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Analysis of Terpene Hydroperoxides by HPLC with Chemiluminescence Detection

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HPLC with Chemiluminescence Detection

Sample Preparation

› Dilute-and-shoot, filtration/centrifugation (x50 into 3/1 IPA/H_2O w 1 mg/mL BHT)

Chromatographic Separation

› Standard C-18 column

› Slightly complex gradient with changing acetonitrile/methanol and water mixtures

› Necessary to get the isomers separated

› The gradient shows many unknown compounds as well

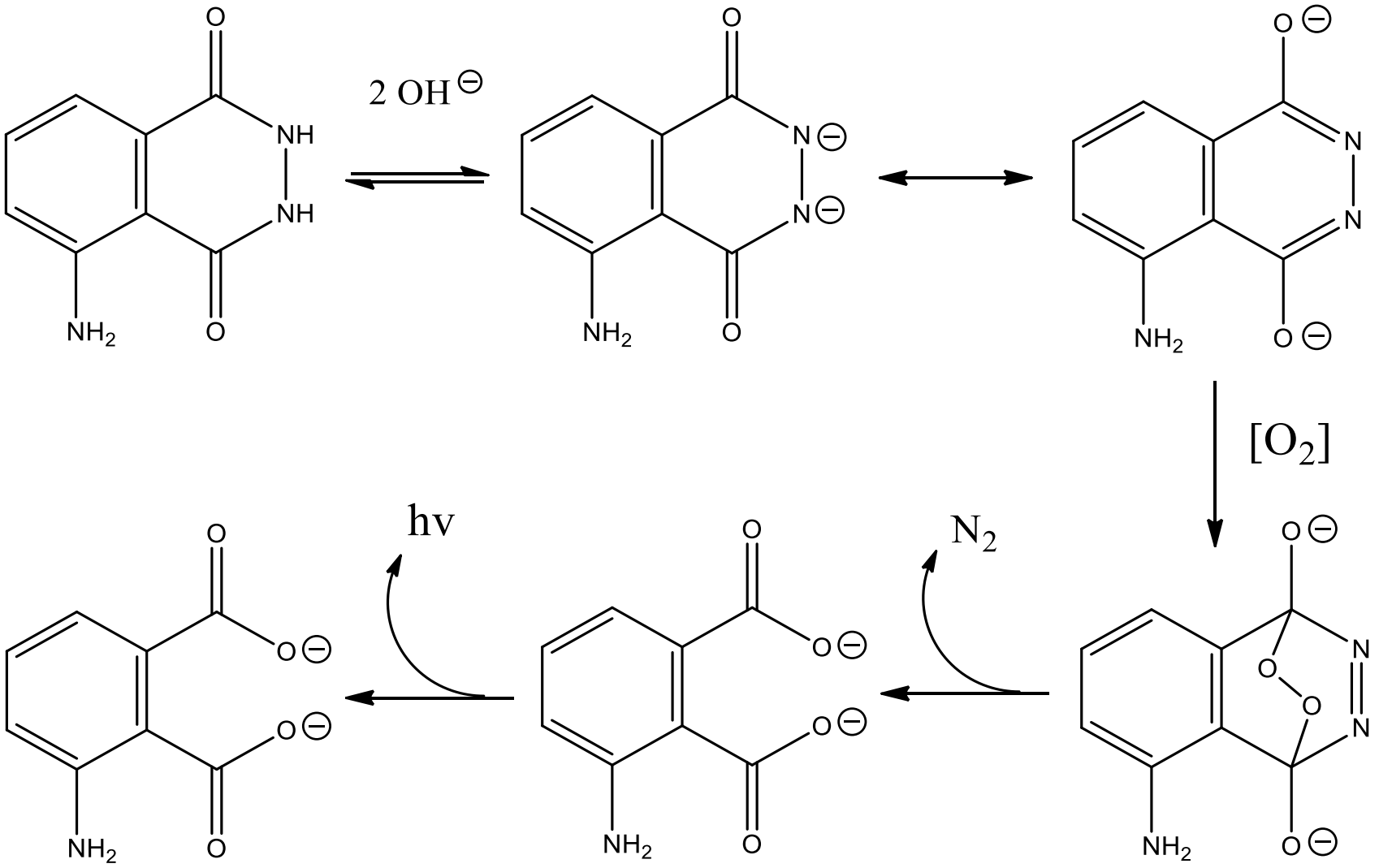
Post Column Reaction with Chemiluminescence Detection

› Cytochrome c catalyzed formation of a reactive oxygen species from R-O-O-H

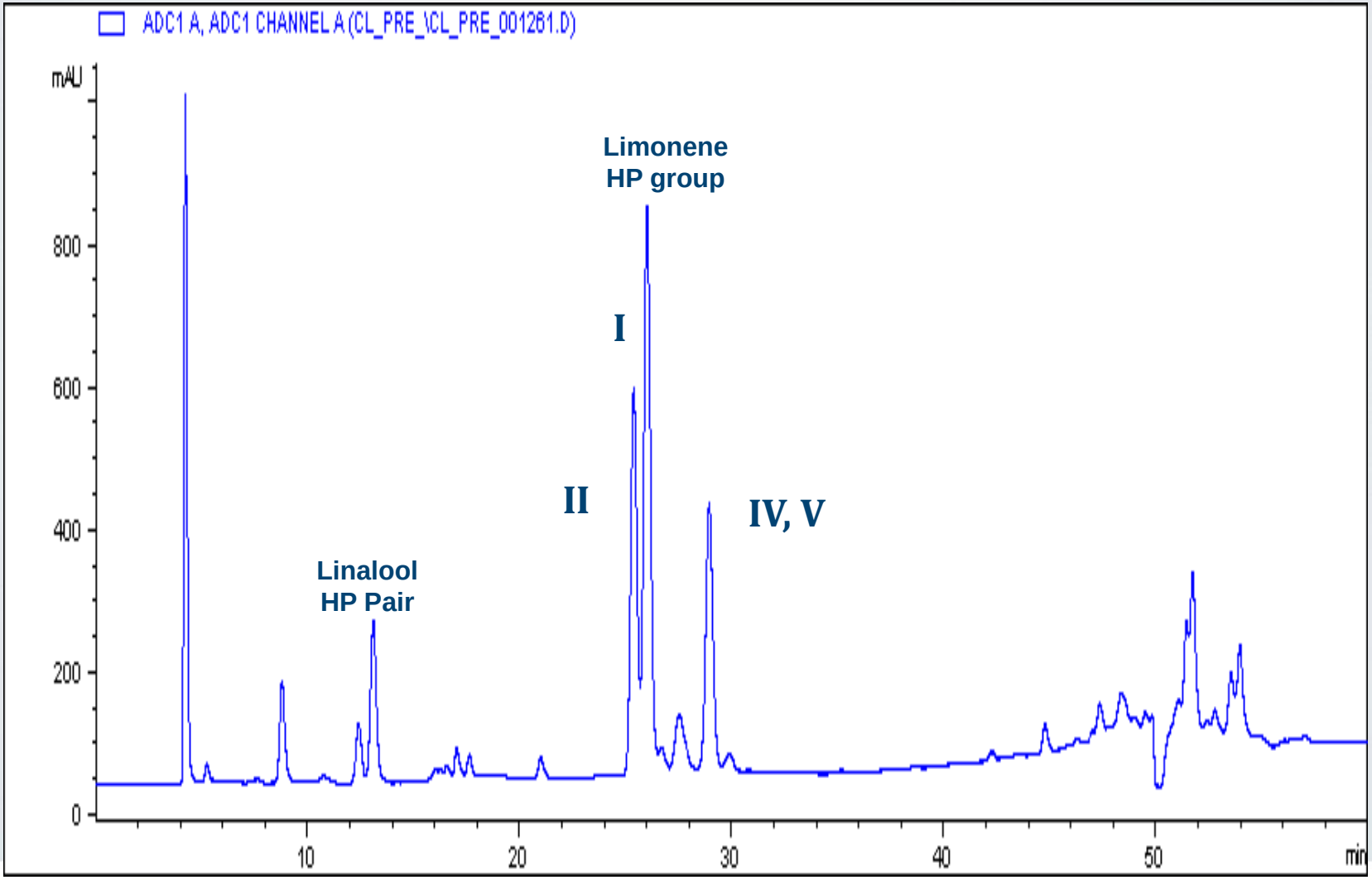
› Luminol oxidation produces light

› **Detection is INHERENTLY SELECTIVE for oxidizing species**

Oxidation of Luminol in Base



Grapefruit and Lime Oil Mixture 1:1 v/v



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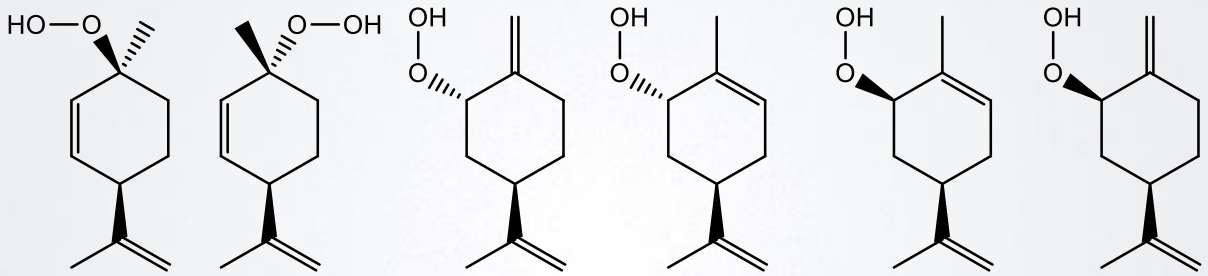
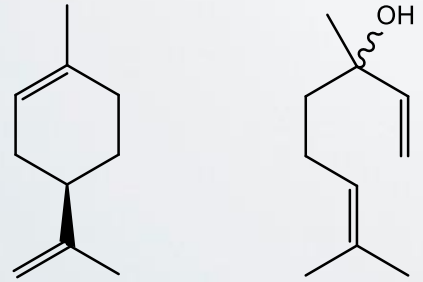
The Main Target Analytes

Limonene Hydroperoxides (HPs) – Six Isomers



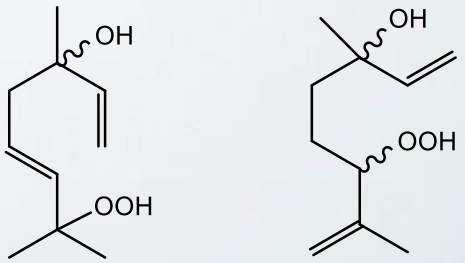
I II III IV V VI

The Parents



d-(+)-Limonene Linalool

Linalool Hydroperoxides (HPs) – Two Isomers



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Effect of Peroxyhemiacetals (PHAs)

- If aldehydes are present, they react with terpene hydroperoxides to form PHAs
- It is a REVERSIBLE reaction (an equilibrium)
- This can cause an apparent low recovery of spiked terpene hydroperoxides
  - Nonpolar environments favor PHA formation
  - Highly polar environments favor PHA dissociation

- The HPLC sample preparation causes slow PHA dissociation
  - 2 – 3 hours: noticable
  - 20 hours: substantially complete, but 43 hours is better

- Increased recovery observed after waiting 20 or 43 hours prior to HPLC injection
  - This implies the presence of PHAs

Recoveries Observed by HPLC-CL

- The “43 hour wait” data was used and gives the best recovery

- 1-Lim-HP isomer was a bit high, but there is a coelution with another Terp-HP
  - Average – **124.3%**

- Other recoveries are reasonable but slightly low
  - 2-Lim-HP: Average – **95.9%** (83.99 – 108.52% range)
  - 6-Lim-HP: Average - **85.1%** (73.26 – 93.54% range)
  - 7-Lin-HP: Average - **92.4%** (89.02 - 99.11% range)
  - Sum 6 & 7: Average - **88.6%** (80.28 - 96.48% range)

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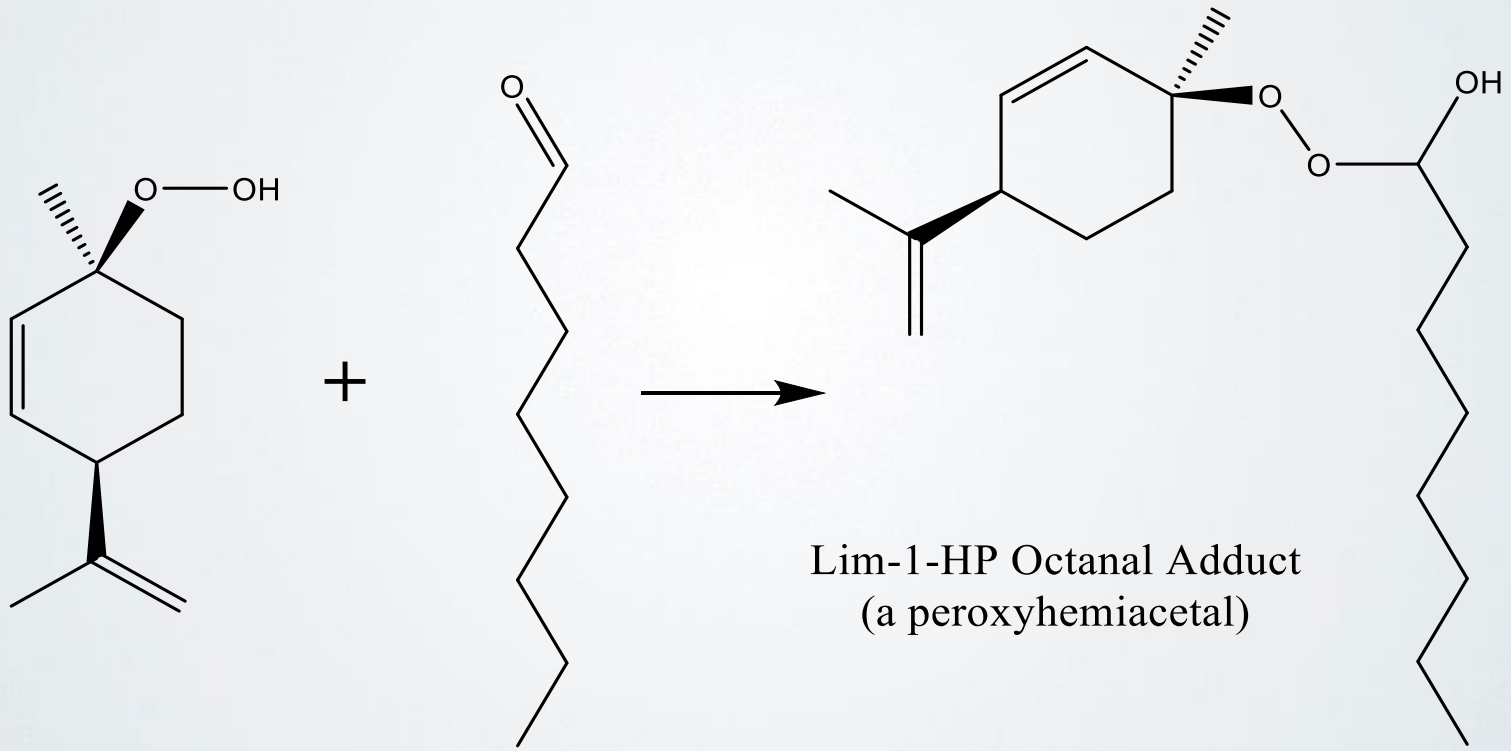
inspiring!

INNOVATIVE CRAFTSMANSHIP IN FRAGRANCES AND FLAVORS SINCE 1895

Reaction of Lim-1-Hydroperoxide with Octanal: An Example of PHA Formation



Lim-1-HP Octanal Adduct
(a peroxyhemiacetal)

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Method Validation Parameters

Reproducibility:

- Linalool hydroperoxides: **4.4% RSD;**
- Limonene hydroperoxides: **2.6% RSD**

Recovery:

- **104.1%** for total linalool hydroperoxides;
- **106.0%** for total limonene hydroperoxides
- Done using reagent-grade limonene, not actual citrus oils

Limit of detection:

- **0.2 – 0.4 pmol** of terpene hydroperoxide

Appendix

- ***Sample preparation solvent:*** Make a 75/25 v/v isopropanol/water mixture, and dissolve in 1.0 mg/ml of BHT to prevent autoxidation during sample preparation.[41,76] Degas this solution with helium gas passed through a sintered glass dispersion tube for 5 min immediately before use; handle to minimize atmospheric exposure/gas reabsorption. ***Sample preparation:*** Place 0.25 ml of neat citrus oil sample into a 10 ml volumetric flask and bring to volume with the sample preparation solvent (a 20-fold dilution). Mix until homogeneous. If any haziness or precipitation becomes apparent, as sometimes occurs mostly with grapefruit oil, centrifuge the sample solution at 2500 rpm for 5 min. Place a portion of the alcohol phase into a dark amber glass autosampler vial (to protect from light). Fill to minimize the atmospheric headspace, or preferably maintain an inert gas headspace within the sampler vial. **HPLC System**: An Agilent 1100 modular HPLC system consisting of a vacuum degasser, quaternary pump, temperature controlled autosampler, column heater, and a diode array detector; connection to the chemiluminescence detector with an Agilent 35900E Interface. Post Column Reactor: Scientific Systems, Inc: Sensivate Elite®, Dual Channel Post Column Reactor, plumbed into the system in a standard flow configuration for post-column reaction. Chemiluminescence Detector: Jasco CL 2027 Plus Chemiluminescence Detector. Column: Phenomenex Luna C18(2), 250 x 4.6 mm, 5 um particle size, Part #00G 4252 E0 Column Oven Temperature: 30º C Re-equilibration Time between Runs: 15 minutes Autosampler Temperature: 5º C Autosampler Vials: Use dark amber vials for protection from light Injection Volume: 5 uL HPLC Pump Flow Rate: 0.8 mL/minute **Preparation of Post-Column Reaction Reagent:** *Sodium Carbonate/Potassium Chloride Solution (approx. 2.0 molar):* Place potassium chloride (ACS reagent grade, 1.0 mole, 74.6 g) and anhydrous sodium carbonate (HPLC grade, 1.0 mole, 105.6 g) into a 500 mL bottle. Add 500 mL of water, and stir until dissolved. Store tightly sealed in a bottle. *Borate Buffer Solution*: A 2000 mL glass beaker is tared, and boric acid (ACS reagent grade, 0.1 mole, 6.2 g) is added to it, along with about 1700 mL of HPLC grade water. Add the Sodium Carbonate/Potassium Chloride Solution slowly with constant stirring while monitoring the pH, until a pH of 10.0 is reached. Add additional water until a total weight of 2000 g is reached for the solution. Store tightly sealed in a bottle. *Luminol Stock Solution:* Place 20 mL of Borate Buffer Solution into a glass vial, and add 25 mg of 3-aminophthalhydrazide, 98+% (“luminol”, 25 mg). Sonicate for 5 – 10 minutes to dissolve the luminol. It can be stored in the refrigerator for two months without any observable loss of effectiveness; beyond that has not been attempted in our hands.

Appendix Continued

- *Luminol/cytochrome c Post-Column Reagent:* Place 500 mL of Borate Buffer Solution into a bottle, and add cytochrome c (from equine heart, 5.0 mg). Stir to dissolve, then add 2.0 mL of Luminol Stock Solution. Store the solution overnight each night in the refrigerator, and degas by sonicating under vacuum for 1 – 2 minutes, then sparging with helium. The solution stays at room temperature only during actual use on the post-column reactor. Discard any remaining solution after one week. Post-Column Reactor Pump A Flow Rate: 0.4 mL/minute of Luminol/cytochrome c Reagent Post-Column Reactor Pump B Flow Rate: Off/Not Used Post-Column Reactor Temperature: 50° C Chemiluminescence Detector Temperature: 50° C

Mobile Phase Gradient:

Time (in minutes)	Acetonitrile	Methanol	Water
0	10	40	50
8	10	40	50
10	45	5	50
30	45	5	50
45	45	50	5
60	45	50	5

- Reproducibility: Linalool hydroperoxides: **4.4% RSD;** Limonene hydroperoxides: **2.6% RSD** Recovery: **104.1%** for total linalool hydroperoxides; **106.0%** for total limonene hydroperoxides. Limit of detection: < **10 pmol** of organic hydroperoxide, based on tert-butyl hydroperoxide.

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