector temperature was held at 250 °C. Injection was performed in the pulsed splitless mode (pulsed to 4 ml min^{-1}until 1.5 min, split (50:1) open at 1.55 min). The oven temperature was programmed as follows: 40 °C for 1 min, from 40 to 250 °C at 3 °C min^{-1} and held at 250 °C for 8 min. The capillary column was connected to the ion source of the mass spectrometer by means of the transfer line maintained at 280 °C. The electron ionization ion source conditions were the following: electron energy 70 eV and temperature 180 °C. GC–MS in the full scan mode was used. The analyses were carried out with a filament multiplier delay of 3 min and the acquisition was performed in a range of m/z 33–600.

GC–FID conditions

Helium was employed as a carrier gas with a constant flow of 1.76 ml min^{-1}. The injector temperature was held at 225 °C. Injection was performed in the split mode (split 10:1). The oven temperature was programmed as follows: 120 °C for 1 min, from 120 to 250 at 2 °C min^{-1} and held at 250 °C for 20 min. The detector temperature was held at 260 °C.

Sample preparation

To a 4 ml screw thread vial, 50 mg of lyophilized algae, 1 ml of chloroform, 50 µl of 5 mg ml^{-1} solution of tricosanoic acid (internal standard) in chloroform, 1 ml of methanol and 100 µl of acetyl chloride were added. The vial was hermetically closed and held at 80 °C for 1 hour for derivatization. Then the mixture was transferred into a 10 ml volume centrifuge tube, washed with 6 ml of distilled water and centrifuged for 1 min at 5000 rpm. The upper aqueous layer was removed. The washing procedure was repeated 3 times, 0.5 ml of an organic layer was transferred into an autosampler vial for GC analysis.

RESULTS AND DISCUSSION

Preliminary gas chromatographic analysis

The first step of the work was to select an extraction solvent capable to extract from algae the biggest quantity of substances. Five solvents, namely, hexane, acetone, methanol, ethyl acetate and chloroform, were tested: to 20 mg of *S. acutus* 1 ml of the solvent was added, the extraction vessel was manually shaken for 5 min and the mixture was centrifuged for 1 min at 5000 rpm. In all the cases the chromatograms were similar and contained only few intense peaks. A chromatogram of the extract in chloroform is presented in Fig. 1. With the help of MS the peaks were identified as diizooctyl phthalate (RT = 66.50 min), 1-eicosanol (RT = 70.68 min) and squalene (RT = 73.77 min). The comparison of the peak areas demonstrated that the biggest extraction efficiency with 2–8 times larger peak areas was achieved using chloroform. Thus, chloroform was selected as an extraction solvent for the further work.

Selection of an acid derivatization catalyst

Although prior to the gas chromatographic analysis fatty acids can be converted into different esters, methylation was selected in this work as FAMEs have a lower boiling point in comparison with the other esters resulting in a faster GC.

The most popular acid catalysts for fatty acid esterification and lipid transesterification are hydrochloric acid and boron trifluoride. Derivatization is normally carried out using HCl at 70–90 °C for 30–120 min, and using 6–20% of BF_3 in methanol at 70–90 °C for 2–60 min [5, 8, 9, 14]. Our scope was to evaluate those acid catalysts and to choose the best ones.

Normally, anhydrous hydrochloric acid in methanol is used. It can be prepared either by bubbling anhydrous hydrogen chloride gas into methanol or by adding liquid acetyl chloride to methanol. The first procedure is quite tedious. On the other hand, according to some authors, the esterification/transesterification reaction using HCl is of low sensitivity to water interference [10]. Thus, we decided to check if the commercially available 32% HCl could be used instead of anhydrous HCl.

The experiment was carried out on *S. acutus*. Tricosanoic acid was used as an internal standard. Three series of the analysis (three samples in each) were carried out. To 50 mg of lyophilized algae 1 ml of chloroform and 50 µl of 5 mg ml^{-1} solution of tricosanoic acid were added. Then 1) 1 ml of methanol and 500 µl of 32% hydrochloric acid were added and the derivatization was held at 80 °C for 120 min; 2) 1 ml of methanol and 100 µl of acetyl chloride were added and the derivatization was held at 80 °C for 120 min; 3) 1 ml of 7% BF_3 metanolic solution was added and the derivatiza-

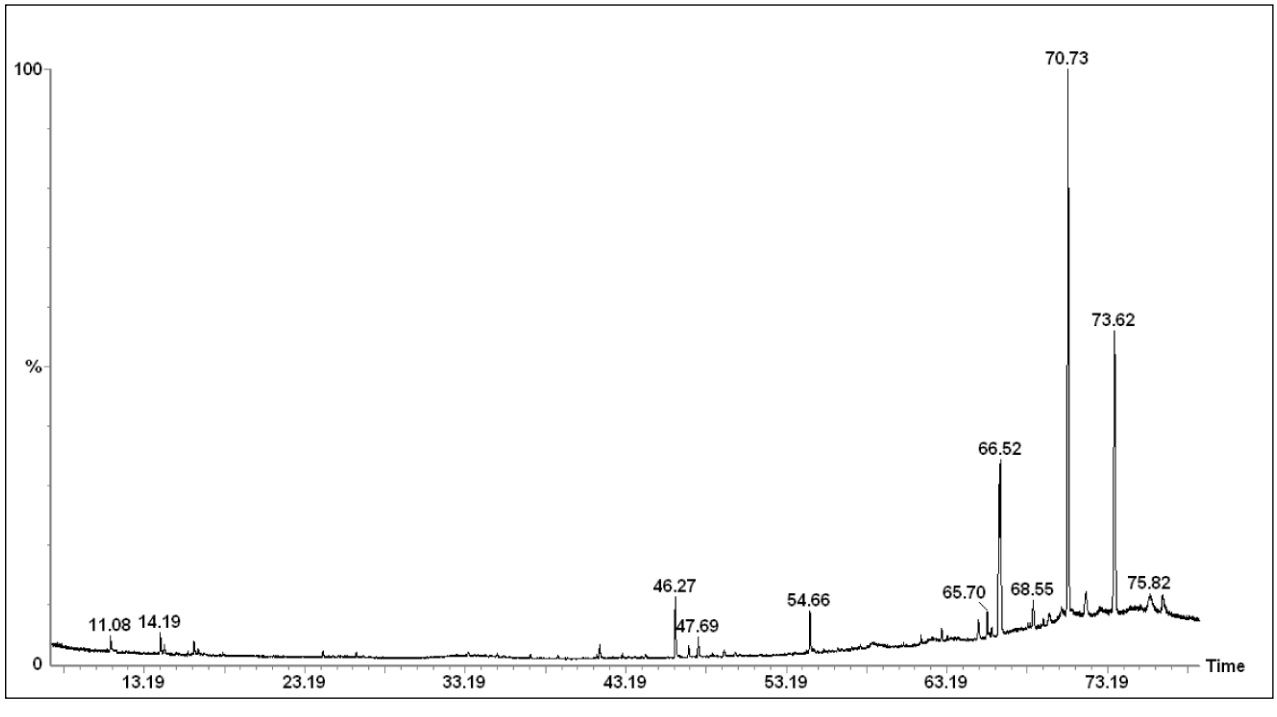

Fig. 1. A chromatogram of underivatized *Scenedesmus acutus* biomass extract in chloroform. For GC–MS conditions, see Experimental

tion was held at 80 °C for 15 min. After the derivatization the mixtures were washed with three portions of distilled water, 6 ml each. After each wash the samples were centrifuged for 1 min at 5000 rpm. One microliter of the organic layer was injected into GC–MS for the analysis. The relative peak areas of the three biggest peaks – hexadecanoic acid ME (16:0 ME) (RT = 49.46 min), 9,12-octadecadienoic acid ME (9,12–18:2 ME) (RT = 54.61 min) and 11-octadecenoic acid ME (11–18:1 ME) (RT = 54.89 min) – were compared (Table 1). The biggest peaks were obtained using acetyl chloride as a catalyst while 32% HCl resulted in 1.4–1.5 times smaller relative peak areas. It should be explained by water influence as we used 32% HCl that contained a significant quantity of water. The worst derivatization efficiency was obtained using BF_3. For 16:0 ME and 11–18:1 ME peak areas were 3.5–3.7 times smaller, for 9,12–18:2 ME they were even 5.2 times smaller than in the case of acetyl chloride. On the other hand, the methylation of free tricosanoic acid (internal standard) using acetyl chloride and BF_3 was nearly the same indicating that the transesterification reactions in the presence of BF_3 are more aggravated than the esterification of free tricosanoic acid. This corresponds with the results obtained by Cavonius et al. [8]. The authors concluded that small recoveries of FAMEs could be caused by a short incubation time (20 min in their case). Thus the further step of our work was to investigate an influence of the transesterification reaction time on the transesterification efficiency using BF_3. The reaction time was increased up to 60 min, however, the derivatization efficiency did not change, the relative peak areas of 16:0 ME, 9,12–18:2 ME and 11–18:1 ME after 15, 30, 45 and 60 min derivatization were very close, their RSDs were only 2.6–4.6%. Probably, because of the short shelf-life the reagent did not act properly and thus the transesterification was incomplete. Considering that BF_3 is hazardous, expensive and has a limited shelf-life, we decided against proceeding with it and selected acetyl chloride as a catalyst for algal lipid derivatization.

Table 1. **Relative peak areas of some FAME (with respect to tricosanoic acid methyl ester) obtained using different acid catalysts**

Catalyst	16:0 ME	9,12–18:2 ME	11–18:1 ME
32% HCl	12.5	10.2	16.1
Acetyl chloride	17.3	15.7	24.2
BF_3	4.9	3.0	6.6

Further, the derivatization time using acetyl chloride was optimized. The derivatization was carried out at 80 °C for 15, 30, 45, 60, 90 and 120 min. The results demonstrated that 60 min derivatization time was sufficient (Fig. 2).

Analysis of cyanobacteria and microalgae compounds

The optimized esterification/transesterification procedure using acetyl chloride as a catalyst was applied for the determination of total fatty acid content in four selected species. To 50 mg of lyophilized algae biomass, 1 ml of chloroform, 50 µl of 5 mg ml^{-1} solution of 23:0 (internal standard) in chloroform, 1 ml of methanol and 100 µl of acetyl chloride were added and the mixture was held at 80 °C for 1 hour for derivatization. Then the mixture was washed with 6 ml of distilled water and centrifuged for 1 min at 5000 rpm. The upper aqueous layer was removed. The washing procedure was repeated 3 times. Chromatograms obtained on GC–MS were much more complicated than those of non derivatized extracts (Fig. 3) indicating the presence of a significant lipid content. However, the GC column used was not able to separate properly some analytes. Thus in order to achieve better separation the 30 m length capillary column was replaced by the 105 m length high polarity capillary column. In addition, instead of MS detection a flame ionisation detector was applied as theoretical FID correction factors for long chain fatty acid (C > 10) methyl esters are quite close (1.122–0.978) [15] and for all the FAMEs can be considered to be equal to unity. This facilitates the quantification of FAMEs allowing to accomplish the calibration using a single standard. The derivatized algae extract chromatograms obtained on GC–FID at optimized conditions are presented in Fig. 4. The identification was made by comparing retention times with those of the FAME standard mixture ME 100.

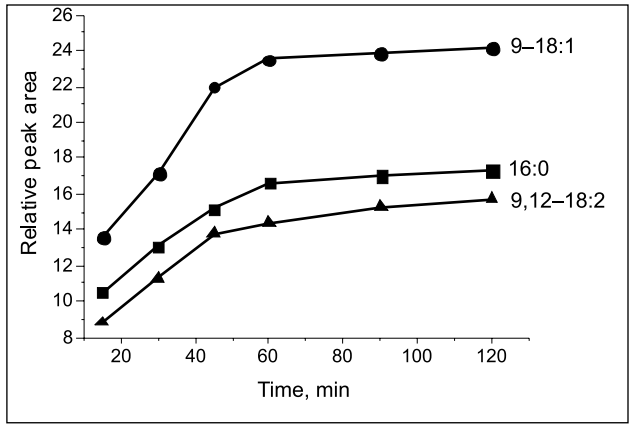

Fig. 2. Influence of the methylation time on the derivatization efficiency. Acetyl chloride was applied as an acid catalyst at 80 °C temperature

All the investigated algae contained significant quantities of palmitic acid (16:0) and α-linolenic acid (9,12,15-octadecatrienoic or 9,12,15–18:3) (Table 2). The oleic (9–18:1) and linoleic (9,12–18:2) unsaturated fatty acids occurred in the highest amounts in green algae *S. acutus* and *M. griffithii*, meanwhile in cyanobacteria the quantities of those compounds are much smaller. On the other hand, cyanobacteria contain higher quantities of palmitoleic acid (9–16:1) in comparison with green algae. Linoleic and α-linolenic acids are essential fatty acids indispensable to human health, but cannot be manufactured in the body and must obtain through diet. Thus, due to the relatively high content of them, freshwater algae are a promising source of nutrition.

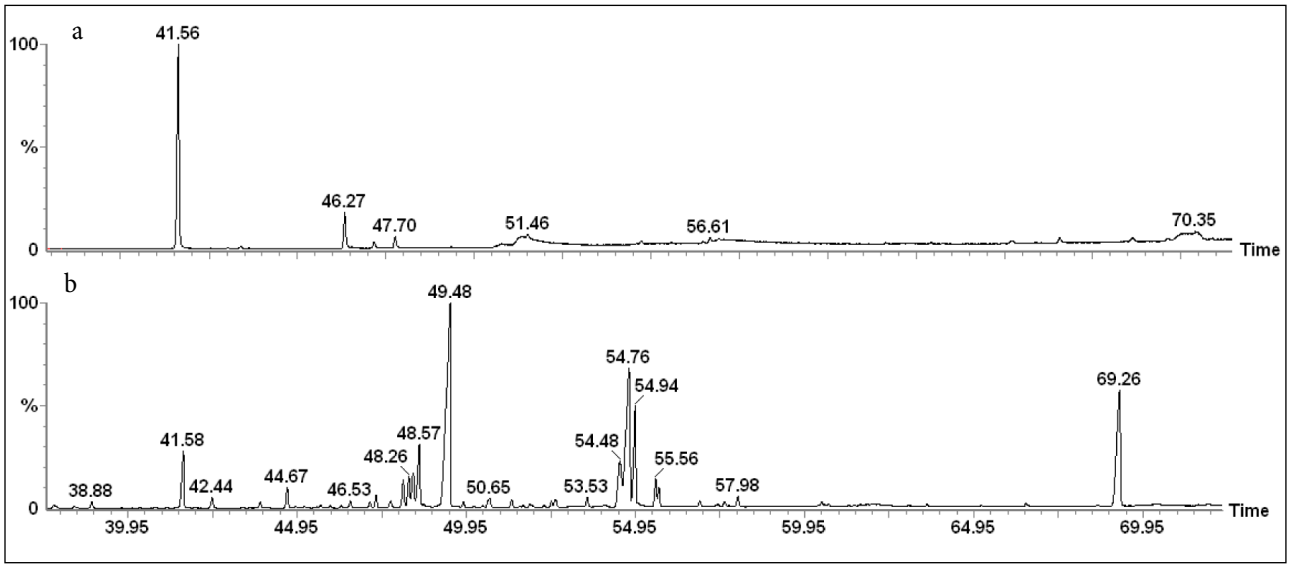

Fig. 3. Chromatograms of underivatized (a) and derivatized (b) *Aphanizomenon flos-aquae* biomass extract in chloroform. For GC-MS conditions, see Experimental

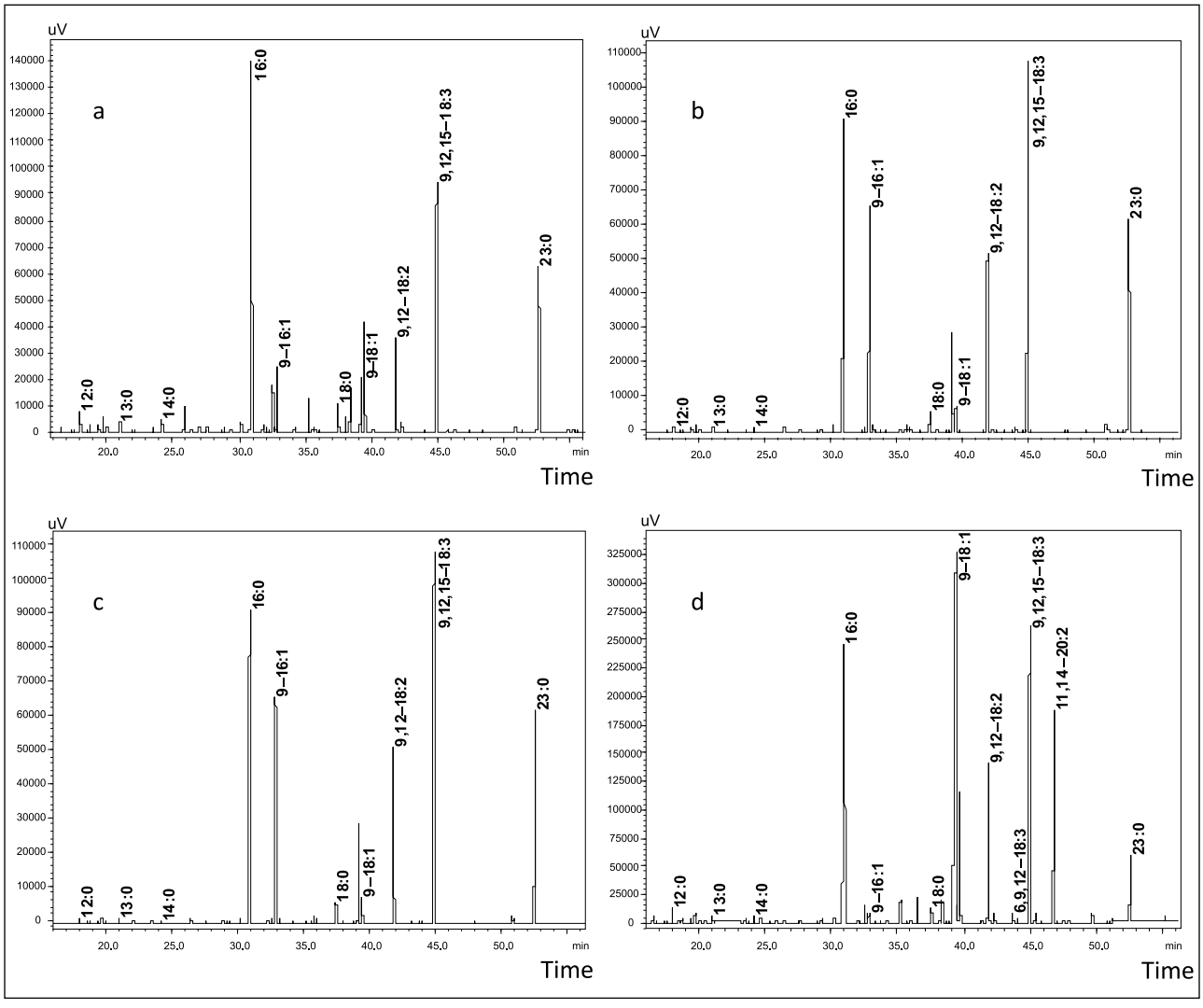

Fig. 4. Chromatograms of derivatized *Aphanizomenon flos-aquae* (a), *Planktothrix agardhii* (b), *Scenedesmus acutus* (c) and *Monoraphidium griffithii* (d) biomass extracts in chloroform. For GC–FID conditions, see Experimental

Table 2. **Fatty acid concentrations (mg g^{-1}) in cyanobacteria and algae biomass**

RT, min	Fatty acid	*A. flos-aquae*	*P. agardhii*	*S. acutus*	*M. griffithii*
18.03	12:0	0.29	–	1.47	0.56
21.04	13:0	0.18	–	0.29	0.29
24.19	14:0	0.21	–	0.16	0.23
30.94	16:0	7.83	6.09	14.4	19.6
32.87	9–16:1	1.08	3.97	0.19	0.37
39.47	9–18:1	0.93	1.73	15.0	31.4
41.83	9, 12–18:2	1.53	3.17	9.62	7.96
43.70	6, 9, 12–18:3	–	–	2.16	0.36
44.90	9, 12, 15–18:3	4.29	6.87	8.04	16.8
46.80	11, 14–20:2	–	–	–	10.1

CONCLUSIONS

Freshwater cyanobacteria and algae are a promising source of fatty acids, can be used for human food and animal feed, thus fast and reliable methods for fatty acids determination are indispensable. In this work, three acid catalysts for the direct esterification/transesterification of algal fatty acids were examined. Acetyl chloride demonstrated the highest derivatization efficiency. Moreover, it is low-cost and simple to use thus can be suggested for fatty acid derivatization for subsequent gas chromatographic analysis.

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RŪGŠTINIO ESTERIFIKACIJOS KATALIZATORIAUS PARINKIMAS BENDRAJAM RIEBALŲ RŪGŠČIŲ KIEKIUI MELSVABAKTERIŲ IR DUMBLIŲ BIOMASĖJE NUSTATYTI DUJŲ CHROMATOGRAFIJOS METODU

S a n t r a u k a

Bendrajam riebalų rūgščių kiekiui melsvabakterėse ir dumbliuose nustatyti dujų chromatografijos metodu buvo išbandyti trys rūgštiniai esterifikacijos / transesterifikacijos katalizatoriai – druskos rūgštis, acetilo chloridas ir boro trifluoridas. Geriausi rezultatai gauti naudojant acetilo chloridą, kuris pasirinktas gėlavandenių melsvabakterių *Aphanizomenon flos-aquae*, *Planktothrix agardhii* ir žaliadumblių *Monoraphidium griffithii*, *Scenedesmus acutus* analizei. Visų tirtųjų rūšių biomasėje buvo nustatyti nemaži palmitino ir α-linoleno rūgšties kiekiai. Didžiausi oleino ir linolo rūgščių kiekiai aptikti žaliadumblių *S. acutus* ir *M. griffithii* biomasėje.