Rapid assay of fatty acid composition using a portable high-performance liquid chromatograph for monitoring aquatic ecosystems ☆

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Abstract

The chromatographic conditions presented allowed the separation of the nitrophenacyl derivatives of standards of eleven free fatty acids (FFA) using a portable high-performance chromatograph, suitable for use aboard a research vessel. A statistically significant linear correlation between UV absorbance and amount of the analytes injected was obtained. The method was tested on FFA from algae cultural media. The method can be used for the ecological monitoring of natural waters.

1. Introduction

The composition of fatty acids in living organisms is known to be a very informative parameter. Recently, the extracellular free fatty acid (FFA) composition of natural waters was also demonstrated to be important for ecological surveys [1,2]. As shown, decoding the FFA composition provides an opportunity to determine the occurrence of limiting of phytoplankton growth and the phase of its seasonal dynamics, the potential kinetics of the ecosystem self-purification, etc. Moreover, the surface film of water (upper microlayer, ca. 50–100 μm) proved to be one of the most informative sampling sites,

because the surface-active FFA exuded by organisms inhabiting the whole water column accumulate in this film owing to flotation and adsorption at the air–water interface. Thus, the surface film is a natural integrator of information on the whole aquatic ecosystem.

To obtain this information on the state of the ecosystem during ecological monitoring of extended natural water bodies, one needs to determine the FFA composition in water samples just after they have been taken, i.e., aboard a research vessel. Thus, two demands can be placed on a method for the determination of the FFA composition. First, it should be a micromethod, because the surface film samples are usually of small volume. Second, the chromatographic equipment should be suitable for operating under field conditions. Standard conventional methods of gas chromatography can hardly be

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used in this environment, whereas a portable
high-performance liquid chromatograph can be
operated successfully, especially if it is suitable
for narrow-bore columns. The determination of
fatty acids by HPLC has recently been reported
[3–6]. The aim of this work was to develop the
method for the determination of FFA using a
portable high-performance liquid chromato-
graph, suitable of use aboard a research vessel.

2. Experimental

2.1. Reagents and chemicals

Acids C10:0, C12:0, C14:0, C14:1 and C-iso-
18:0 were purchased from Sigma (St. Louis,
MO, USA) and C18:1, C18:2, C17:0, C16:0 and
C18:0 from Merck (Darmstadt, Germany). P-
Nitrophenacyl bromide and N,N-diisopropyl-
ethylamine were purchased from Aldrich (Mil-
waukee, WI, USA). Acetonitrile, ethanol and
dimethylformamide (Reakhim, Angarsk, Rus-
sian Federation) were distilled before use. Water
used for preparing eluents was doubly distilled.
Mixtures of solvents used as the eluents were
degassed under vacuum.

2.2. Preparation of fatty acid derivatives

Derivatization was carried out according Smith
and Thompson [7] with some modifications.
Briefly, a fresh solution containing 20 μmol of
p-nitrophenacyl bromide and 40 μmol of di-
isopropylethylamine per 1 ml of dimethylform-
amide was added to dried FFA. The volume of
the solution added depended on the approximate
amount of FFA and ranged from 10 to 500 μl.
FFA from the medium of a laboratory culture of
the blue-green alga *Spirulina platensis* were ex-
tracted as described previously [2]. Briefly, lipids
were extracted with chloroform and separated by
TLC and FFA were eluted from silica gel with
diethyl ether.

The reaction vial was closed and heated at
65°C for 15 min. After the reaction was com-
pleted, dimethylformamide was removed by ro-

tary evaporation. This removal was applied
because dimethylformamide had considerable
absorbance at the wavelength used for FFA
detection. Then the fatty acid derivatives were
dissolved in a small volume of ethanol ranging
from 50 to 1000 μl. Aliquots of the solution were
injected directly into the chromatograph.

2.3. HPLC and GC analysis

Analyses were carried out using a Milikhrom-1
portable high-performance liquid chromatograph
(Nauchpribor, Oriol, Russian Federation). The
chromatograph was equipped with a 2600-μl
syringe pump with flow-rate capabilities from 2
to 600 μl/min. The operating pressure was up to
5 MPa. The volumes injected ranged from 5 to
20 μl. The variable-wavelength UV detector
monitored the absorbance from 190 to 360 nm.
The absorbance range of the detector was from
0.05 to 12.8 AUFS. The detector was a single-
beam instrument with a vibrating mirror that
deflected the light beam passing through the
sample and reference flow cells. The time con-
stant was selectable in the range 0.15–20 s. The
unique optical design of the detector allowed its
successful operation under field conditions. Both
flow cells (1.5 μl) had 1.5-mm light paths. The
reference cell was filled with ethanol. The values
measured were normalized by the electronics to
the equivalent absorbance with a 10-mm light
path and presented as an analogue output on the
recorder. The accuracy of absorbance measure-
ments was stated by the manufacturer to be 3%.

The derivatives were separated with a Separon
C$_{18}$ column (64 × 2 mm I.D., particle size 5 μm)
(Nauchpribor) using step-gradient elution at a
flow-rate of 50 μl/min. The gradient elution
system consisted of following discrete steps:
acetonitrile–water (70:30) and ethanol–water
(82:18, 83:17, 84:16, 84.5:15.5 and 85:15, v/v).
Each step was equal to 300 μl. The overall time
of the chromatography did not exceed 45 min
(including a reconditioning step). If necessary,
the elution could be interrupted and a quasi-
smooth spectrum could be registered with a
wavelength increment of 2 nm.

The FFA composition of the oil of the berry of sea buckthorn (*Hippophae rhamnoides*) was studied by GC. For this purpose, methyl esters of FFA (FAME) were prepared. A detailed description of the preparation of FAME and GC was published previously [1]. Briefly, FAME were analysed using a Chrom-5 gas chromatograph (Laboratórní Přístroje, Prague, Czech Republic) with a glass column (3 m × 3 mm I.D.). Helium was used as the carrier gas and polyethylene glycol adipate on Celite 545 as the stationary phase. The column temperature was maintained at 170°C and a flame ionization detector was used.

2.4. Quantitative measurements

An ethanol solution containing 1 mg/ml each of myristic acid, oleic acid and of stearic acid was prepared. Then ten samples containing 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 μg of each acid were prepared and subjected to the derivatization procedure. After the reaction was completed and dimethylformamide had been removed, each sample was dissolved in 3 ml of ethanol and aliquots of 10 μl were chromatographed.

The areas of the peaks in the chromatograms were calculated by multiplying the heights of the peaks by the widths at half-height. The areas of the peaks was preferred to the heights because widths of the peaks of different fatty acids were not the same. The dependence of the peak area on the amount injected was fitted by linear least-squares for each fatty acid. The standard errors (*SE*) of the linear fits were calculated as follows:

$$SE_{yx} = s_y \sqrt{1 - r^2}$$

where s_y is the standard deviation of y (area) values and r is the correlation coefficient [8]. The precision of the method was estimated as the relative standard deviation (s_r):

$$S_r(\%) = SE_{yx}/M \cdot 100$$

where M is the mean of the y values.

3. Results and discussion

In order to determine the optimum wavelength of detection, the absorption spectra the derivatives of some fatty acids were recorded. The absorption spectrum of the derivative of palmitic acid is presented in Fig. 1; the spectra of the derivatives of the other fatty acids were similar. The length of the carbon chain and the number of double bonds for the different fatty acids did not affect the pattern of the spectrum. Therefore, the wavelength of maximum absorption (230 nm) was chosen for detection.

The fatty acids that were found to dominate in natural waters (saturated, monoenic, some dienic and branched acids) were taken as standards for the development of the separation procedure. Initially, for the separation of the FFA derivatives an isocratic mobile phase was used containing ethanol–acetonitrile–water (82:9:9, v/v/v) according to Smith and Thompson [7]. However, under these conditions most of the acids
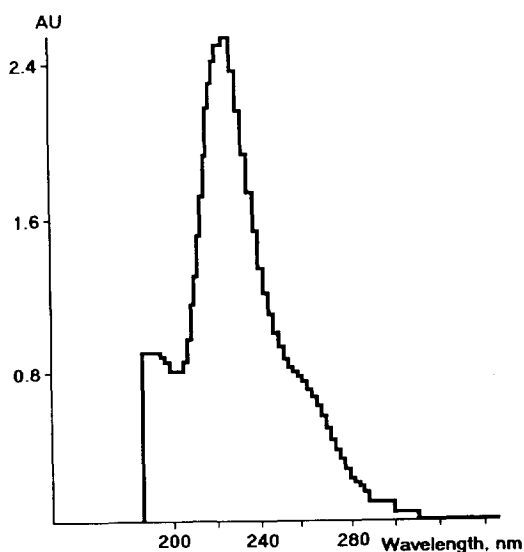

Fig. 1. Quasi-smooth spectrum of UV absorbance of nitrophenacyl derivative of palmitic acid obtained with the spectrophotometric detector of the portable HPLC equipment used in the stopped-flow mode. Amount injected, 1.48 nmol; volume injected, is 5 μl.

did not separate and the peaks emerged as wide and asymmetric bands. Much better resolution was obtained using gradient elution with ethanol–water mixtures, but palmitic and oleic acids were not resolved. The described six-step gradient (see Experimental) allowed a satisfactory resolution of this critical pair. Fig. 2 shows a chromatogram of the derivatives of eleven standard FFA separated with this elution system.

An essential factor in the separation was the acetonitrile–water step before the ethanol–water gradient. Acetonitrile and ethanol are known to have almost equal eluent strengths, but they show different selectivities because of the different types of molecular interactions between the solvents, stationary phase and analytes [9]. Hence it is possible that the successive effects of the solvents with two different selectivities played a key role in this critical separation.

For quantitative purposes the linearity of the method was investigated in the range 0.5–7.5 nmol each of myristic, oleic and stearic acids.

Fig. 3. Relationships between amounts of fatty acids injected and areas of the peaks in the chromatograms. Straight lines are linear least-squares fits. For coefficients of the fits, see Table 1. ● = C14:0; ○ = C18:1; □ = C18:0.

The relationships between amounts of fatty acids injected and the peaks areas in the chromatograms are shown in Fig. 3. As can be seen, the relationships between amount of fatty acid and peak area for the given ranges were satisfactorily linear, as was confirmed by the linear least-squares fits, which provided statistically significant coefficients (Table 1).

There were statistically significant differences between the values of the angle coefficients α for the pairs of acids C14:0–C18:1 (Student's test, $t = 3.17$) and C14:0–C18:0 ($t = 4.32$), whereas for the pair C18:1–C18:0 there was no significant difference ($t = 1.64$). Hence the length of the carbon chain of the acids may be of importance with regard to the absorption coefficients of the derivatives rather than the presence of double bonds. Differences in the slopes could result in inaccurate determination of the different acids if a single standard is used. However, as can be seen from Fig. 3, this inaccuracy would be negligible if the amount injected did not exceed 5 nmol of each fatty acid. Hence, for accurate determinations when a single standard is used, one should carry out measurements within this range of amounts. The relative standard deviations for the linear fits are given in Table 1.
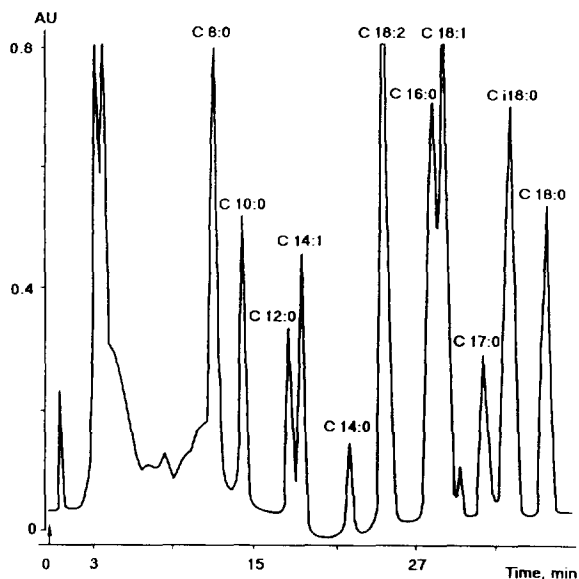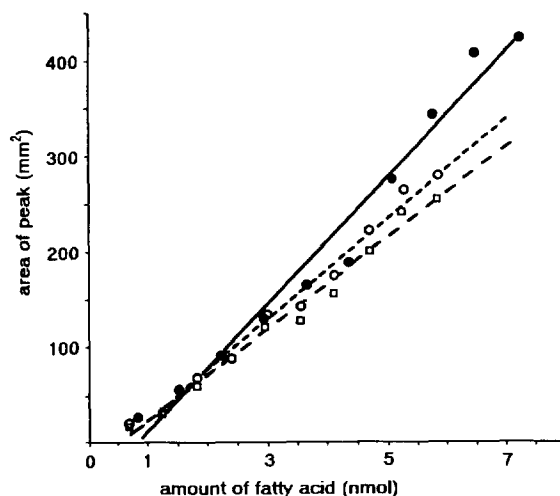

Fig. 2. Chromatogram of nitrophenacyl derivatives of standards of eleven fatty acids separated by gradient elution: acetonitrile–water (70:30, v/v) 6 min; ethanol–water (82:18, v/v) 6 min; (83:17, v/v) 6 min; (84:16, v/v) 6 min; (84.5:15.5, v/v) 6 min; (85:15 v/v) 6 min. Flow-rate, 50 μl/min; UV detection at 230 nm. The total amount of fatty acids injected, ca. 3 nmol; volume injected, 10 μl.

Table 1
Calibration coefficients of linear equations $y = ax + b$, extracted by least-squares fit, where x is amount of FFA injected (nmol), y is area of peak of FFA in chromatograms (mm^2)

Acid	$a \pm SE$	t_a	$b \pm SE$	t_b	$M \pm SE_{yx}$	R.S.D. (%)
C14:0	63.7 ± 3.63	17.5	−47.9 ± 16.5	2.9	208.3 ± 22.7	10.9
C18:1	50.5 ± 2.03	24.9	−23.1 ± 7.4	3.1	141.0 ± 10.3	7.3
C18:0	46.0 ± 1.94	23.8	−19.7 ± 7.1	2.7	128.6 ± 9.8	7.6

SE = standard error; t = Student's t-value (degrees of freedom = 8); M = mean of y values; SE_{yx} = standard error for linear fits; R.S.D. = relative standard deviation ($n = 10$).

3.1. Application

The method was tested using the oil of the berry of sea buckthorn (*Hippophae rhamnoides*), which was chosen because its fatty acid composition is well known and fairly simple. The GC analysis of the FAME of the same sample was carried out simultaneously. Relative contents (percentage of the total FFA) of the acids obtained with HPLC and GC are presented in Table 2. These data were used for the identification of C18:3, C16:1 and C16:2 fatty acids. Although the relative contents of the fatty acids obtained with HPLC and GC were not identical (Table 2), the small number of acids (seven) in the sample and the regular sequence of their elution allowed their identification.

The compositions and concentrations of FFA in the medium for laboratory cultures of algae were demonstrated to be comparable to those of natural waters [2]. Thus, the medium of the laboratory culture of the blue-green alga *Spirulina platensis* was used for testing the method. Fig. 4 shows a typical chromatogram of FFA
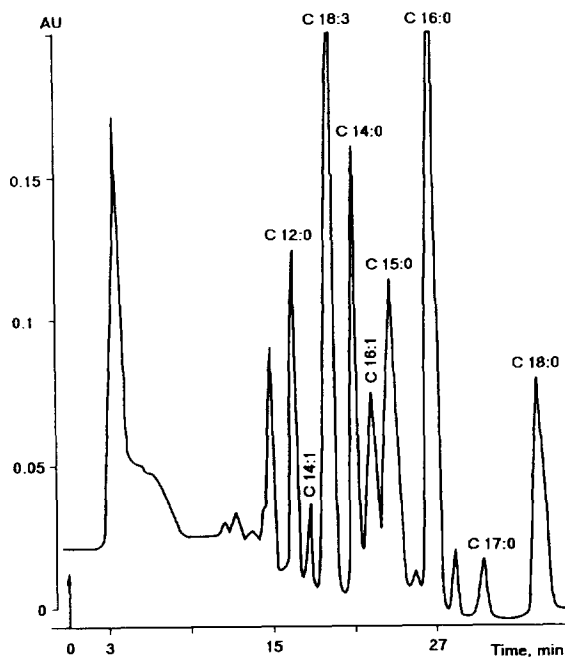

Fig. 4. Chromatogram of nitrophenacyl derivatives of FFA obtained from the medium of the laboratory culture of the blue-green alga *Spirulina platensis*. Chromatographic conditions as in Fig. 2.

Table 2
Relative contents of fatty acids (percentage of total) in the oil of the berry of sea buckthorn (*Hippophae rhamnoides*) obtained by HPLC and GC (data for a single measurement)

Method	Acid						
	16:0	16:1	16:2	18:0	18:1	18:2	18:3
HPLC	15.5	14.7	1.6	2.2	15.7	27.0	22.9
GC	13.8	9.8	0.5	2.1	14.5	32.2	27.0

obtained. The amount of FFA injected into the chromatograph was of the order of several pico-moles.

4. Conclusions

A method for the determination of fatty acids was developed for a Milikhrom portable high-performance liquid chromatograph which is suitable for operating under field conditions. The gradient used allowed the separation of fourteen species of fatty acids including the critical pair C16:0–C18:1. Under the conditions adopted the retention time of the fatty acids derivatives decreased with decrease in the length of the carbon chain and with increase in the degree of unsaturation. Regularities in the elution obtained for the standards of fatty acids were successfully used for the identification of the fatty acids in natural samples. The method can be applied for both qualitative and quantitative measurements of FFA. For carrying out quantitative measurements the amount injected should not exceed 5 nmol for each fatty acid. Quantitative analyses need further thorough elaboration using diverse FFA species. The method can be used for ecological monitoring of natural waters.

Acknowledgement

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