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A Biochemical GC–MS Application for the Organic Chemistry Laboratory: Determination of Fatty Acid Composition of *Arabidopsis thaliana* Lipids

Jared D. Bender, Arthur J. Catino III, Kenneth R. Hess, Michael E. Lassman, Phyllis A. Leber,* Michael D. Reinard, and Neil A. Strotman

Department of Chemistry, Franklin & Marshall College, Lancaster, PA 17604-3003; *P_Leber@acad.fandm.edu

Carl S. Pike

Department of Biology, Franklin & Marshall College, Lancaster, PA 17604

With the advent of inexpensive modular GC–MS units amenable to student use, there is a trend toward inclusion of this powerful instrumental technique in organic laboratories. As an indication of this, a survey of the *JCE Index* (online) reveals that more than ten experiments related to GC–MS analysis have appeared in this *Journal* since 1990. However, only three of these deal with biochemical applications (*1*–*3*) and only one of these three describes the use of GC–MS for fatty acid analysis (*1*).

The relatively fast analysis time, interactive software, and internal databases characteristic of GC–MS instruments contribute substantially to their ease of use. But what are the pedagogical reasons for incorporating GC–MS into teaching labs? This instrumental technique combines two powerful laboratory operations—separation and identification. It is also ideal for interdisciplinary work, especially at the biochemical interface. Now that the ACS Committee on Professional Training has mandated that an ACS-approved curriculum will provide a strong biochemical component either by inclusion of a separate course in the curriculum or by integration of biochemistry throughout the curriculum, there is even greater incentive to develop biochemical laboratory experiments.

We have given a preliminary report of our attempts to develop experimental biochemical modules for the organic laboratory sequence based on the theme of assays of plant constituents (*4*). We have devised one module to determine the qualitative and quantitative fatty acid composition of lipids from leaf samples. The analysis can be performed with routine leaf samples such as spinach. Here, we present a variation that enables students to compare the fatty acid composition of *Arabidopsis thaliana* (the major genetic model system in the plant kingdom) wild type (WT) with four fatty acid biosynthetic mutants (*fadA–fadD*).

There are four facets of this project: (i) synthesis and characterization of individual fatty acid methyl esters (FAMEs) as standards for GC–MS analysis, (ii) isolation of the fatty acids of *A. thaliana* leaves, both WT and mutants, as FAMEs, (iii) GC–MS analysis of the leaf extracts for the fatty acid composition of leaf lipids, and (iv) comparison of the class results with the literature data for WT and the four mutants and with a biochemical model of two pathways for lipid biosynthesis (Fig. 1) in *A. thaliana* leaves (*5*). Because this experimental paradigm links organic synthesis and spectral characterization by IR and NMR, both ^{1}H and ^{13}C, with separation and identification via GC–MS analysis, all of the key areas of laboratory procedure are encompassed in this single project.

Experimental Design

Materials

Methanolic HCl (3 N) is purchased from Supelco. The lipid standard, which is supplied by Alltech as a 1:1:1:1:1 by weight mixture of methyl palmitate (16:0), methyl stearate (18:0), methyl oleate (18:1), methyl linoleate (18:2), and methyl linolenate (18:3), is stored in the freezer at 0 °C.

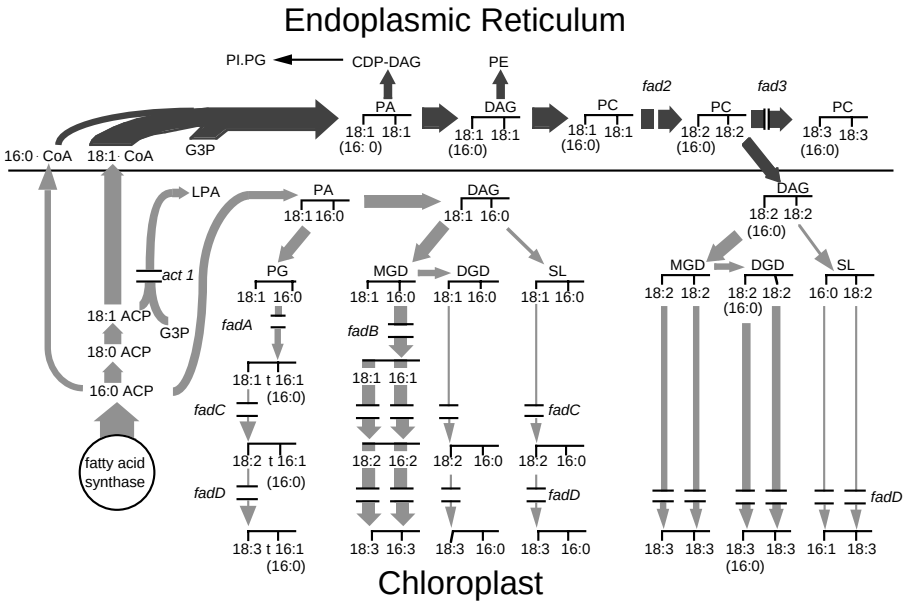

Figure 1. An abbreviated scheme showing the two pathways for glycerolipid synthesis in *Arabidopsis* leaves. (Used with permission of the American Society of Plant Physiologists.)

Growth of Arabidopsis thaliana

Seeds of *Arabidopsis thaliana L.* (available from Arabidopsis Biological Resource Center, Ohio State University) are sown in greenhouse potting soil moistened with $^1/_4$-strength Hoagland nutrient solution. The pots are placed at 4 °C for 2–3 days and then transferred to a plant growth chamber. (Alternatively, plants can be grown in a greenhouse under low light.) The growth conditions are constant temperature of 24 °C, 14-h photoperiod, and illumination with mixed fluorescent–incandescent bulbs providing photosynthetically active radiation of 100 µmol photons m^{-2} s^{-1}. Seedlings are thinned to about 2 cm apart. Nutrient solution and distilled water are provided alternately by subirrigation. Leaves are harvested about 3–4 weeks after planting, before flowering occurs.

Equipment

An HP GCD System (Model G1800A) equipped with an electron ionization detector and fitted with a Supelco SP-2330 capillary column (30 m × 0.25 mm i.d.) is utilized for the FAME analysis. The temperature program is 180–187 °C at a rate of 1 °C/min, and the flow rate of the helium carrier gas is 1 mL/min. There is a 2-min solvent delay, and the peak elution time is less than 6 min.

Synthesis of FAME Standards

A textbook procedure for the synthesis of ethyl laurate is used as a point of reference (*6*). A sample of the assigned fatty acid is refluxed in the presence of an excess of methanol and a catalytic amount of acetyl chloride, which reacts with methanol to generate the actual HCl catalyst in situ.

An alternate convenient method for esterifying fatty acids is to treat the fatty acid with excess methanol and chlorotrimethylsilane at ambient temperature (*2*). The experimental conditions are mild, and the procedure affords high yields of the FAME product in short reaction time.

Isolation of FAMEs from Plant Material

Approximately 100 mg of crushed plant leaf tissue is heated at 80 °C for 1 h in 1 mL of 3 N methanolic HCl, a reagent that effects both tissue digestion and transesterification. The cooled plant extract is then treated with 1 mL of 0.9% NaCl(aq) and extracted with 2 mL of hexane. The hexane layer is separated and concentrated to ca. 0.1 mL. Where an internal standard is required, 3 µL of methyl heptadecanoate (17:0) is added to exactly 100 µL of this solution.

GC–MS Analysis

A 1-µL injection made in split mode provides sufficient ion count to obtain reproducible analyses. The printout yields a chromatogram of total ion count (abundance) versus retention time and a tabular display of peak area percent for each component and peak identification from the library database search. Molecular ion peaks are observable in the mass spectra.

Results and Discussion

This experiment has been conducted by three different laboratory sections of approximately 20 students each during two successive laboratory periods. The synthesis of the FAME standard is performed during the first 4-h lab period, and the plant extraction, preparation of FAMEs, and the GC–MS analysis are accomplished during the second. When fresh acetyl chloride is used, average yields of 80% are obtained. Similar yields are also achieved using *p*-toluenesulfonic acid and boron trifluoride–methanol complex as alternative catalysts.

The ^{1}H NMR spectra are sufficiently resolved that most deshielded hydrogens can be readily identified. However, several nonequivalent methylene hydrogens overlap in the upfield region around 1.3 ppm. Virtually all ^{13}C NMR spectra are easily interpretable. In a few cases, some peak overlap due to near equivalent methylene carbons occurs in the upfield region around 29–30 ppm. Representative NMR data acquired on a Varian Unity 300 spectrometer operating at 300 MHz for ^{1}H and 75.4 MHz for ^{13}C are available online as supplemental material for those who do not have access to a high-field NMR spectrometer.[W]

Identification of each individual FAME is achieved both by GC retention time comparison with the prepared standards and by MS database match (from composite class data). The latter is especially important for FAME identification in those cases where the precursor fatty acid is not readily available commercially.

A comparison of our data (Table 1) with literature values (Table 2) for *A. thaliana* (*11*), both WT and the mutants, reveals that experimental values have relative deviations as low

Table 1. GC–MS Fatty Acid Composition of Leaf Lipids of *Arabidopsis thaliana* WT and Mutants ("New" Samples)

Retention Time/min	FAME[a]	Fatty Acid Content (%)				
		WT	Mutant			
			fadA	*fadB*	*fadC*	*fadD*
2.5	16:0	21.3	26.9	31.5	18.5	18.2
2.7	16:1	5.7	—	—	16.4	4.1
3.1	16:2	—	—	—	—	7.4
3.6	16:3	10.9	9.7	—	—	—
3.4	18:0	—	—	—	—	—
3.7	18:1	—	—	—	24.7	—
4.3	18:2	13.4	12.4	14.1	11.7	33.3
5.2	18:3	48.8	51.0	54.4	28.8	36.9

NOTE: "New" samples were stored in a freezer (-20 °C) for 1 month.
[a]The first integer represents the number of carbon atoms in the corresponding fatty acid; the second integer indicates the number of sites of unsaturation in the carbon chain.

Table 2. Normalized Literature Values for Fatty Acid Composition of Leaf Lipids of *Arabidopsis thaliana* WT and Mutants

FAME	Fatty Acid Content (%)				
	WT (*7*)	Mutant			
		fadA (*8*)	*fadB* (*9*)	*fadC* (*10*)	*fadD* (*11*)
16:0	15.9	19.0	26.4	14.1	17.3
16:1	4.0	—	—	15.2	4.0
16:2	—	—	—	—	5.3
16:3	14.6	12.0	—	—	—
18:0	—	—	—	—	—
18:1	—	—	—	16.3	—
18:2	16.6	19.1	18.7	16.8	32.3
18:3	48.7	48.9	54.9	37.6	39.7

Note: Excluding FAMEs present in amounts not detectable by GC–MS.

Table 3. GC–MS Fatty Acid Composition of Leaf Lipids of *Arabidopsis thaliana* WT and Mutants ("Old" Samples)

FAME	Fatty Acid Content (%)			
	WT	Mutant		
		fadA	*fadB*	*fadC*
16:0	28.3	37.0	45.5	33.3
16:1	—	—	—	14.1
16:2	—	—	—	—
16:3	7.2	4.1	—	—
18:0	—	—	4.8	—
18:1	—	5.0	—	28.4
18:2	22.6	25.1	19.7	13.6
18:3	41.9	28.9	30	10.7

NOTE: "Old" samples were stored in a freezer (–20 °C) for 10 months.

as 1% and, in a few cases, as high as 25%. However, the trends within the series are as expected: (i) *fadA* differs from WT by the lack of 16:1 and a modest increase in 16:0 due to the partial interruption of the 16:0 → 16:1 conversion; (ii) *fadB* has appreciably more 16:0 than WT owing to more extensive interference with the 16:0 → 16:1 desaturase; (iii) compared to WT, *fadC* contains more 16:1 owing to some blockade of the 16:1 → 16:2 conversion and substantially more 18:1 accompanied by significantly less 18:3 owing to the prevention of 18:1 → 18:2 dehydrogenation; and (iv) *fadD*, unlike WT, contains detectable amounts of 16:2 due to inhibition of the 16:2 → 16:3 desaturase and substantially more 18:2 accompanied by somewhat less 18:3 owing to suppression of the 18:2 → 18:3 conversion. All of the trends are consistent with the Browse model, Figure 1 (*5*).

Variations on the Theme

The plant extraction procedure has been adapted from a published protocol (*12*) for the extraction of FAMEs from spinach leaves. When it is not feasible to grow *A. thaliana* plants from seed, the availability of spinach enhances the versatility of the experiment. The experimental results with spinach leaves can also be compared with the literature values (*12*).

We have also analyzed *A. thaliana* leaves to determine the effect of long-term storage or freezing on the FAME composition. As shown in Table 3, long-term storage in the freezer causes a marked increase in 16:0 (and 18:2 in WT and *fadA*) and an appreciable decrease in 18:3. These observations are consistent with those reported when dehydration of alfalfa leaves via lyophilization was delayed for 24–48 h (*13*).

This experiment permits a myriad of hypothesis-testing variations. Plants can be grown at different temperatures and for different lengths of time to determine if and how fatty acid composition varies in *A. thaliana* as a function of growth temperature or age. Different types of plant leaves can be examined to ascertain if each plant (e.g., *A. thaliana* vs spinach) has a unique FAME fingerprint.

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[W]Supplemental Material

Student instructions and instructor notes are available as supplemental material in this issue of *JCE Online*.

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