Comparison of Fatty Acid Composition of Cell Homogenates and Isolated Chloroplasts in *Acetabularia crenulata* (Lamouroux)

Lory Z. Santiago-Vázquez[a,b,*] and Robert S. Jacobs[a]

[a] Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, CA 93106, USA

[b] Current address: Florida Atlantic University, Department of Chemistry and Biochemistry, 777 Glades Road, Boca Raton, FL 33431, USA. Fax: (561)297-2759. E-mail: santiago@fau.edu

* Author for correspondence and reprint requests

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Algal preparations from *Acetabularia crenulata* were analyzed for their fatty acid composition to establish the suitability of this alga as a model to study fatty acid oxidation and oxylipin biosynthesis. The work was based on two goals. The first goal of this study was to determine the contribution of fatty acids from contaminating bacteria and how this influenced the total fatty acid composition of cell homogenates of *A. crenulata* collected in the wild as compared to specimens cultured in sterile conditions. The major fatty acids detected for both specimens were palmitic (C16:0), palmitoleic (C16:1*n*-7), oleic (C18:1*n*-9), linoleic (C18:2*n*-6), linolenic (C18:3*n*-3), and octadecatetraenoic acid (C18:4*n*-3). Significant amounts of odd-chain fatty acids common to bacteria were not detected in either sample. Furthermore, branched-chain fatty acids, typical bacterial biomarkers, were not detected in either sample. Data suggest that bacteria do not greatly contribute to the total fatty acid pool of *A. crenulata*.

The second goal was to compare the fatty acid composition of cell homogenates with that of isolated chloroplasts. Comparatively speaking palmitoleic and octadecatetraenoic acid were found at significantly lower concentrations in the chloroplast whereas oleic and linolenic acid were found at significantly higher amounts in this organelle. Furthermore, the amount of hexadecatrienoic acid (C16:3), a fatty acid commonly esterified to monogalactosyldiacylglycerol (MGDG; lipid present at high concentrations inside the chloroplasts of algae), was present at very low concentrations in these plastids (0.7%). Typically green algal follow the "prokaryotic pathway" for MGDG biosynthesis where C18:3 is esterified at the s*n*-1 position of the glycerol backbone and C18:3 or C16:3 at the s*n*-2 position, making C16:3 a major fatty acid inside chloroplasts. Interestingly, our results suggest that chloroplasts of *A. crenulata* appear to follow the "eukaryotic pathway" for MGDG biosynthesis where C18:3 is both at the s*n*-1 and s*n*-2 position of MGDG. Taking into account the exceptions noted, the fatty acid composition for *A. crenulata* is similar to that reported for most chlorophytes.

Key words: Acetabularia crenulata, Fatty Acids, MGDG

Introduction

The tropical coenocytic alga *Acetabularia crenulata* has been the subject of extensive developmental biology studies (reviewed in Berger and Kaever, 1992; Bonotto, 1988). Its relative abundance in nature, ease of collection, established laboratory culture conditions (Berger and Kaever, 1992), and published lipid data of related species such as *Acetabularia mediterranea* (Moore and Tschimadia, 1977) can make this an ideal model to study fatty acid composition and oxylipin biosynthesis. Fatty acids are important components of cells and fatty acid composition has been used as a taxonomical tool for a variety of organisms (Carballeira *et al.*, 1997; Krüger *et al.*, 1995). More importantly, these compounds are the precursors to oxylipins. Oxylipins are members of the eicosanoid family of compounds, important secondary metabolites involved in the inflammation cascade of mammals. These compounds are also thought to be important defense molecules in plants and algae (Blée, 1998).

The fatty acid composition of green algae is similar to that of higher plants. The main constituents are members of the C16 and C18 families such as palmitic acid (C16:0), hexadecatetraenoic acid (C16:4*n*-3), oleic acid (C18:1*n*-9), linoleic acid (C18:2*n*-6), linolenic acid (C18:3*n*-3), and octade-

Abbreviations: AA, arachidonic acid; PUFA, polyunsaturated fatty acid; SAFA, saturated fatty acid; MGDG, monogalactosyldiacylglycerol.

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catetraenoic acid (C18:4*n*-3). C20 fatty acids such as arachinoic acid (AA) (C20:4*n*-6) are present in small amounts (Jamieson and Reid, 1972; Khotimchenko, 1995; Wiltshire *et al.*, 2000; Xu *et al.*, 1998). Exceptions are *Anadyomene stellata* (Bemis *et al.*, 2000) and *Parietochloris incisa* (Bigogno *et al.*, 2002). Both organisms have a high concentration of AA. The fatty acid C16:3*n*-3 is ubiquitous in Chlorophytes. This fatty acid is commonly esterified to monogalactosyldiacylglycerol (MGDG) in alga that follow the "prokaryotic pathway" of MGDG biosynthesis.

The *Acetabularia mediterranea* fatty acid composition at different morphological stages was analyzed (Dubacq, 1972, 1973). In summary, the young cells were found to be rich in oleic and palmitic acid and very poor in polyunsaturated fatty acids (PUFAs), which only accounted for 24% of total fatty acid content. As cells mature, there was an increase in PUFA content in lipids such as MGDG, with high amounts of linoleic (30%) and linolenic acid (15%). This resembles the fatty acid composition of other Chlorophytes. During the maturation of the alga's cysts the total lipid and fatty acid content of the alga decreases, suggesting a possible role of fatty acids in the growth and reproduction of *A. mediterranea.* A general fatty acid study performed in *A. crenulata* reported high amounts of AA, C16:2, and C16:4 (Brush and Percival, 1972). The same study reported smaller amounts of C20:1 and C20:2. To our knowledge, there are no detailed reports investigating the relative fatty acid composition and subcellular distribution in *A. crenulata*.

In our efforts to better understand the oxylipin biosynthetic pathway in primitive algae, our group has successfully used isolated chloroplasts of the alga *Anadyomene stellata* (Mikhailova *et al.*, 1995) and cell homogenates of *Euglena gracilis* (Santiago-Vázquez *et al.*, 2004) to study the oxidation of fatty acids into oxylipins. We want to expand our studies to include *Acetabularia crenulata* and to establish this alga as a suitable model to study oxylipin biosynthesis by analyzing its fatty acid content in cell homogenates and in isolated chloroplasts. Our first goal was to determine the contribution of bacterial fatty acid contamination to *A. crenulata's* fatty acid content by comparing cell homogenates of wild alga to those of cultured alga grown in sterile conditions. The second goal was to compare the fatty acid content of isolated chloroplasts to that of cell homogenates preparations. Fatty acid biosynthesis occurs inside the chloroplasts and it is important to analyze these for potentially significant differences. These studies allowed us to determine which preparations are better suited for the study of oxylipin biosynthesis.

Results and Discussion

Total fatty acid composition of the cell homogenates

A detailed study of fatty acid composition is the first step necessary to establish a sound base for oxylipin metabolism in *A. crenulata.* Many factors such as growth stage and time of day (Dubacq, 1972, 1973; Vanden Driessche *et al.*, 1997), and the presence of microorganisms that contaminate the field (wild) collections can greatly influence the fatty acid composition of an organism. For this reason, we decided to compare the fatty acid composition of cell homogenates of algae collected in the wild with cell homogenates of algae cultured under sterile conditions to determine if the culture conditions were a contributing factor to the total fatty acid content of *A. crenulata.* Cell homogenates have been previously shown to be useful for the study of oxylipin biosynthesis (Santiago-Vázquez *et al.*, 2004).

The major fatty acids in both wild and cultured algae were composed of palmitic (C16:0), palmitoleic (C16:1*n*-7), oleic (C18:1*n*-9), linoleic (C18:2*n*-6), linolenic (C18:3*n*-3), and octadecatetraenoic acid (C18:4*n*-3; Table I). None of the major fatty acids listed above were found to be significantly different from each sample by one-way ANOVA analysis of variance (Table I). However, statistically significant differences were noted in minor fatty acids (each present at less than 1%) such as C20:3*n*-6. These fatty acids did not significantly contribute to the total fatty acid composition of *A. crenulata.* Odd-chain fatty acids were found to consist of 0.73% of the total fatty acid content in wild alga when compared to 2.44% in cultured alga. We were expecting to find a higher incidence of odd-chain fatty acids in the wild specimens when compared to cultured specimens. These odd-chain fatty acids are usually common among bacteria (Alvarez *et al.*, 2001; Hall *et al.*, 1998) and uncommon among algae. Branched-chain fatty acids, typical bacteria biomarkers (Hopkins and MacFarlane, 2000; Sebastian and Larsson, 2003), were not detected in either preparation. These data suggest microorganisms do not

Table I. FAME composition of *A. crenulata* preparations and one-way analysis of variance. All pairwise multiple comparison procedures were done by using the Holm-Sidak method. The major fatty acids percentages are shown in italics.

FA families	FAMEs	Percentage of the total fatty acids (%)		
		Wild alga $n = 8$	Cultured alga $n = 3$	Chloroplasts $n = 4$
C12	C12:0	0.20 ± 0.09	0.25 ± 0.14	0.45 ± 0.45
C14	C14:0	1.65 ± 0.31	1.13 ± 0.28	1.97 ± 0.21
C15	C15:0	0.26 ± 0.13	0.16 ± 0.09	0.00 ± 0.00
C16	C16:3[#]	*4.26 ± 0.82*	*5.89 ± 1.61*	*0.74 ± 0.54**
	C16:2[#]	2.61 ± 0.63	3.29 ± 0.38	1.46 ± 0.19
	C16:1*n*-7	*9.56 ± 1.31*	*9.86 ± 1.14*	*4.37 ± 0.57**
	C16:0	*13.37 ± 1.91*	*10.04 ± 2.63*	*16.11 ± 3.55*
C17	C17:2[#]	0.03 ± 0.03	0.24 ± 0.12*	0.00 ± 0.00
	C17:1[#]	0.02 ± 0.02	0.34 ± 0.22*	0.00 ± 0.00
	C17:0	0.42 ± 0.23	0.27 ± 0.15	0.07 ± 0.09
C18	C18:4*n*-3	*10.84 ± 2.14*	*10.03 ± 2.77*	*2.82 ± 1.78*
	C18:3*n*-6	*3.06 ± 0.46*	*6.51 ± 2.35*	*2.99 ± 1.63*
	C18:3*n*-3	*19.33 ± 1.31*	*14.50 ± 4.23*	*28.38 ± 3.26**
	C18:2*n*-6	*18.30 ± 1.99*	*15.44 ± 2.01*	*17.26 ± 1.95*
	C18:1*n*-9	*6.64 ± 0.60*	*6.06 ± 1.37*	*12.10 ± 1.72**
	C18:0	1.33 ± 0.29	2.57 ± 0.26	2.69 ± 0.82
C19	C19:2[#]	0.00 ± 0.00	0.64 ± 0.48*	0.00 ± 0.00
	C19:1[#]	0.00 ± 0.00	0.79 ± 0.55*	0.00 ± 0.00
C20	C20:5*n*-3	2.50 ± 0.49	3.06 ± 0.33	2.11 ± 0.56
	C20:4*n*-6	1.02 ± 0.21	2.76 ± 1.00	1.26 ± 0.43
	C20:4[#]	1.10 ± 0.24	1.44 ± 0.37	1.50 ± 0.49
	C20:3*n*-6	0.12 ± 0.06	0.98 ± 0.49*	0.00 ± 0.00
	C20:2[#]	0.04 ± 0.04	0.55 ± 0.28*	0.09 ± 0.12
	C20:1*n*-12	1.13 ± 0.44	0.82 ± 0.42	1.52 ± 0.55
	C20:0	0.04 ± 0.04	0.20 ± 0.11	0.00 ± 0.00
C22	C22:5[#]	1.80 ± 0.41	2.18 ± 0.95	1.86 ± 0.77
	C22:1*n*-9	0.37 ± 0.37	0.00 ± 0.00	0.25 ± 0.25
	SAFA	17.27	14.62	21.29
	PUFA	82.73	85.38	79.29
	Ratio 18:3 to 18:1	2.91	2.39	2.35

[#] Location of the double bond is unknown.

* Analysis of variance, $p < 0.05$, difference between this and the other two groups is significant.

greatly affect the total fatty acid composition of *A. crenulata.* These results also suggest that *A. crenulata* is capable of using substrates other than acetate, such as propionate (C3 acid), as a substrate to synthesize odd-chain fatty acids. Both cell homogenates were found to be suitable for oxylipin biosynthesis by having a large presence of C18 fatty acids and a relatively small presence of C20 fatty acids, both of which can be involved in the biosynthesis of these compounds. Overall, the fatty acid composition of the two preparations of cell homogenates of alga collected in the wild and of alga cultured in the lab was found to be very similar (Table I).

Cell homogenates were also saponified and methylated in acidic methanol following the methodology of Sasser (1997; data not shown). Because results were not significantly different from those obtained from samples methylated by diazomethane, they were not included in this paper.

Chloroplast fatty acid composition compared to cell homogenate fatty acid composition

Our second goal was to compare the total fatty acid composition of the chloroplast to the total fatty acid composition of cell homogenates because fatty acid biosynthesis occurs mainly inside this organelle (Stumpf, 1987).

The major fatty acids in the chloroplast preparation were composed of palmitic, oleic, linoleic and linolenic acid, with linolenic acid reaching almost 30% of total fatty acid composition (Table I). In broad terms, the fatty acid composition of the chloroplast is similar to that of cell homogenates.

However, key differences were noted. The major fatty acids linolenic (C18:3*n*-3) and oleic acid (C18:1*n*-9), isolated from chloroplasts, were found at statistically significant higher percentages when compared to cell homogenates. Palmitoleic (C16:1*n*-7) and hexadecatrienoic acid (C16:3), major fatty acids in cell homogenates, were found at smaller percentages in the fatty acids of the chloroplast ($p < 0.05$). *A. crenulata* chloroplasts had the highest oleic acid percentage and the second highest palmitic acid percentage when compared to cell homogenates (Table I). The overall percentage for the major fatty acids palmitic, oleic, linoleic and linolenic acid in chloroplasts correlates well with that reported by Dubacq (1972, 1973) for fatty acid composition of neutral lipids of mature cells of *A. mediterranea.* Since there was not much difference between the fatty acids of cells collected from the wild and those cultured in the lab, we decided to use cultured cells for the isolation of chloroplasts and determination of their fatty acid content.

Chloroplasts of algae are believed to synthesize MGDG through the "prokaryotic pathway" where C18:3 is esterified to the *sn*-1 position of the glycerol backbone and C16:3 or C18:3 is esterified to the s*n*-2 position. Organisms that follow the "eukaryotic pathway" of MGDG biosynthesis have C18:3 esterified at both the s*n*-1 and s*n*-2 position. MGDG is one of the most abundant lipids in the chloroplasts of *Acetabularia* (Moore and Tschimadia, 1977), and *Acetabularia* is rich in chloroplasts (Maréchal *et al.*, 1997; Vanden Driessche, 1973). From this statement one could assume that the fatty acids C18:3 and C16:3 should be present at very high concentrations inside the chloroplasts of *A. crenulata.* While C18:3 makes up almost 30% of the total fatty acid content of the chloroplast, C16:3 is present as a minor fatty acid (0.7%) inside the chloroplast. This is a much lower percentage from what would be expected if the "prokaryotic pathway" for MGDG biosynthesis was occurring in the chloroplasts of *A. crenulata.* Interestingly, it seems that C16:3 is more abundant outside the chloroplast since cell homogenates of cultured specimens had almost 6% of this fatty acid. From these results we propose that a "eukaryotic pathway" of MGDG biosynthesis appears to be prevalent in chloroplasts of *A. crenulata*.

The polyunsaturated fatty acid (PUFA) content in *A. crenulata* ranged from 75 to 85%, much higher than the saturated fatty acid (SAFA) content. In other green algae such as *Codium* the total PUFA content varied from 17 to 54% (Xu *et al.*, 1998). In *Caulerpa* the major fatty acids were not PUFAs but SAFAs ranging from 82–92% (Khotimchenko, 1995). The genera *Enteromorpha* sp., *Ulva* sp., and *Cladophora* sp. seem to have PUFA levels comparable to those found in *A. crenulata.*

In summary, the major fatty acids of *A. crenulata* chloroplasts and cell homogenates were composed of monounsaturated and polyunsaturated fatty

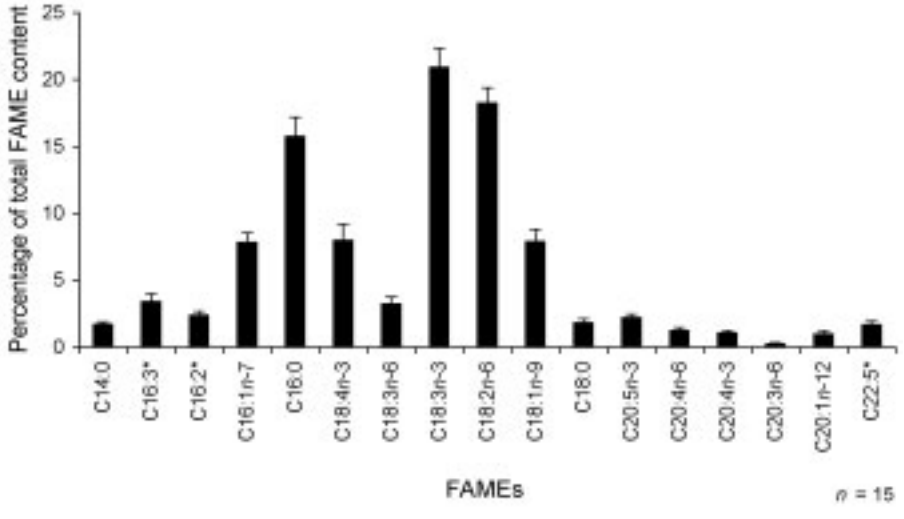

Fig. 1. Average total fatty acid composition for all the preparations of *A. crenulata* included in this study. This graph only displays the major fatty acids.
*, Location of the double bond is unknown.

acids of C16 and C18 families (Fig. 1). In all samples C20 and C22 fatty acids were observed but not at high percentages when compared to the C16 and C18 families. Eicosapentaenoic acid (EPA, C20:5*n*-3) was always the most prominent of the C20 group. Our data contradicts previous papers (Brush and Percival, 1972) that reported that *A. crenulata* had high concentrations of AA, C16:2 and C16:4. In our study AA and C16:2 were found at very low percentages and C16:4 was not detected. We did report small percentages for C20:1 and C20:2 as suggested in the previous study. Differences might be due to different technologies available at the time or the growth stage and different preparations used for both studies. As mentioned previously, our choice of preparations correlated well with previous experiments by our group and with the future studies in oxylipin metabolisms planned.

Experimental

Materials, collection sites and algal culture

Phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), leupeptin, pepstatin A, *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (diazald), polyvinylpyrrolidone (PVPP), trizma hydrochloride [tris-(hydroxymethyl)aminomethane hydrochloride], Percoll, and cell culture chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Fatty acid standards were obtained from Matreya, Inc. (Pleasant Gap, PA). HPLC-grade organic solvents, Miracloth, and all filters were obtained from Fisher Scientific (Pittsburgh, PA). Plastic containers for cell culture (PLANTCON) were obtained from ICN (Aurora, OH).

Samples of mature cells of *A. crenulata* were collected at Long Key and at Sugar Loaf Key in the Florida Keys during the months of June to September from 1997 to 2001. *A. crenulata* was usually found in very shallow water (less than 0.5 m) attached to rocks that were easily transported to the laboratory. The alga was cleaned of epiphytes and debris in fresh seawater at the Keys Marine Laboratory, Long Key, Florida, immediately flash frozen in liquid N_2, packed in dry ice, and flown overnight to Santa Barbara, California, where the alga was stored at – 70 °C until used.

Samples of living young cells of *A. crenulata* were obtained from the Max Planck Institute for Cell Biology in Ladenburg, Germany. Cells were placed in sterile plastic containers (ICN) with Müller's medium (Schweiger *et al.*, 1977) and exposed to a 12 h:12 h light:dark cycle at 190 ft-c (footcandles, lumens per square foot) at a temperature of 28 °C (Berger and Kaever, 1992).

Preparation of cell homogenates

A. crenulata cells (approx. 100–130 g) were homogenized in chilled phosphate buffer, pH 7.8 (2 mM EDTA, 2 mM DTT, 1 mM PMSF, 1μg/ml leupeptin and 1μg/ml of pepstatin A). After the cell free extract was made, PVPP was added to bind phenolic constituents. The extract was filtered through Miracloth, centrifuged at 1000 × *g* on a refrigerated (4 °C) TJ-6 Beckman centrifuge to eliminate any remaining debris, and the supernatant was flash frozen in liquid N_2, and stored at – 70 °C until used.

Isolation of intact chloroplasts

Cells (~ 130 g) were homogenized in isolation buffer made of 100 mM trizma hydrochloride, pH 7.8, 600 mM sorbitol, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 2 mM EDTA, 2 mM DTT, and 1 mM PMSF following the procedure by Tymms and Schweiger (1985). In summary, the extract was passed through Miracloth and centrifuged in a Sorvall SS-34 rotor (Sorvall RC-5B Refrigerated Superspeed Centrifuge, DuPont Instruments, Newton, CT). The supernatant was decanted and the pellet was manually separated from starch, re-suspended in 100 ml of isolation buffer, filtered through 11 μm mesh nylon cloth, and re-filtered through a 5 μm polycarbonate membrane filter. The filtrate was centrifuged in a Sorvall HS-4 rotor. Pellets were re-suspended in isolation buffer and loaded on a 5–80% linear Percoll gradient prepared with a gradient mixer. The gradient was centrifuged at 1300 × *g* for 30 min in a Sorvall HS-4 rotor (4 °C). The lower dark green band composed of mostly intact chloroplasts was collected and separated from Percoll. Chloroplast pellets were re-suspended in isolation medium, flash frozen and stored at – 70 °C until used. Protein concentration for cell homogenates and isolated chloroplasts was determined by a Bradford protein assay using a Bio-Rad Protein Assay Kit (Hercules, CA). Typical concentrations ranged from 50–500 μg of protein per ml of buffer.

Preparation of methyl esters and sample preparation for chromatography

Lipids of algal preparations were extracted twice in chloroform/methanol ($CHCl_3$/MeOH 2:1 v/v). The organic layers were pooled together the volume was reduced *in vacuo* and samples were re-suspended in methylene chloride (CH_2Cl_2) in preparation for diazomethane methylation. Chloroplasts were homogenized in the extraction solvent and treated as explained above.

Organic extracts were methylated using etheral diazomethane (Lombardi, 1990). Samples were filtered through 0.2 μm nylon membrane HPLC filters (Acrodisc 13 mm Syringe Filter), dried, weighed, and re-suspended to 40 μg/μl in MeOH. For fatty acid methyl ester (FAME) analysis, the samples were concentrated to 100 ng/μl in hexanes. Samples were stored under N_2 at −70 °C.

FAME chromatography

FAMEs were separated from other lipids and pigments using Sep-Pak Light Silica Cartridges (Waters, Milford, MA) for solid phase extraction (MacPherson *et al.*, 1998). Lipids were fractionated with increasing percentages of ethyl acetate (EtAc) in hexanes starting from 100% hexanes and ending in 100% EtAc. FAMEs usually eluted between 99.5% and 99.0% hexanes in EtAc (fractions 2 and 3). Fractions were spotted in analytical aluminum-backed silica gel TLC plates (Whatman, Fischer) using a solvent system of hexanes/EtAc (90:10 v/v). Fatty acids were viewed by spraying the plate with 3% vanillin (w/v, 0.5% glacial acetic acid) in 200 proof ethanol (EtOH). FAMEs were localized by comparing Rf and spot color with authentic standards. The fraction containing FAMEs was weighed, diluted to 100 ng/μl in hexanes, and stored at − 70 °C until analyzed. Other fractions were analyzed by GC-MS but these contained no significant amounts of fatty acids and were contaminated with other components not relevant to this study.

FAMEs were analyzed on a Hewlett-Packard 5890 Gas Chromatograph coupled to a HP5970 mass selective detector (MSD) operating on EI mode following established procedures by Bemis *et al.* (2000) and MacPherson *et al.* (1998). The detector transfer line was 280 °C. The carrier gas used was helium. The MSD was scanned from m/z 40–500 at a scan rate of 1.75 scans/s. The capillary column used was a DB-5MS column, 30 m × 0.25 mm i.d. (J & W Scientific, Folsom, CA). The flow rate is not directly controlled on the gas chromatograph, but the column head pressure was set to 5 psi. The injector had split/splitless capabilities and was operated in splitless mode. A 1 to 2 μl aliquot of the FAME sample was injected. The column was held at 50 °C for 1 min, ramped to 125 °C at 18°/min, and then ramped to 255 °C at 3 °C/min. The FAMEs were identified by comparing the mass, retention times, and fragmentation patterns to a mix of authentic FAME standards (Matreya). Hewlett-Packard Chemstation Enhancement Software was used for data analysis. The suitability of this system was tested and confirmed by running authentic standards and comparing results with those provided by the manufacturer (Matreya).

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