Rapid determination of the molecular weight distribution of total cellular fatty acids using chemical ionization mass spectrometry

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Abstract A new method for the qualitative and quantitative determination of the molecular weight distribution of total cellular fatty acids is described. The method includes a simple extraction–saponification followed by chemical ionization–mass spectrometric analysis of the saponifiable matter. This technique requires small quantities of cell material which, combined with the rapidity and precision of the analysis, makes it attractive to the biologist interested in changes in the fatty acid composition of growing cells. As an example, an application of this method to the fatty acid determination of marine diatoms at different growth stages is presented.

Supplementary key words saponification · algae · marine phytoplankton

The lipid components of many microorganisms change in response to changes in environmental conditions and in the physiological state of the organisms. Because organisms are not constant entities in a constant world, these and other biochemical parameters can be studied as reflections of dynamic processes. Studies of the biochemical changes in a cell may provide explanations for that cell's behavior under various conditions.

Many marine phytoplankters change their biochemical composition in response to certain environmental influences, though the degree to which the algal fatty acids change has been little studied to date. Research in this area has been difficult to pursue because studies of this kind involve many analyses of small quantities of sample material over relatively short time periods and the present methods used are not optimal for this purpose.

In our studies of marine phytoplankton, numerous samples must be analyzed in order to monitor changes in the algal fatty acids with growth. For this purpose, it is desirable to generate a quick overall picture of cellular fatty acids and to produce the data in a form that can be easily stored and processed.

We have therefore developed a method to extract and analyze rapidly the fatty acids from small quantities of cells, so a qualitative and quantitative picture of the total fatty acid composition of cells can be determined. This method includes a simple extraction–saponification of whole cells followed by direct chemical ionization–mass spectrometric analyses of the saponifiable matter. Data acquisition and processing are readily performed by a minicomputer that is coupled to the mass spectrometer. The data are stored on a tape cassette and are available for further computer manipulation. This report describes the details of our method and provides a sample of its application to a study of algal fatty acids. We feel the general attributes of this technique should make it particularly suitable for any fatty acid studies of dynamic systems.

EXPERIMENTAL METHODS

Instrumentation

A Finnigan Model 3200 MS was used for all mass spectrometric analyses. The instrument is equipped with a Data System 6000, including a disc drive and a tape cassette unit. The source conditions were source temperature, 150°C; electron energy, 130 eV; emission current, 0.3 mA; *iso*-butane pressure, 750 μm; and methane pressure, 1000 μm. The EI–MS was recorded at 70 eV.

The samples were introduced into the ion source of the MS with the cooled probe described by Sawdo and Blumer (1). This is accomplished by first transferring the sample (typically 0.5–1.0 μg) into a small glass capillary, which is then attached to the tip of the probe, cooled with Freon, and inserted into the heated MS ion source.

For the GLC analysis, the sample was esterified with BF_3–CH_3OH and analyzed on a Carlo Erba Model

Abbreviations: EI–MS, electron impact mass spectrometry; CI–MS, chemical ionization mass spectrometry; GLC, gas–liquid chromatography.

2150 gas chromatograph equipped with an SE 52 glass capillary column (25 m × 0.35 mm ID). The conditions were carrier gas He 3 ml/min.; temperature program 100–240°C at 3°/min.

Algal species

Cells of two clones of *Thalassiosira pseudonana*, a small, centric diatom, were axenically cultured at 18°C in f/2 medium (2) prepared from Woods Hole seawater; clone "13-1", an isolate from the Sargasso Sea, and clone "3H", an isolate from a Long Island estuary, were obtained from Dr. Robert R. L. Guillard of the Woods Hole Oceanographic Institution. Cultures of both clones were sampled simultaneously and growth was monitored with a Speirs-Levy eosinophil counter.

Extraction procedure

Between 1.5 and 5.0 mg dry weight of algal cells (determined on separate aliquots by the technique of Fisher and Schwarzenbach (3)) were filtered out of each culture onto baked-out (450°C) 25-mm glass-fiber filters. Each filter was folded in half and immediately transferred to a screw cap glass vial (17 × 60 mm, 8 ml capacity; cap lined with two Teflon liners) containing a small Teflon-coated magnetic stir bar and 3.5 ml of 5% KOH in methanol–water 7:3 preheated to 60°C. In this way the algal enzymes were deactivated before they could alter the cell's fatty acids. The sample was then thoroughly flushed with N_2, sealed, wrapped with aluminum foil to prevent photooxidation of the unsaturated acids, and placed atop a magnetic stirrer–heater where it was gently boiled for 2 hr. After this extraction–saponification step, the vial was cooled to room temperature and the solution was passed through a funnel containing a plug of cotton (pre-extracted with methanol–benzene 1:1) into another vial. After 2 ml of redistilled pentane was added to the solution, the vial was vigorously agitated with a Vortex-genie mixer, the two layers were allowed to separate, and the pentane fraction was removed. After three such extractions to remove the alcohols, hydrocarbons, and other pentane-extractable compounds, the vial was placed in an ice bath where it received 2 ml of 2.3 N HCl. The fatty acids were then extracted with three 2-ml portions of pentane. These pentane fractions were pooled and washed with 2 ml of a saturated NaCl solution, dried over anhydrous Na_2SO_4 (pre-extracted with methanol–benzene 1:1), and evaporated to dryness at 40°C. The residue was dissolved in a known amount of methylene chloride. An aliquot was removed for weighing on a Cahn Electrobalance and another aliquot, containing about 500 ng of sample, was used for the MS analysis. The remaining sample was stored under N_2 at −18°C in the dark.

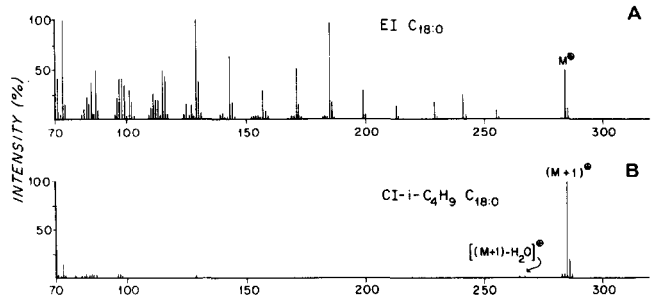

Fig. 1. Mass spectra of stearic acid. (*A*) EI spectrum of stearic acid; (*B*) CI spectrum of stearic acid with *iso*-butane as reactant gas.

Analysis of fatty acids by chemical ionization mass spectrometry

It is possible to display the molecular weight distribution of a fatty acid mixture by distilling the compounds directly into the ion source of a MS if, for each fatty acid of a given molecular weight, very few (preferably one) ion species are produced that can serve unambiguously as markers for the qualitative and quantitative analysis of this particular fatty acid. The high degree of fragmentation of the fatty acid molecules in traditional electron impact–mass spectrometry (EI–MS), however, makes this technique unsuitable for this purpose because fragments of different acids coincide and each signal contains only a small percentage of the total intensity (see **Fig. 1A**).

In 1965, a new technique, chemical ionization–mass spectrometry (CI–MS) was invented (4, 5). Murata, Takahashi, and Takeda (6) have shown that, by using *iso*-butane as reactant gas, this CI–MS technique is suitable for the analysis of fatty acids because essentially only one ion is formed from each fatty acid. This ion is produced by the protonation of the fatty acid molecule, resulting in a signal at mass (M + 1), where M denotes the molecular weight of the fatty acid molecule. This signal gives the molecular weight of the fatty acid and can serve as a marker for that particular fatty acid.

For the analysis of a fatty acid mixture using the probe distillation technique, the sample is heated slowly and each individual fatty acid distills into the ion source at a different time according to its boiling point. For the duration of the distillation period, mass spectra are continuously recorded and stored in the computer. **Fig. 2** illustrates the change in signal intensity at (M + 1)(*m/e* 285) of stearic acid during the distillation period. By plotting the signal intensity over time for each mass unit, a three-dimensional picture is obtained (**Fig. 3**). For the total signal intensity at each given mass unit, the sum of all the individual signals for that mass over the distillation period is calculated

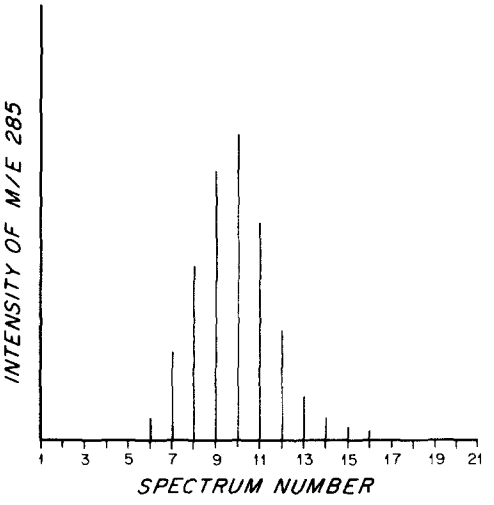

Fig. 2. Changes in the (M + 1) signal intensity of stearic acid over the distillation period.

with a computer program. The resulting "total mass spectrum" is then used for the qualitative and quantitative analysis of the fatty acid mixture, where the signal at the mass value (M + 1) enables detection of the molecular weight of an individual fatty acid present in the mixture, and the intensity of the signal gives a measure of its abundance in the mixture.

Differentiating between signals from the sample and the background of the MS ion source can be accomplished by examining the pattern of intensity changes of the corresponding signals over time. The presence of an erratic pattern indicates background whereas a smooth distillation curve (e.g. see Fig. 2) indicates a signal deriving from the sample material. If necessary, it is possible to distinguish between fatty acids and other material in the sample exhibiting signals in the same mass range by checking the position of the maximum of the distillation curve. As an example, **Fig. 4** presents the "total mass spectrum" obtained

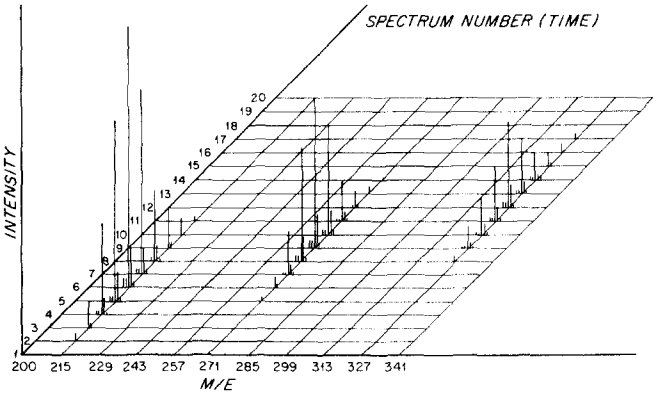

Fig. 3. Three-dimensional representation of the CI–MS data obtained from the distillation of a mixture of n-$C_{13:0}$, n-$C_{17:0}$, and n-$C_{21:0}$ fatty acids with *iso*-butane as reagent gas.

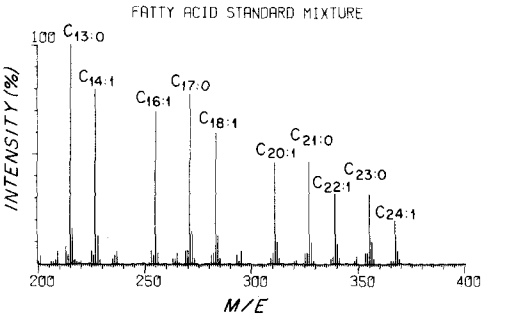

Fig. 4. "Total mass spectrum" of a fatty acid mixture containing equimolar concentrations of four saturated and six monounsaturated fatty acids.

from the distillation of a standard fatty acid mixture containing equimolar concentrations of four saturated and six mono-unsaturated fatty acids, spanning the molecular weight range of 200–400. Because the (M + 1) signal is used as the marker for each acid, it is only necessary to record signals in this range. It is, of course, advantageous to keep the mass range to be scanned to a minimum, thereby allowing for a maximum number of spectra during the distillation.

Though there is an equal number of molecules of each fatty acid in the mixture, examination of the spectrum in Fig. 4 shows that there is a drop in intensity of the signals as the molecular weights of the fatty acids increase. This decline in signal intensities can primarily be attributed to two factors. First, with increasing C number, a higher percentage of the molecules contains one ^{13}C atom, resulting in an increase in signal at the position (M + 2) and a decrease at the (M + 1) position; the second, and more prominent, factor results from an instrumental artifact of the quadrupole MS, which discriminates against ions with higher masses. It is noteworthy that the intensities of the (M + 1) signals of the monounsaturated fatty acids, regardless of the double bond positions, are lower than the signals from the corresponding saturated fatty acids. This also holds true for the polyunsaturated fatty acids, because of the higher degree of fragmentation of these compounds. However, the decline in signal intensity with increasing molecular weight and the lower relative intensity of the (M + 1) signals from the unsaturated fatty acids can, if desired, be easily corrected for by a computer program using a calibration curve established from a standard fatty acid mixture.

RESULTS AND DISCUSSION

The "total MS" of the two algal clones at different stages of growth are presented in **Fig. 5**. Because the (M + 1) signals used for the identification of the algal

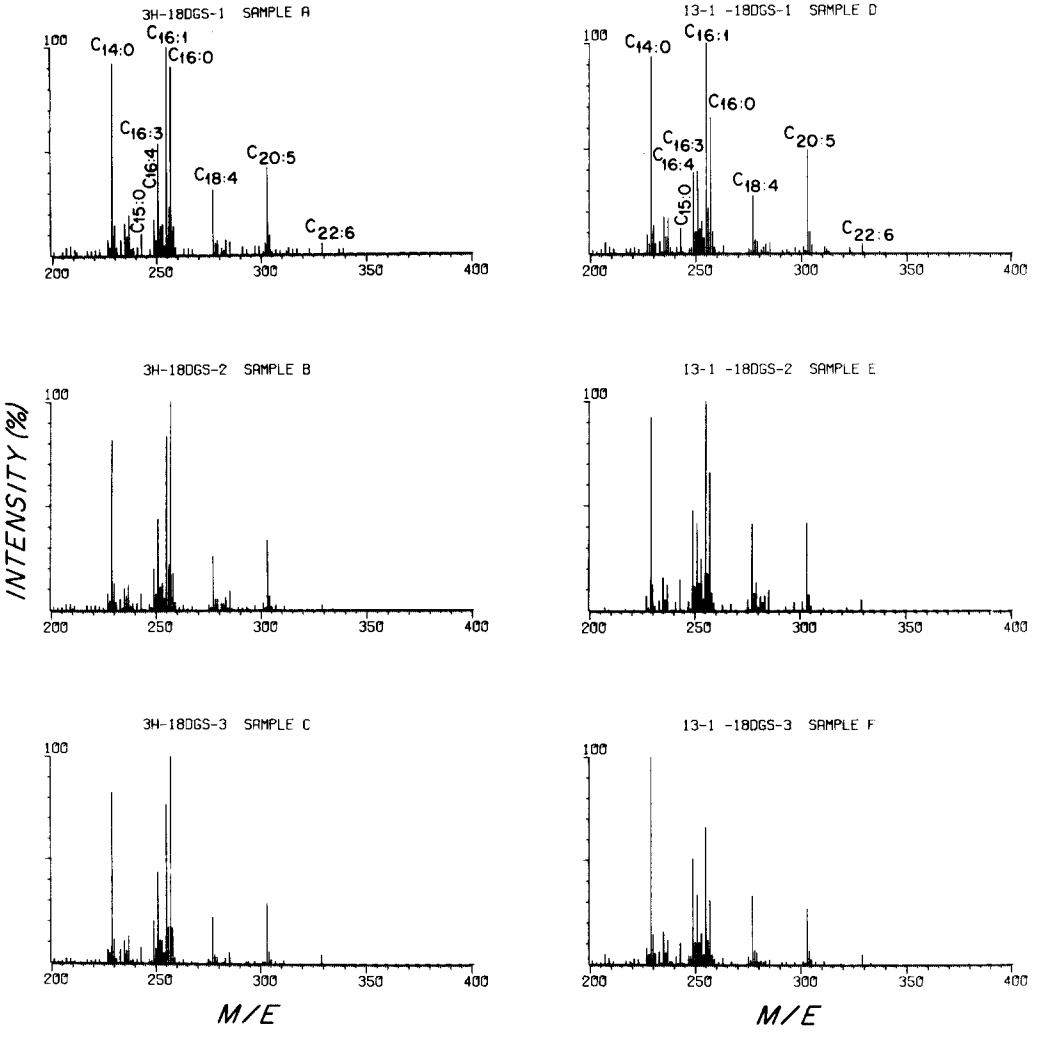

Fig. 5. "Total mass spectra" of the fatty acid composition of *Thalassiosira pseudonana*, clone 3H (*A–C*) and clone 13-1 (*D–F*). Cultures *A* and *D*, early exponential growth phase; cultures *B* and *E*, end of exponential growth phase; cultures *C* and *F*, stationary growth phase.

fatty acids lay in the range of 200–400, only this range was scanned. Each CI–MS run, including data acquisition and processing, was completed within 15 min. The CI–MS analyses of the algal fatty acids (Fig. 5) indicate that both clones contain fatty acids with corresponding molecular weights, and therefore with the same C number and degree of saturation. The major fatty acids of both clones are $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{16:3}$, $C_{16:4}$, $C_{18:4}$, and $C_{20:5}$. Minor components of both clones are $C_{15:0}$, $C_{16:2}$, $C_{18:0}$, $C_{18:1}$, $C_{18:3}$, and $C_{22:6}$.

The intensity of the signals of each MS displayed in Fig. 5 is normalized on the most intense signal of that spectrum. If desired, the computer can normalize the spectrum on any other signal or given absolute value. (This, for example, would be very useful in clarifying changes of minor fatty acid components and could be done by setting their most intense signal at 100%.) To each sample represented by a spectrum in Fig. 5, a mixture of internal standards containing equimolar amounts of $C_{13:0}$, $C_{17:0}$, $C_{21:0}$, and $C_{23:0}$ was added before analysis. The signals of these standard compounds were used to correct for the decline in signal intensities with increasing molecular weights and were then automatically deleted from the spectrum by the computer. This correction was necessary because the magnitude of the decline due to instrumental artifact varies with some instrumental parameters. The lower response at the (M + 1) signals of the unsaturated fatty acids was not corrected because it is not substantially affected by instrumental parameters (provided that source conditions are kept more or less constant) and because, in this study, only relative changes in fatty acid composition were of interest.

Fig. 5 shows that, although the two clones have the same major and minor fatty acid compounds, they differ with respect to the relative amounts of these com-

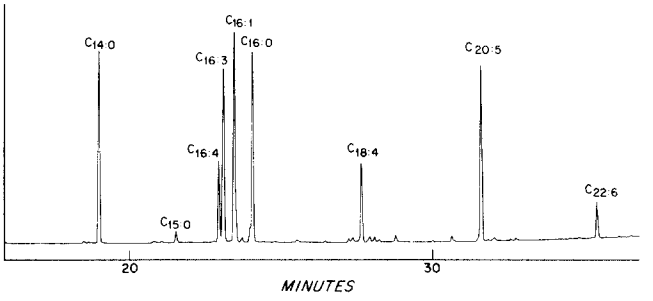

Fig. 6. Gas chromatogram of the methyl esters from algal sample *C* (see Figure 5).

pounds. The results further indicate that the fatty acid composition of clone 3H changes much less with the age of the culture than does the composition of clone 13-1, especially with regard to the C_{16} acids. A discussion of the biochemical significance of these results will be presented elsewhere (3). The spectra shown here simply demonstrate the suitability of this method for inter- and intra-specific comparisons as well as for monitoring fatty acid changes with growth.

To ascertain that any fatty acid differences observed are not attributable to methodological artifacts, the precision of the different steps was determined. The CI–MS precision was determined by measuring the "total MS" of an algal sample four times over a 2-week period. Each sample aliquot contained an internal standard for the correction of the decline in signal intensity with increasing molecular weight. The average coefficient of variation ($s/\bar{Y} \times 100\%$) of the major signal intensities was 5.5%, with a range of 2–9%. During this 2-week period, the ion source was taken apart and reassembled, with no substantial effect on the precision. For all measurements, the same source conditions prevailed. It should be noted that careful control of the ion source temperature, electron energy, and fit of the probe to the ion source are essential for obtaining reproducible results. The pressure of the reactant gas, the amount of material analyzed in each sample, and the scan rate of the MS are not critical for maintaining reproducibility, if they are kept within reasonable limits (i.e., 900–1000 μm, 0.2–2 μg of sample material, $\geq$8 scans for each acid during the distillation period, respectively). During the 2-week storage period there was no discernible loss of the polyunsaturated fatty acids.

The fatty acid mixture obtained by the extraction procedure outlined above was compared with a mixture obtained with the standard Soxhlet extraction procedure described by Chuecas and Riley (7) and was found to be identical. Grinding of an algal sample on a filter by a Vir-Tis tissue grinder before the extraction–saponification step had no effect on the results. A check on the fatty acid recovery of our procedure with a standard fatty acid mixture (containing saturated and unsaturated fatty acids in the range of C_{12} to C_{23}) showed that roughly 91% of the total material was recovered. Losses were primarily of the higher molecular weight ($>C_{21}$) saturated fatty acids. When dealing with long chain saturated fatty acids ($>C_{21}$), pentane no longer seems to be an adequate solvent. However, most marine phytoplankters do not contain saturated fatty acids of molecular weight $>C_{21}$ (8). Replicate samples from the cultures showed that the entire method, beginning with the cells suspended in their medium and ending with the final analyses, is as precise as the CI–MS analysis alone.

To compare the CI–MS method with the GLC method, a GLC analysis of an aliquot of sample *C* (see Fig. 5) was performed (**Fig. 6**). The results produced with the GLC are qualitatively consistent with the results obtained with the MS. For a quantitative comparison of the two methods (see **Table 1**), the peak areas of the GLC signals were manually determined and divided by the C number of the corresponding fatty acid. The expected differences between the two methods were observed; that is, when using the (M + 1) signals as markers, the CI–MS method generally underestimates the relative abundance of the unsaturated (especially polyunsaturated) fatty acids with regard to the saturated fatty acids. This phenomenon is primarily attributable to the greater fragmentation of the polyunsaturated fatty acids in the MS. If the exact absolute values for each fatty acid are required, a simple computer program using a calibration table (similar to the use of response factors in GLC) could be employed for the appropriate corrections. However, in our (and similar) studies concerning relative fatty acid changes in dynamic systems such corrections are not necessary.

TABLE 1. Quantitative comparison of the fatty acid analyses of sample C (see Figure 5) by GLC and CI–MS (*iso*-C_4H_{10})[a]

Fatty Acid	Method	
	GLC	CI–MS
$C_{14:0}$	90	110
$C_{15:0}$	8	10
$C_{16:0}$	96	128
$C_{16:1}$	100	100
$C_{16:3}$	83	54
$C_{16:4}$	41	26
$C_{18:4}$	39	28
$C_{20:5}$	66	42
$C_{22:6}$	12	7

[a] Values of each analysis are normalized on $C_{16:1}$ (= 100).

CONCLUSIONS

This method, including the extraction of cellular fatty acids and the CI–MS analysis, is very well-suited for rapid determination of the molecular weight distribution of cellular fatty acids. Because the CI–MS analysis of fatty acids does not require modification or extensive purification of samples, only a simple cellular extraction procedure is necessary. One person could comfortably extract and analyze up to eight samples in 8 hr; this efficiency compares favorably with past methods. The precision of the method enables the detection of minor fatty acid changes in dynamic systems. Only small quantities of material (10–20 μg of saponifiable matter) are needed; this, and the rapidity and precision of the analysis make it attractive to biologists interested in following changes in the fatty acid composition of growing cells. In addition, this method should facilitate the detection of other saponifiable material, such as hydroxy fatty acids and dicarboxylic acids, which are difficult to detect by GLC due to their low abundance and high polarity. It should be emphasized, though, that the CI–MS analysis leads to the determination of the molecular weight distribution of a fatty acid mixture. If a complete analysis of all the fatty acids present is required, including isomers of the same molecular weight, the traditional GLC and GLC–MS methods are the appropriate analytical tools. There are, however, few isomers of algal fatty acids (8; Fig. 6), thus minimizing the disadvantages of CI–MS for fatty acid studies of marine phytoplankton.

One important aspect of the method is that the raw data produced are already in a computer-readable, easily interpretable form. This allows the analyst to transform the data readily into the most convenient form for any given study without an extensive computer program. The compact quality of the data facilitates data storage in small computers. When dealing with large numbers of samples, access to such a data storage system employing a computer coupled to the analytical instrument offers a definite advantage, in that many sets of data can be retrieved quickly for examination and comparison. Most modern mass spectrometers have a minicomputer system and chemical ionization capability, so our method is of general applicability.

The CI–MS analysis of fatty acid mixtures presented in this report is, of course, not restricted to research concerning unicellular algae, but can be applied to any studies involving fatty acid analysis (e.g., biomedical applications and environmental research). Further, the CI–MS method should be applicable to the study of other compounds or compound classes, such as sterols or fatty alcohols.

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