

## A Gas Chromatographic–Mass Spectrometric Method Using a PoraPLOT Column for the Detection of Hydroperoxide Lyase in *Chlorella pyrenoidosa*

**ABSTRACT:** A gas chromatographic–mass spectrometric (GC–MS) method using a PoraPLOT Q column was developed for the analysis and identification of the volatile products produced by the action of hydroperoxide lyase (HPLS) upon 13-hydroperoxylinoleic or 13-hydroperoxylinolenic acids. The developed procedure required no derivatization, was not affected by the presence of water, did not require cryogenic conditions to be maintained during injection, and allowed for the quantitation of most products. An acetone powder preparation of *Chlorella pyrenoidosa* cells was triturated with borate buffer pH = 8.0, and the mixture centrifuged at 12,000 × g. The supernatant and pellet were assayed for HPLS activity by GC–MS analysis of the volatile products given by linoleic acid hydroperoxide. The data showed that the majority of HPLS activity resides in the pellet fraction, and that the primary volatile component was pentane, with smaller amounts of 2-(Z)-pentene and 1-pentene being produced. The fact that HPLS activity resides in the water-insoluble fraction of the acetone powder suggests that HPLS from *Chlorella* is a membrane-associated enzyme. This investigation also determined that a spectrophotometric assay using alcohol dehydrogenase for measuring HPLS activity was not specific, but measured enzymatic activity other than HPLS.

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Membrane-associated enzyme fatty acid hydroperoxide lyase (HPLS) cleaves linolenic or linoleic acid hydroperoxy derivatives **1** and **2**, respectively. HPLS from higher plants cleaves at the C–C bond adjacent to the hydroperoxide function that is proximal to the carboxy carbon to produce C<sub>12</sub> oxo-aldehyde **3** and hexanal **4**, and hexenals **6** and **8** (Scheme 1). In crude systems the corresponding reduced hexanol **5** or hexenols **7** and **9** often are detected. HPLS derived from algae, mushroom, and grass cleaves fatty acid hydroperoxides at the C–C bond adjacent to the hydroperoxide function distal to the carboxyl carbon to form C<sub>13</sub> oxo-aldehyde **10** and a C<sub>5</sub> fragment that varies with enzyme source (1). For exam-

ple, HPLS from the microalga *Chlorella* will produce pentane **11** and 2-(Z)-pentene **14** (2), whereas HPLS from the blue-green microalga *Oscillatoria* and from grass produces pentanol, **12**, and 1-pentene-3-ol, **13** (3,4). Whereas HPLS in higher plants is membrane-associated, HPLS in *Chlorella* and *Oscillatoria* is reported to be water-soluble (2–4).

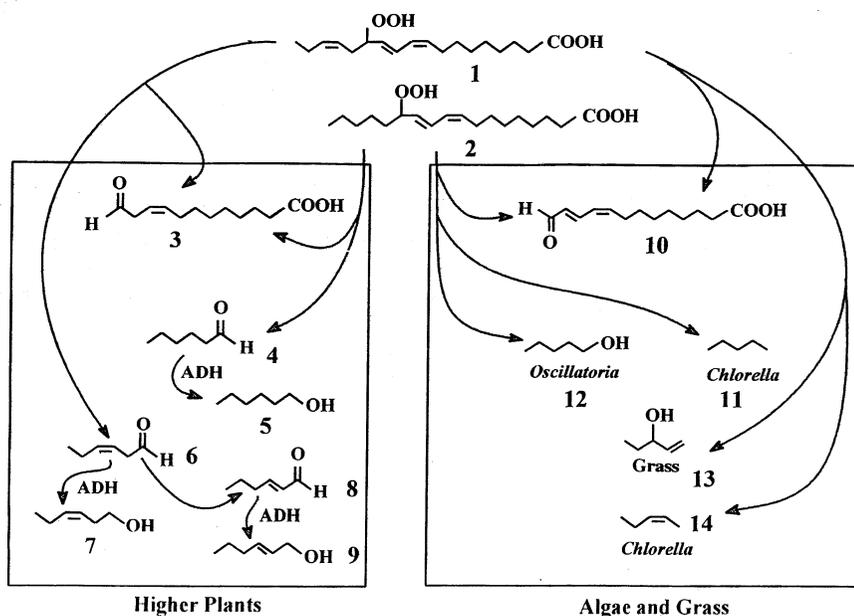
Although most studies involving HPLS are directed toward an understanding of lipid metabolism and the physiological role of HPLS products, there are also potential industrial uses for this enzyme. For example, the short-chain volatile products from the above reactions are major contributors to the characteristic fresh odor of many green-leaf plants and fruits that are of importance to the fragrance and flavor industry. On the other hand, the long-chain HPLS products, oxo-carboxylic acids, can be oxidized to dicarboxylic acids and used to produce polyamides similar to nylon 13,13.

Recently, we reported a procedure for immobilizing HPLS that was isolated from a partially purified aqueous extract of *Chlorella* (5). HPLS activity was assayed by a published method using **2** as substrate (6). This method is based on the ability of aldehyde **10** to serve as a substrate for yeast alcohol dehydrogenase (ADH) and thereby cause the oxidation of the ADH cofactor NADH. By following the oxidation of NADH spectrophotometrically, a crude or a partially purified enzyme extract could be assayed for HPLS activity. Doubts, however, arose about the actual presence of *Chlorella* HPLS when the expected oxo-carboxylic acid product **10** could not be identified. Accordingly, an alternative method was devised for the identification, isolation, and quantification of the volatile short-chain oxidation products formed from **2** by HPLS.

There are several prior reports concerning the analysis of short-chain HPLS oxidation products. One method measured volatile aldehydes by high-performance liquid chromatographic (HPLC) analysis of their 2,4-dinitrophenylhydrazone derivatives (7). Identification of the short-chain HPLS reaction products directly has been achieved by GC analysis of headspace volatiles, while the semivolatile products required solvent extraction (2–4,7–9). GC analysis was done with columns that separated the products based on their polarity. Accordingly, reported polar and apolar volatile products such as those expected from HPLS cleavage (Scheme 1) were poorly resolved.

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Abbreviations: ADH, alcohol dehydrogenase; GC, gas chromatography/chromatographic; HPLC, high-performance liquid chromatography; HPLS, hydroperoxide lyase; MS, mass spectrometer/spectrometry/spectrometric; NMR, nuclear magnetic resonance.



SCHEME 1

In this study, a GC-MS method using a porous polymer column (PoraPLOT Q) was evaluated for its ability to separate standard compounds of different polarity and volatility that are expected from the action of HPLS on hydroperoxides **1** or **2**. The main advantage of this column is that compounds are separated primarily by size, rather than polarity or boiling point. Accordingly, both polar and apolar HPLS volatile oxidation products were resolved. After determining optimal conditions for separating the expected products, the method was used to assay HPLS activity in water-soluble and insoluble protein fractions from *Chlorella pyrenoidosa*.

## MATERIALS AND METHODS

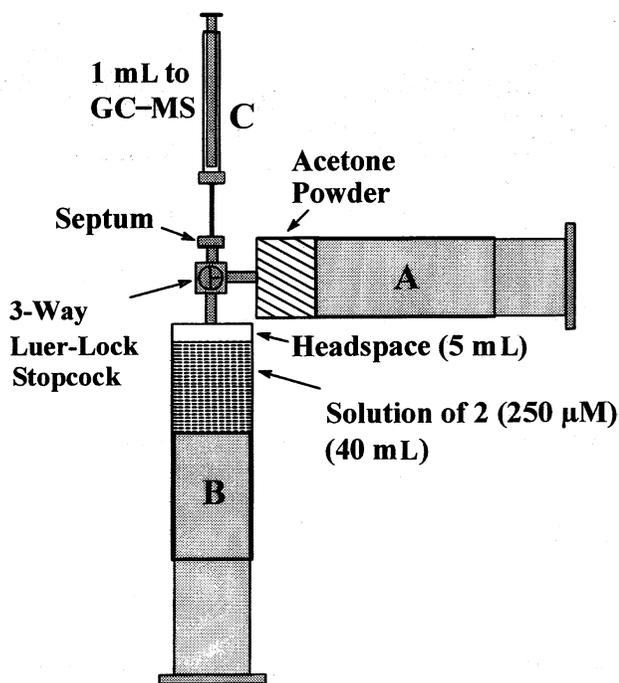
**Chemicals and materials.** Soybean lipoxygenase Type 1-B, linoleic acid, ADH, and NADH were purchased from Sigma Chemical (St. Louis, MO). Water was purified to a resistance of 18 M $\Omega$ -cm using a Barnstead (Dubuque, IA) NANOpure system. Pentane, 1-pentene, pentanol, 1-hexene, 1-penten-3-ol, hexanal, 2-(*E*)-hexenal, and hexanol were purchased from Aldrich Chemical (Milwaukee, WI). All other reagents were of the highest purity available. Hydroperoxide **2** was produced as described previously (5). *Chlorella* cells were disrupted using cold acetone to give an acetone powder as reported previously (10).

**Enzyme extraction and purification.** Water-soluble enzymes were extracted and partially purified from the acetone powder as reported earlier (5). Further purification and molecular weight estimation of the partially purified enzyme extract was done with a Sephadex G-75 column (2.5  $\times$  48 cm) eluted with potassium phosphate 50 mM (pH 7.0), collecting 2.8-mL fractions. The molecular weight standards (Sigma) were: aprotin (6.5 kDa), cytochrome c (12.4 kDa), carbonic

anhydrase (29 kDa), albumin (66 kDa), and blue dextran (2,000 kDa).

**Headspace analysis.** Typically, 4 g of acetone powder obtained from *Chlorella* cells was mixed with 20 mL 0.2 M borate buffer (pH 8.0) and centrifuged at 12,000  $\times$  g. The supernatant was removed, and the pellet was resuspended and centrifuged (12,000  $\times$  g) five additional times. The pellet was divided equally, and each portion was suspended in 20 mL of buffer. One portion was boiled for 10 min to give the control preparation. The fraction to be assayed for HPLS activity was loaded into a 60-mL gas syringe and connected to a closed three-way Luer-lock stopcock (syringe A on Scheme 2). A 40-mL aliquot of an aqueous solution of **2** (250  $\mu$ M) was loaded into a second 60-mL gas syringe (syringe B on Scheme 2), leaving a headspace of 5 mL. Before allowing the contents of both syringes to mix, a 10- $\mu$ L aliquot of hexane in methanol (1.5  $\mu$ L hexane/mL methanol) was added through the septum on the three-way stopcock (see Scheme 2) as an internal standard. The contents of syringe A were dispensed into syringe B through the stopcock, and the mixture was allowed to stand for 30 min at room temperature. Afterward, 1 mL of gas headspace was removed by syringe through the septum and manually injected into the GC-MS. After analysis, 2 mL of hexane was added to the reaction mixture for extraction of less volatile products, and 1  $\mu$ L of the organic layer was injected into the GC-MS.

**GC-MS of volatiles.** Analysis of headspace volatiles was performed with a Hewlett-Packard (HP, San Fernando, CA) 5890 Series II Plus GC equipped with an HP 5972 Series mass detector set to scan from  $m/z$  35 to  $m/z$  400 at 1.2 scans per second and a PoraPLOT Q column coated with styrene-divinylbenzene polymer (25 m  $\times$  0.32 mm  $\times$  8  $\mu$ m) (Chrompack, Raritan, NJ). The following oven temperature profile was used to elute the products: 50 (3 min) to 160 $^{\circ}$ C (5 min)



SCHEME 2

at 15°C/min; to a final temperature of 250°C (5 min) at 15°C/min. All injections were splitless with the injector and detector set at 250°C, using He as carrier gas at 1 mL/min. For GC-MS analyses of the products in the hexane layer the oven temperature profile was: 180 (10 min) to 250°C (5 min) at 15°C/min.

**Isolation and analysis of 10.** Semipreparative purification of the C<sub>13</sub>-oxo carboxylic acid methyl ester was done on a Waters (Milford, MA) LC Module 1 HPLC equipped with a Lichrosorb 5 μ Diol Column (25 × 1 cm) from Phenomenex (Torrance, CA) using a linear gradient of hexane/0.5% isopropanol to hexane/2% isopropanol (30 min). Detection of products was made with a Waters 996 Photodio Array Detector set to monitor at 276 and 233 nm and a Varex MK III Light Scattering Detector from Alltech Associates (Deerfield, IL). The fractions with absorption at 276 nm were collected and analyzed by GC-MS: column HP-5MS (30 m × 0.25 mm); 80 (5 min) to 230°C (10 min) at 10°C/min. The <sup>1</sup>H nuclear magnetic resonance (NMR) spectra of the products were obtained using a Varian (Palo Alto, CA) Unity + 400 MHz NMR instrument.

## RESULTS AND DISCUSSION

**GC-MS assay.** One unique property of the porous polymer column used in this study is its ability to separate polar and apolar products. Separation of C<sub>1</sub> to C<sub>7</sub> compounds is accomplished with little or no influence of compound polarity; compounds are separated primarily on the basis of size. However, column elution temperatures are much higher than the boiling points of the volatiles and because of this, products in the C<sub>3</sub>-C<sub>5</sub> range are trapped on the column head even at a 50°C column temperature. Accordingly, multiple injections of large sample volumes can be made without compromising compound resolution until the minimum sensitivity of the instrument is reached, approximately 10<sup>-10</sup> g of sample. Figure 1

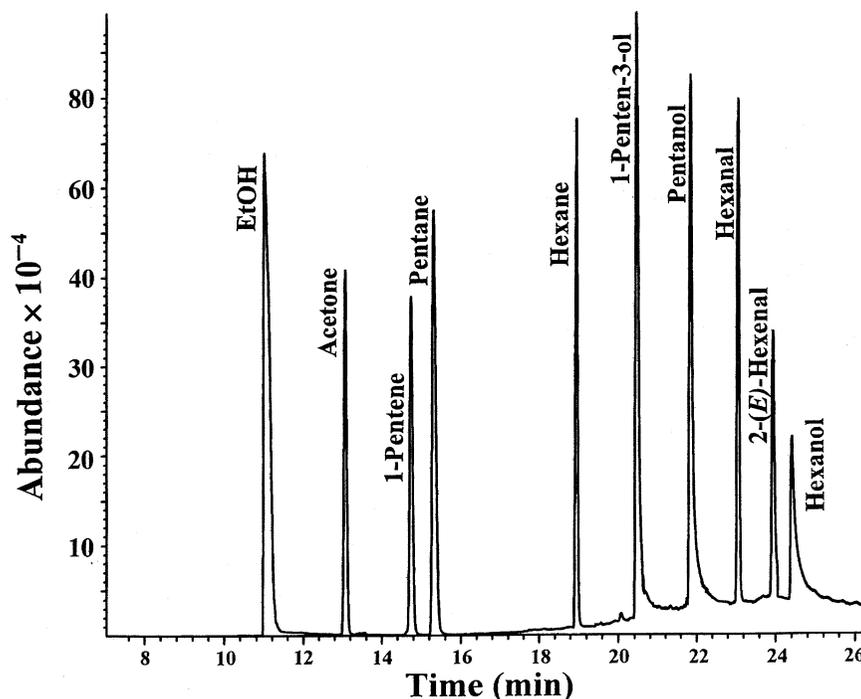


FIG. 1. Gas chromatography-mass spectrometry (GC-MS) of the headspace volatiles in a standard mixture (see the Results and Discussion section) using a PoraPLOT Q column with a temperature program described in the Materials and Methods section.

shows a chromatogram for a headspace injection of a mixture of standard compounds similar to the short-chain products expected from the oxidation of **1** or **2** with HPLS. Because the standards had different volatilities, the standards were injected by taking different amounts of headspace from three separate sample vials in order to obtain similar peak sizes. In the first vial 1  $\mu\text{L}$  of acetone, ethanol, 1-pentene, pentane, and hexane were dissolved in 1 mL methanol. From the headspace of this vial was taken 100  $\mu\text{L}$  for injection. The second vial contained 1  $\mu\text{L}$  of pure 1-penten-3-ol, hexanal, and 2-(*E*)-hexenal, and 200  $\mu\text{L}$  of the headspace was taken for injection from this vial. The third vial contained 1  $\mu\text{L}$  of pure 1-pentanol and 1-hexanol, and 400  $\mu\text{L}$  of the headspace was injected. Peak shape and retention times of the standards from consecutive injections of the three mixtures were identical to those obtained from a single injection of the same volume of each vial. Acetone and ethanol were included because traces were present in the acetone powder and partially purified enzyme extracts. Among the compounds injected, pentanol and hexanol were the more difficult to detect because they tend to elute as broad peaks at low concentration. For the same reason, the resolution of 2-(*E*)-hexenal and 2-(*E*)-hexenol also was poor.

Previous work (2) identified pentane as the major volatile product from the action of *Chlorella* HPLS on **2** by comparison of its retention time with a standard. In that study an active alumina column was used for GC headspace analysis. This column gave effective separation of light hydrocarbons ( $\text{C}_1\text{--C}_5$ ), whereas large hydrocarbons or polar material eluted only with difficulty. Because water interacts strongly with alumina columns, precautions need to be taken with regard to the water content of samples to avoid variations in eluate retention times. In another study (3), pentanol was the only

short-chain product reported from the reaction of *Oscillatoria* HPLS with **2**. In that study a Carbowax 20M column was used for the detection of pentanol. Other GC analyses of volatiles from grass and germinating soybean seedling HPLS cleavage of **2** also used polar or intermediate polarity columns (4,8). These latter columns, however, require a minimum operating temperature of at least 40°C, but at this temperature apolar short hydrocarbon fragments, such as pentane, are not retained. More recently Salch *et al.* (9) used an HP-5MS low-polarity GC column with a mass detector for the detection of pentane and 2-(*Z*)-penten-1-ol and 1-penten-3-ol. This column can elute both polar and apolar compounds, but we found that ethanol and pentane were not resolved even when cryogenic conditions were used. Also, injection of large sample amounts required cryogenic cooling to avoid peak broadening. As Figure 1 shows, a PoraPLOT column can resolve the mixture of polar and apolar  $\text{C}_5$  and  $\text{C}_6$  volatile products such as those expected from the interaction of HPLS with **1** or **2** with no need for subambient or cryogenic cooling when large volumes are injected. An added benefit of the column is that it is not affected by water or oxygen, which allows for direct injection of aqueous headspace samples.

By using the air-tight system described in the Materials and Methods section, shown in Scheme 2, the headspace of an active HPLS acetone powder, a heat-treated powder control, and the supernatant from a buffer wash of the active powder were assayed for HPLS activity using the PoraPLOT Q column. Injection of the headspace from the active acetone powder reaction, with hexane as internal standard, showed the presence of three peaks with retention times and mass spectra that corresponded to pentane, 2-(*Z*)-pentene, and 1-pentene (Fig. 2A and inset). Pentane yields were between 5

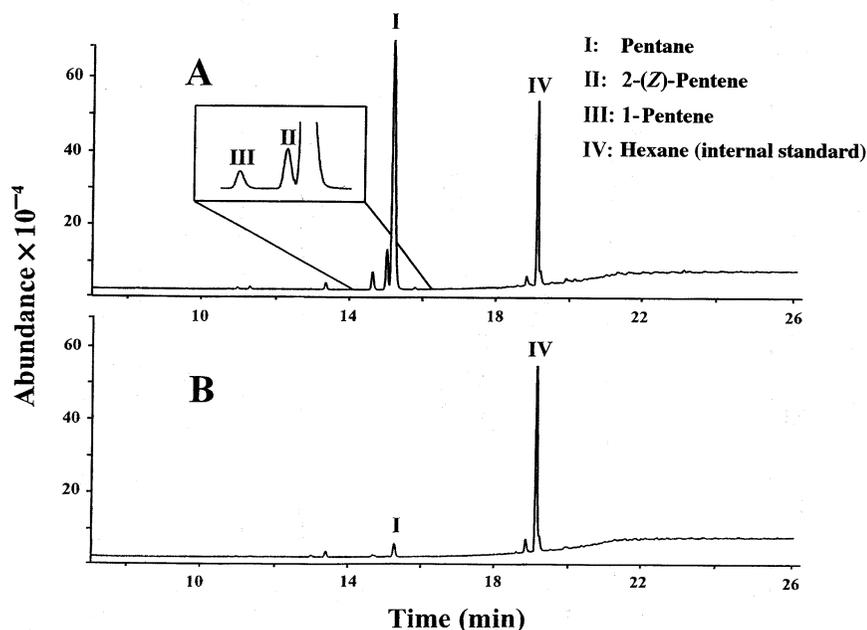


FIG. 2. (A) GC-MS of the headspace volatiles from the reaction of 40 mL of **2** (250  $\mu\text{M}$ ) with 2 g of hydroperoxide lyase-active acetone powder from *Chlorella pyrenoidosa*; (B) control reaction. For structure of **2** see Scheme 1; for abbreviation see Figure 1.

and 10%. Although detectable amounts of pentane also are present in the heat-treated powder control (Fig. 1B) and the buffer-washed supernatant (data not shown), the control levels of pentane were always below 3% of the amount found using the HPLS-active acetone powder. In our product analysis the presence of 2-(Z)-pentene could be attributed to small amounts of **1** contaminating **2**. However, the source of the 1-pentene is unclear. 2-(Z)-Pentene and a third unidentified product that was presumed to be 2-(E)-pentene were detected previously in *Chlorella* HPLS reactions when the substrate was **1** (2). Our analysis of the headspace showed that no E-isomer was formed.

To determine whether less volatile and more polar alcohols or aldehydes were produced, the reaction mixture was extracted with hexane, and 1  $\mu$ L of the hexane phase was injected in the GC-MS. No alcohol products were detected. Although small amounts of hexanal were found in the product mixtures, this compound also was detected in the control and is presumed to be formed by nonenzymatic degradation of **2**.

Additional confirmation for the presence of the products resulting from HPLS activity in the acetone powder pellet was obtained by semipreparative HPLC isolation of the non-volatile products of the reaction. The latter were extracted from the aqueous phase with ether and then methylated. A collected HPLC fraction with ultraviolet absorption at 276 nm gave a single peak by GC-MS with the following mass spectrum: 238 [M]<sup>+</sup>; 207 [M - CH<sub>3</sub>O]<sup>+</sup>; 206 [M - CH<sub>3</sub>OH]<sup>+</sup>; 188 [M - H<sub>2</sub>O - CH<sub>3</sub>OH]<sup>+</sup>; 178; 149; 121; 119; 109; 95; [CH<sub>2</sub>CH=CHCH=CHCHO]<sup>+</sup>; 81 [CH=CHCH=CHCHO]<sup>+</sup>; 68; and 55. This spectrum corresponds to the published spectrum of **10** (2,3,9,11). <sup>1</sup>H NMR of the same fraction furnished the following chemical shifts in ppm. In parentheses are the number of protons, multiplicity, coupling constants, and carbon assignments:  $\delta$  = 9.60 (1H, *d*,  $J_{12,13}$  = 7.90 Hz, C13); 6.15 (1H, *dd*,  $J_{11,12}$  = 15.2 Hz, C12);  $\delta$  = 7.43 (1H, *ddd*,  $J_{10,11}$  = 11.53 Hz,  $J_{9,11}$  = 0.92 Hz, C11),  $\delta$  = 6.26 (1H, *ddt*,  $J_{9,10}$  = 10.80 Hz,  $J_{8,10}$  = 0.55 Hz, C10);  $\delta$  = 5.98 (1H, *dt*,  $J_{8,9}$  = 7.87 Hz, C9);  $\delta$  = 3.70 (3H, *s*, ester methyl);  $\delta$  = 2.30 (4H, *m*, C2,8);  $\delta$  = 1.62 (2H, *m*, C3);  $\delta$  = 1.45 (2H, *m*, C7);  $\delta$  = 1.34 (6H, *m*, C4-6). These data match the spectrum recently reported by Salch *et al.* (9) for 13-oxo-9-(Z), 11-(E)-trideca-dienoic acid.

A recent report showed that under anaerobic conditions lipoxigenase can cause the cleavage of **1** to produce **10** and a mixture of 2-(Z)-penten-1-ol and 1-pentene-3-ol. However, no pentane was found in this reaction (9). In our studies with *Chlorella*, pentane was the major volatile detected from the acetone powder pellet. Whether the reaction was conducted under an atmosphere of oxygen or nitrogen, no difference in the products was observed (results not shown). These data suggest that HPLS activity is the major route to the oxo-product **10** and pentane.

**Spectrophotometric assay.** HPLS activity has been monitored spectrophotometrically at 234 nm by following the loss of the conjugated diene chromophore of **2** or the increase at 280 nm when oxo-acid **10** is the main product. To improve

the specificity of this assay a linked assay has been devised that couples ADH to HPLS. ADH reduces the aldehydes produced by HPLS to alcohols and in the process oxidizes NADH. Thus HPLS activity can be followed by the decrease in absorption at 340 nm (6). We reported earlier that a partially purified water-soluble protein fraction derived from the supernatant of *C. pyrenoidosa* and *fusca* gave the expected decrease in absorption at 234 nm and the decrease at 340 nm when **2** and ADH/NADH were present (5,7). However, a more careful examination of the ADH/NADH assay revealed that the reaction of the water-soluble protein fraction with **2** and NADH has the same kinetic profile in the presence or absence of ADH. Because only traces of the ADH/NADH-reduced alcohol derivative of **10** can be detected in organic extracts of this reaction and because the level of volatile organics is comparable to the background levels in heat-treated controls, these results indicate that the spectrophotometric assay was detecting an enzyme activity that was not HPLS. These results were obtained even after further purification of the water soluble extract from *C. pyrenoidosa* by DEAE Sepharose CL-6B columns and gel filtration, where the major protein present had a molecular weight of 46 kDa and maximal activity at pH 6.5, properties similar to those reported previously for HPLS from *C. pyrenoidosa* and *Oscillatoria* (2,3).

In conclusion, the use of GC-MS with a PorapLOT Q column allowed the development of a method that requires no derivatization, was not affected by the presence of water, did not require cryogenic conditions to be maintained during injection, and allowed for the quantitation of most of the expected volatile products from the action of HPLS upon **1** or **2**. Moreover, the data obtained by using this method show that HPLS resides in the water-insoluble fraction obtained from *C. pyrenoidosa* acetone powder. HPLS activity in an aqueous extract of *C. pyrenoidosa* acetone powder could not be confirmed by the ADH/NADH assay, and the nature of the products arising from the degradation of **2** by enzymatic activity in the aqueous is under study.

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