ANALYSIS OF METHANOL, ETHANOL AND PROPANOL IN AQUEOUS
ENVIRONMENTAL MATRICES

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ABSTRACT

A method for analysis of methanol, ethanol, and propanol at submicromolar levels in aqueous samples has been developed. It involves enzymatic oxidation of the alcohols to their corresponding aldehydes using alcohol oxidase. The resulting aldehydes are derivatized to their respective hydrazones using 2,4-dinitrophenylhydrazine (DNPH). These hydrazones are separated and quantified using high performance liquid chromatography (HPLC) at 370nm. Comparison of samples with and without enzyme, allows for indirect determination of the alcohol concentration by reversed phase HPLC. The optimum conditions for the enzyme reaction were 40 minutes at $40 \pm 0.1°C$, pH 8.5, and addition of 100 μL of alcohol oxidase (2 units mL^{-1}). Alcohol concentrations in enzyme blanks were 0.44 ± 0.01 μM for methanol, 0.050 ± 0.004 μM for ethanol, and 0.15 ± 0.04 μM for propanol. Limits of detection were 0.03 μM, 0.02μM, and 0.14 μM for methanol, ethanol, and propanol respectively. Percent conversions under optimum conditions were $90.1\% \pm 2\%$ RSD for methanol, $40\% \pm 3\%$ RSD for ethanol, and $7\% \pm 3\%$ RSD for propanol. Alcohol concentrations in rain events in southeastern NC were dependent on storm origin and varied from 0.08-0.91 μM for methanol and 0.08-0.23 μM for ethanol. Conditions for propanol analysis are still being optimized. Percentages of carbon from methanol and ethanol in dissolved organic carbon in rain events 570A, 573, and 574 were 3.1%, 0.4%, and 3.5% respectively.

ACKNOWLEDGMENTS

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INTRODUCTION

Low molecular weight straight chain alcohols are important precursors to more highly

oxidized forms of carbon and may play an important role in the global biogeochemical cycle of

carbon. Methanol and other saturated straight chain alcohols enter the environment through a

variety of anthropogenic and biogenic sources. Anthropogenic sources include alternative fuels,

gasoline additives, aerosol sprays, and paint strippers, whereas biogenic sources of methanol

include decomposition of waste, volcanic gasses, vegetation, microbes and insects [1]. Methods

for analyzing gas phase alcohols in the atmosphere and in biological fluids at high concentrations

have been developed. Analysis of gas phase alcohols in the atmosphere is not applicable to

water due to high limits of detection and/or reagent reactions with alcohols and water [2].

Detection of alcohols in biological fluids has been accomplished at higher limits of detection but

with no distinction between alcohols [3].

A method for analysis of methanol in water using membrane introduction mass spectrometry [1]

has also been developed comparing poly(dimethylsiloxane) and allyl alcohol membranes,

however the reported limits of detection were 1.5×10^{-4} M and 6.25×10^{-5} M respectively, which is

insufficient for environmental analysis.

Despite the significance of low molecular weight alcohols in the environment, there is no

method for their quantitative analysis in environmental aqueous samples at micro- and sub-

micromolar levels. Saturated straight chain alcohols such as methanol, ethanol, and propanol,

are difficult to quantify in aqueous environmental matrices because they are in very low

concentrations, structurally similar to water, have poor molar absorptivities, and are hard to

derivatize. Once the alcohols have been oxidized to their respective aldehydes, they can easily

be derivatized and analyzed. However, normal chemical processes, which oxidize alcohols in water, typically continue to oxidize to the more thermodynamically favored carboxylic acid. Analysis of aldehydes is preferred over analysis of carboxylic acids because, lower concentrations (sub-micromolar) can be detected and the experimental procedure is less involved, which reduces possible introduction of error.

A method exists for analysis of nanomolar concentrations of aldehydes and ketones using 2,4-dinitrophenylhydrazine (DNPH) to form their hydrazones, which are then separated and quantitated by HPLC [4]. By oxidizing an alcohol to its corresponding aldehyde without any conversion to carboxylic acid, the alcohol concentration could be measured indirectly. Two types of enzymes are capable of terminal oxidation of alcohols to aldehydes, alcohol dehydrogenase and alcohol oxidase.

Alcohol dehydrogenase (ADG) is an enzyme present in plants, yeast, and bacteria that reversibly catalyzes oxidation of C_2 to C_{10} aliphatic alcohols [3] according to the following equation:

$$RCH_2OH + NAD^+ \xrightleftharpoons{ADG} RCHO + NADH + H^+ \qquad (1)$$
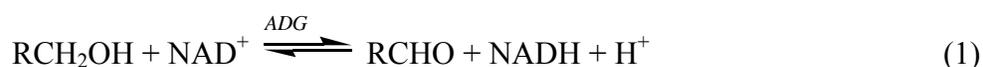
Addition of the coenzyme nicotinamide adenine dinucleotide (NAD^+) to the alcohol in the presence of alcohol dehydrogenase produces the corresponding aldehyde, the reduced form of the coenzyme (NADH) and a proton [3]. This enzyme exhibits maximum activity for the conversion of ethanol to acetaldehyde. This enzyme was not chosen for this project because of the need to add NAD^+. By introducing another reagent into the experimental procedure, there is more possibility for error.

Alcohol oxidase (AO) catalyzes oxidation of C_1 to C_6 saturated straight chain alcohols to their corresponding aldehyde via equation 2 below:

$$RCH_2OH + O_2 \xrightarrow{AO} RCHO + H_2O_2 \qquad (2)$$

This enzyme was chosen because it displays maximum activity for oxidation of methanol to

formaldehyde and because the oxidant is oxygen [3]. The cofactor FADH was bound to the

alcohol oxidase and does not need to be added separately. A possible drawback to using alcohol

oxidase is that the aldehyde can be further converted to its corresponding carboxylic acid

according to equation 3:

$$RCHO + O_2 + H_2O \xrightarrow{AO} HCOOH + H_2O_2 \qquad (3)$$

Specifically, formaldehyde forms stable hydrates in water that are similar in appearance to

methanol. This makes further conversion to formic acid more likely. The likelihood of

subsequent conversion to the acid from the aldehyde decreases as the number of carbons

increases [3].

Following oxidation of alcohol to aldehyde by alcohol oxidase enzyme, reaction of the

aldehyde with DNPH produces the respective hydrazone [4] via equation 4:

$$(4)$$
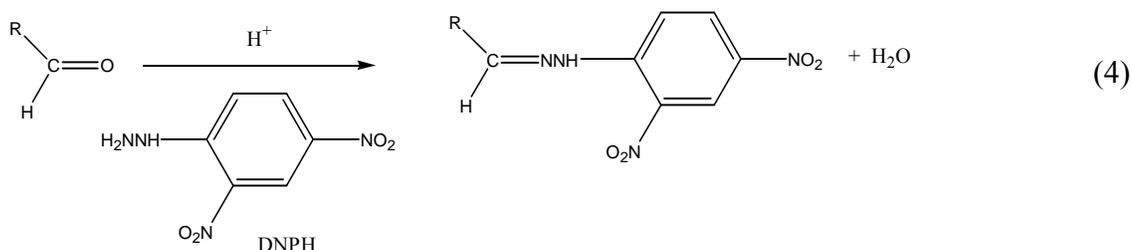
Addition of the acidic DNPH reagent immediately denatures the enzyme and converts aldehydes

to their respective hydrazones. These are then separated, identified, and quantified using

reversed phase high performance liquid chromatography (HPLC) to indirectly analyze alcohols

at submicromolar concentrations.

METHODS

Reagents and Standards

Alcohol oxidase (100 units) from the yeast *Hansenula sp* was purchased from Sigma (St. Louis, MO). Water was purified using a Millipore Q-water system (Millipore Corp., Bedford, MA) and used to prepare all solutions. Reagent grade 2,4-dinitrophenylhydrazine (DNPH) was purchased from Sigma (St. Louis, MO), triply recrystallized from acetonitrile and kept refrigerated in the dark. Acetonitrile (HPLC grade, Burdick and Jackson, Muskegon, MI), 12 M hydrochloric acid (Reagent Grade, VWR International, West Chester, PA), and carbon tetrachloride (HPLC grade 99.9%, Sigma, St. Louis, MO) were used in preparation and purification of DNPH reagent solution.

Formaldehyde (37.69% CH_2O, 12.37% MeOH) and paraformaldehyde (94.19%, containing no methanol) were obtained from Wright Chemical Company (Wilmington, NC). Acetaldehyde was reagent grade and obtained from Fisher Scientific (Fair Lawn, NJ). Propionaldehyde (98%) was from Alfa Aesar (Ward Hill, MA). A mixed standard solution, 1 M in each of C_1-C_3 aldehydes, was prepared on a weekly basis. Methanol (HPLC grade, Burdick and Jackson, Muskegon, MI), ethanol (200 proof, AAPER Alcohol and Chemical Co., Shelbyville, KY), 1-propanol (Fisher Scientific, Fair Lawn, NJ) and Milli-Q water were used to prepare a 1 M mixed alcohol standard solution on a weekly basis. ACS grade (99.0%) potassium dihydrogen phosphate and reagent grade potassium hydrogen phosphate (Alfa Aesar, Ward Hill, MA) were used in preparation of all buffer solutions.

General Procedures

Polyethylene disposable gloves (VWR International, #32915-188) were worn when handling all reagents, buffers, and samples. Gloves were changed between all aldehyde and alcohol samples, (including stock, dilution, and sample preparation) as well as prior to enzyme handling, to avoid cross-contamination. All alcohol, aldehyde, buffer, and DNPH reagent solutions were stored in different locations to prevent contamination.

All digital pipet tips (1-200 μL, 200-1000 μL, and 1-5 mL, VWR International) were placed in a 10% hydrochloric acid bath (700 mL 12 M HCl diluted with 7.0 L of DI water) and allowed to soak for four hours. After soaking, tips were rinsed thoroughly with Milli-Q water in a class 100 clean room, allowed to dry under positive flow hoods, packaged in acid rinsed Ziploc Bags, and stored in a room free of alcohol and aldehyde contamination. Pipet tips were changed between samples of varying concentrations and between use of aldehyde and alcohol solutions. All volumetric glassware and caps were rinsed several times with Milli-Q water prior to making solutions. All HPLC vials were heated in a muffle furnace for 6 hours at 550°C prior to use. On days when lab work was conducted, no perfume, nail polish, hair products, or any other sources of alcohols or aldehydes were worn to prevent contamination. Alcohol was not consumed on days prior to conducting lab work.

Dinitrophenylhydrazine Reagent Preparation

Reagent preparation was done according to Kieber and Mopper [4]. The 2,4-dinitrophenylhydrazine (DNPH) reagent was prepared on a weekly basis in a 30 mL Teflon vial by dissolving 20 mg of triply recrystallized DNPH in 4 mL of 12 M hydrochloric acid (HCl), 10 mL of Milli-Q water, and 2 mL of acetonitrile (ACN). The DNPH

reagent was then shaken for 1 hour on a wrist action shaker. To reduce the background signal, the reagent was extracted with 2 mL of carbon tetrachloride, shaken for 10 minutes on a wrist action shaker, and centrifuged for 2 minutes. After the initial extraction, the organic layer was removed and the process repeated. After the second extraction, the organic layer was left in the reagent vial and removed prior to successive extractions. DNPH reagent extraction was done twice on the first day of use, and once each subsequent day, for up to one week.

Hydrazone Preparation

To determine aldehyde peak retention times using reversed phase HPLC, DNPH derivatives of the C_1-C_3 aldehydes were prepared separately [5]. Specifically, 1.00 g of triply recrystallized DNPH was weighed and ground using a mortar and pestle. The DNPH reagent was made in an Erlenmeyer flask by adding 10 mL of 12 M HCl to 7 mL of Milli-Q and 50 mL of 95% ethanol while stirring. The hydrazone derivatives were made by adding 0.3991 g of 37.58% CH_2O, 0.2202 g of CH_3CHO, or 0.2904 g of CH_3CH_2CHO to 2 mL of 95% ethanol and 2 mL of the DNPH reagent. The mixtures were shaken vigorously and precipitate formation was instantaneous. The solutions were placed in the refrigerator overnight to aid in crystal formation. The following day, the products were rinsed with cold deionized water and cold 95% ethanol, filtered using a Büchner funnel, and allowed to dry. Hydrazone samples were labeled and stored in the refrigerator.

Hydrazone stock solutions (1 mM) were prepared for each of the derivatives by taking 0.0105 g of the CH_2O derivative, 0.0112 g of the CH_3CHO derivative, and 0.0119 g of the CH_3CH_2CHO derivative, diluting with 50% acetonitrile and 50% Milli-Q in 50 mL volumetric flasks, and sonicating until dissolved. Dilutions (1μM) of each were made and analyzed by

reversed phase HPLC. Based on retention times of the known hydrazone C_1-C_3 derivatives, the formaldehyde peak was found to elute at 6.0 minutes, the acetaldehyde peak at 8.4 minutes, and the propionaldehyde peak at 14.3 minutes.

Buffer Preparation

Potassium phosphate buffer (KPB, 0.1 M) at pH 9.0 was prepared in a 1 L volumetric flask by adding 0.2177 g of potassium dihydrogen phosphate (KH_2PO_4) and 22.4578 g of potassium hydrogen phosphate ($K_2HPO_4\bullet3H_2O$) and diluting to 1 L with Milli-Q water. This solution was stored in a refrigerator free of alcohol and aldehydes. The buffer was made at pH 9.0 because during the enzyme reaction the pH dropped to 8.5.

Enzyme Solution Preparation

Twenty, 25 mL high-density polyethylene (HDPE) enzyme vials and caps were rinsed several times with Milli-Q water in a class 100 clean room and allowed to dry under a positive flow hood before use. Alcohol oxidase (100 units) from *Hansenula sp* was suspended in 5mL of 0.1 M KPB at pH 9.0 resulting in a concentration of 20 units mL^{-1}. Since alcohol oxidase does not readily dissolve small aliquots of 5mL of 0.1 M KPB at pH 9.0 were added to the enzyme separately by digital pipet. Each portion was drawn up into a digital pipet and dispensed into a 25 mL HDPE vial several times, quickly to dissolve the oxidase. Nineteen 250 µL aliquots were removed from this initial vial and placed in separate 25 mL HDPE vials, in an ice water bath, labeled, and then frozen at –80˚C. The resulting quantity of enzyme in each 25 mL vial was 5 units. One vial of enzyme was removed from the freezer on a daily basis and diluted with 2.5 mL of 0.1 M KPB at pH 9.0 resulting in an enzyme concentration of 2 units mL^{-1}. The final

enzyme concentration in each HPLC sample was 0.18 units mL^{-1}. Alcohol oxidase was stored in separate HDPE vials to be used on an experimental basis. This minimized the number of freeze-thaw cycles, which in turn minimized enzyme degradation.

Sample Preparation

C_1-C_3 aldehyde and alcohol working stock solutions (1.0 M) were prepared on a weekly basis in 50 mL volumetric flasks. For the aldehyde working stock, 3.9949 g (37.58% CH_2O) or 3.983 g (37.69% CH_2O), 2.025 g acetaldehyde (CH_3CHO), and 2.904 g propionaldehyde (CH_3CH_2CHO) were weighed in separate Teflon containers. Aldehydes were combined in the flask and diluted to 50 mL with Milli-Q water. The alcohol working stock solution was prepared by combining 1.6021 g methanol (MeOH), 2.3034 g ethanol (EtOH), and 3.0050 g propanol (PrOH) in a 50 mL volumetric flask and diluting with Milli-Q. Again, separate Teflon containers were used to weigh each of the reagent stock solutions. Milli-Q was added to the Teflon containers prior to weighing the alcohols to minimize evaporation.

Aldehyde and alcohol dilutions were made in a separate area that was free of alcohol and aldehyde vapors. Separate Teflon containers were used for each concentration of working stock or dilution solution for both aldehydes and alcohols. To make a 1.0 mM solution, 100 μL of the 1.0 M C_1-C_3 aldehyde (or alcohol) working stock was dispensed into a 100 mL volumetric flask, and diluted with Milli-Q. Aldehyde (and alcohol) solutions (10 μM) were made by removing 1000 μL of the 1.0 mM solution and diluting with Milli-Q in a 100 mL volumetric flask. Solutions (1.0 μM) were prepared in an HPLC vial from 100 μL of 10 μM aldehyde (alcohol) solution and 900 μL of 1.0 mM KPB at optimum pH. All HPLC samples were 1.0 μM unless otherwise noted.

Reagent blanks consisted of 1000 µL of 1.0 mM KPB at pH 9.0. HPLC samples containing 1.0 µM C_1-C_3 aldehydes were combined with 10 µL of DNPH reagent. Enzyme blanks were made using 1000 µL of 1.0 mM KPB at pH 9.0, 100 µL of enzyme, and 10 µL of DNPH reagent. 1.0 µM C_1-C_3 alcohol samples were also inoculated with 100 µL of enzyme, and 10 µL of DNPH reagent. All results were corrected for dilution.

Rain Sample Preparation

Rain samples were collected at the University of North Carolina Wilmington rain collection site from May 6, 2005 to May 21, 2005 on an event basis (events 570A, 573, and 574). The collection site is located at 34º 13.9'N, 77º 52.7'W and is about 8.5 km from the Atlantic Ocean. Since the collection site is near the laboratory, rain samples were analyzed within hours of collection, minimizing loss of alcohols.

Rainwater samples were collected using four Aerochem-Metrics (ACM) Model 301 Automatic Sensing Wet/Dry Precipitation Collectors. Rain for alcohol analysis was collected in sample collectors consisting of a Teflon funnel connected by Tygon tubing to a 2 L trace metal cleaned Teflon bottle. Rainwater for alcohol analysis was then poured into a clean 30 mL high-density polyethylene (HDPE) container, labeled with the event number, and placed in the refrigerator. HDPE vials were rinsed well with Milli-Q and allowed to dry in a positive flow hood prior to use. Information such as pH, hydrogen peroxide concentration, rain amount, and storm origin were recorded for each event. Real time precipitation maps were used to indicate the beginning and end of each rain event.

Rainwater was analyzed for C_1-C_3 aldehydes and alcohols. To quantify the aldehydes, 1000 µL of rain was combined with 10 µL of 0.1 M KPB at pH 9.0 and 10 µL of DNPH and

allowed to react for 30 minutes before HPLC analysis. To test for the presence of alcohols in

rainwater, 1000 µL of rain was combined with 10 µL of 0.1 M KPB at pH 9.0, 100 µL of

enzyme, and allowed to react at optimum conditions before addition of 10 µL of DNPH. The

peak areas from rain samples with DNPH and the enzyme blanks were subtracted from those in

rain samples with enzyme to obtain the signal generated from alcohols alone. Rain samples were

also spiked with 1 µM C_1-C_3 alcohols. This was done by combination of 1000 µL of rain, 20 µL

of 0.1 M KPB at pH 9.0, 100 µL of the C_1-C_3 10 µM alcohols, and 100 µL of enzyme, and was

allowed to react at optimum conditions prior to addition of 10 µL of DNPH. Resulting peak

areas for this sample contained the aldehydes and alcohols present in the rain as well as the 1 µM

alcohol spike. By taking the difference between signals generated from this sample and the

sample containing rain with enzyme, the resulting peak area was that of the 1 µM C_1-C_3 alcohol

spike alone. Peak areas of the spike were compared to the signal from 1 µM alcohols in buffer,

to test for method accuracy and interference. All results were corrected for dilution. Dissolved

organic carbon (DOC) was analyzed for each rain event [6] and percentages of carbon from each

of the C_1-C_3 alcohols combined, were calculated and compared to total DOC concentrations.

Preliminary Identification of Other Compounds in Rain

Succinic semialdehyde (15% by weight, Aldrich), nitrite (sodium nitrite, Reagent grade,

Fisher), a 5 carbon oxo-acid (synthesized according to [7]), glyoxal (40% wt., Aldrich), and

pyruvic aldehyde (40% wt., Aldrich) were all preliminarily identified by spike recovery.

Standards of each of the compounds were used to make 1 µM dilutions. The solutions were

analyzed by reversed phase HPLC under optimum conditions to determine retention times.

Solutions (~8-9 µM) of each were used to spike rain samples. These were reacted with DNPH

and confirmation of the compounds was evidenced by retention time and an increase in peak area.

HPLC Instrumentation and Conditions

The HPLC used was a Hewlett-Packard (Agilent) Model 1100 Series, equipped with an autosampler, autoinjector, and a thermostatted column compartment. Agilent ChemStation software for LC and LC/MS systems was used. Integration parameters were as follows; slope sensitivity set at 0.3402, peak width set at 0.1086, an area reject of 0.0282, a height reject of 0.0323, no shoulders, and an integration start time of 3.00 minutes with baseline integration at valleys from 3.2 to 7.322 min. A reversed phase Luna 100 mm x 4.60 mm 3 μ C18(2) Phenomenex column with a pore size of 100 Angstroms was used. HPLC temperature studies were conducted from 10-35˚C to determine optimum column temperature giving the best resolution between rain peaks. Different ratios of Milli-Q and filtered acetonitrile (both containing 0.1% trifluoroacetic acid) were also tested isocratically on rain samples to determine conditions for optimum peak resolution. Optimum conditions were 50:50 Milli-Q and filtered acetonitrile (both 0.1% in trifluoroacetic acid), with a 1.00 mL min^{-1} flow rate, and a 100 μL injection volume. The column temperature was set at 10.0 ± 0.15˚C and detection was at 370 nm using a variable wavelength absorbance detector. These conditions gave the best resolution between peaks in rainwater samples. Run times were 16 minutes for all aldehyde and alcohol samples and 21 minutes for all rain samples. HPLC grade acetonitrile was filtered for use in the mobile phase. This was done by vacuum filtration using a 0.2 micron filter soaked in Milli-Q.

After each days set of runs the column was thoroughly flushed with a mobile phase gradient program containing two solutions (A) Milli-Q with 0.1% trifluoroacetic acid and (B)

filtered acetonitrile, at a flow rate of 1.250 mL/min, 370 nm detection, $10 \pm 0.15°C$ column temperature, for 30 minutes. The gradient was set up as follows: isocratic in 60% B for 6 minutes, 60% B to 80% B in 1.5 minutes, isocratic in 80% B for 6 minutes, 80% B to 100% B in 1.5 minutes, and then isocratic at 100% B for 15 additional minutes.

RESULTS AND DISCUSSION

Temperature Studies

The effect of temperature on percent conversion of alcohol to aldehyde was studied on samples (n=3) reacted with enzyme over a range of 30 to 45°C. Enzyme blanks and 1 μM alcohol samples were reacted with 100 μL of enzyme at pH 8 in HPLC vials, in a hot water bath at the experimental temperature for 10 minutes. Following the enzyme reaction, 10 μL of DNPH was added and allowed to react for 30 minutes at room temperature prior to HPLC separation. The optimum enzyme reaction temperature was 40 ± 0.1°C (Figure 1), which is similar to the 45°C optimum determined at higher concentrations [8]. Percent conversions of each alcohol were low because the enzyme reaction time was 10 minutes. A 10 minute reaction time was used so a change in percent conversion could be seen over the temperature range studied. A reaction time of 45 minutes was not chosen because percent conversion of methanol to formaldehyde decreased. This decrease may be a result of further oxidation of formaldehyde to formic acid. Standard deviations of methanol at 40 and 45°C were the same at the 95% confidence level using the F test [9]. Percent conversions at these temperatures were then compared using a T test [9] and were statistically different at the 95% confidence level.
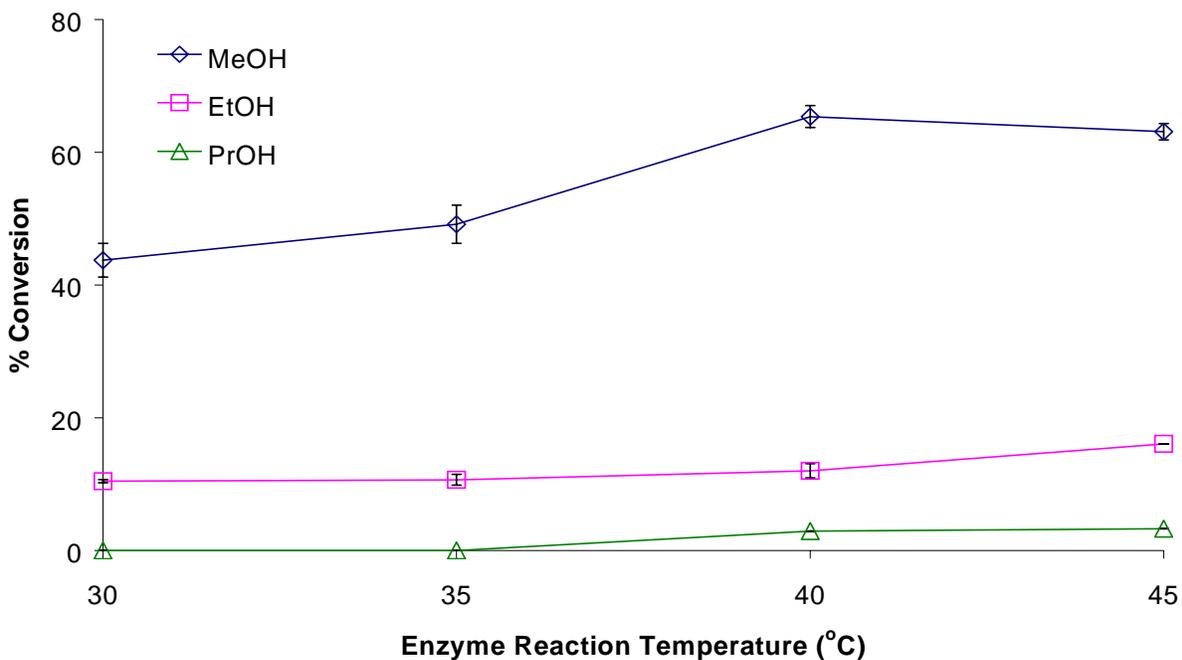
Figure 1: Percent conversion of C_1-C_3 alcohols versus enzyme reaction temperature at pH 8 when reacted with 100 µL of alcohol oxidase for 10 minutes (n=3).

pH Studies

Experiments (n=3) were done to determine optimum enzyme reaction pH. An enzyme

reaction pH range of 6.74 to 8.80 was studied. Alcohol samples consisted of 900 µL of 1 mM

potassium phosphate buffer at each pH studied, 100 µL of the 10 µM C_1-C_3 alcohols, 100 µL of

enzyme, and 10 µL of DNPH. Enzyme blanks were prepared in analogous fashion except, 1000

µL of 1 mM buffer at the respective pH was used in place of the 900 µL of buffer and 100 µL of

sample. Enzyme blanks and 1 µM C_1-C_3 alcohols were allowed to react with enzyme for 30

minutes at $40 \pm 0.1°C$. Phosphate buffer concentrations were high enough to allow enzyme

reaction but to then allow pH to drop to approximately 2 upon addition of DNPH reagent for

conversion of aldehydes to their hydrazones. The optimum enzyme reaction pH was 8.5,

resulting in percent conversions of 96% with a relative standard deviation (RSD) of 3% for

methanol, 41% with a RSD of 4% for ethanol, and 9.0% for propanol with a RSD of 9% (Figure

2). Experiments were repeated 4 times (n=3) at pH 8.5 to ensure percent conversion was correct.

Standard deviations for methanol at pH 8.5 and 8.8 were the same at the 95% confidence interval

based on the F test [9]. Percent conversions of methanol at pH 8.5 and 8.8 were statistically

different at the 95% confidence interval using a T test [9]. Potassium phosphate buffers (0.1 M

and 1 mM) were both made at pH 9.0, because the pH dropped to roughly 8.5 during the enzyme

reaction as a result of the buffered enzyme pH. This experimental value is consistent with the

literature [8] optimum pH value of 8.5 for alcohol oxidase from the yeast *Hansenula* in 50 mM

potassium phosphate buffer. Although the trend is not obvious, it is the same for ethanol and

propanol because percent conversions at 8.5 were statistically greater than at other pHs.
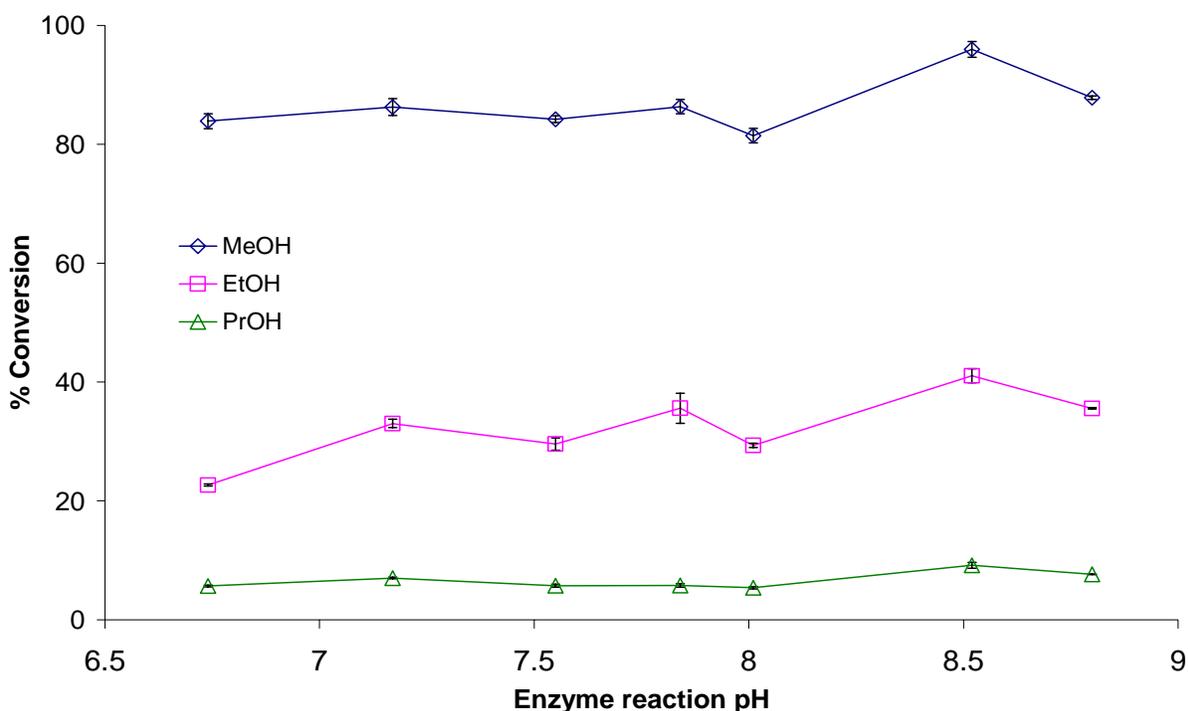

Figure 2: Percent conversion of C_1-C_3 alcohols as a function of the enzyme reaction pH when
reacted for 30 minutes at $40 \pm 0.1°C$ (n=3).

Buffer Concentration

Peak area responses for the 1 μM C_1-C_3 alcohols (n=3) were compared using 1 mM and 50 mM buffered Milli-Q to see if the buffer concentration had an effect on the reaction. Reagent blank and enzyme blank peak areas increased with increasing buffer concentration. There was no significant difference in peak areas for the 1 μM aldehydes with enzyme and alcohols. The pH of both the 1 mM and 50 mM buffered solutions dropped to pH 2.0 upon addition of DNPH. Therefore the 1 mM buffer was used for all samples because of the lower blanks.

Enzyme Reaction Time

To determine optimum enzyme reaction time, 1 μM C_1-C_3 alcohol samples (n=3) were reacted over a period of 5 to 50 minutes, at pH 8.4 and 40 ± 0.1˚C (Figure 3). A pH of 8.4 was used because this was initially thought to be the optimum enzyme reaction pH based on preliminary experiments. Maximum percent conversion of methanol (91.4%) occurred after 40 minutes with a RSD of 1%. Percent conversion of methanol decreased after 40 minutes possibly due to conversion of the alcohols to formic acid. Maximum conversion of ethanol (46%) and propanol (11%) was at 50 minutes with RSDs of 5% and 7% respectively. Percent conversions of ethanol and propanol were low because the activity of alcohol oxidase decreases as the number of carbons increases, suggesting the ethanol and propanol do not fit as well into the active site on the enzyme compared to methanol. Percent conversions of ethanol and propanol were not studied at longer enzyme reaction times but may possibly be increasing with time according to experimental data.
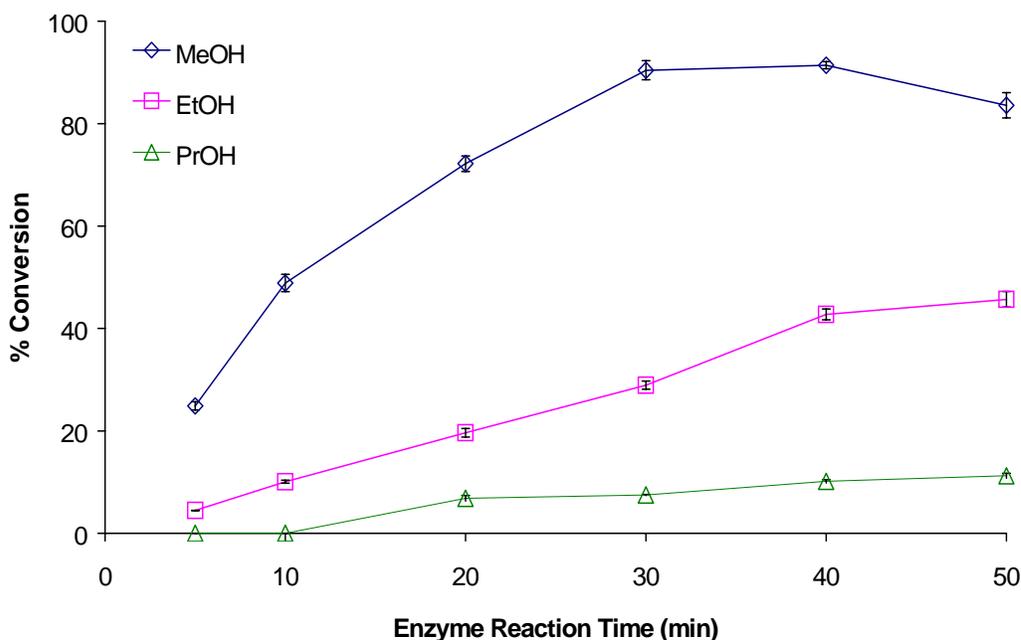
Figure 3: Percent conversion of methanol, ethanol, and propanol as a function of enzyme reaction time studied at pH 8.4 and $40 \pm 0.1^{\circ}C$ (n=3).

Conversion to Formic Acid

Recall, the drawback of using alcohol oxidase was possible further oxidation of formaldehyde to formic acid. First, to determine if conversion of formaldehyde to formic acid was occurring, a methanol-free formaldehyde source was needed to compare with the 37.69% CH_2O with methanol stabilizer. Paraformaldehyde was chosen because it was known to be free of methanol. Paraformaldehyde was only used in experiments requiring a methanol free source of formaldehyde because it was difficult to get into solution. An initial experiment was conducted to ensure the paraformaldehyde source was methanol free. This was done by comparing peak area response of paraformaldehyde reacted with DNPH alone with that of paraformaldehyde reacted with enzyme and DNPH at pH 8.5, $40 \pm 0.1^{\circ}C$, for 40 minutes (n=4). Based on the F-test [9], standard deviations for paraformaldehyde with and without enzyme over the entire concentration range were statistically equivalent at a 95% confidence interval. Peak

areas, corrected for dilution, of paraformaldehyde were the same between samples with and

without enzyme over the concentration range of 0.5-3.0 µM at the 99.9% confidence level using

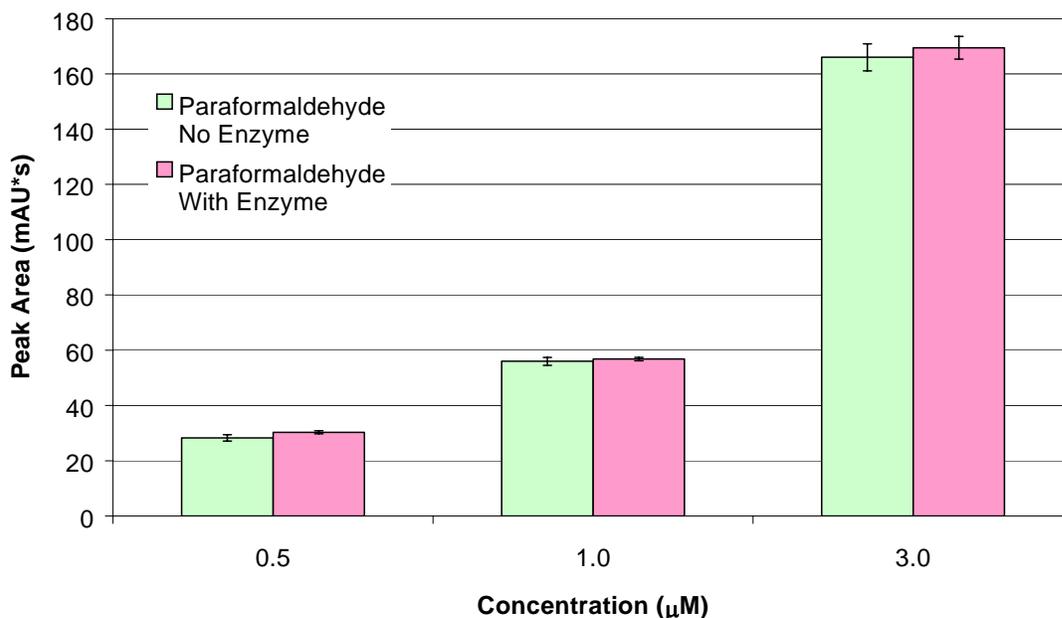a T-test [9], indicating that no methanol was present (Figure 4).

Figure 4: Comparison of peak areas for paraformaldehyde with and without enzyme at pH 8.5, 40 ± 0.1°C, for 40 minutes (n=4).

Conversion of formaldehyde to formic acid was tested over a concentration range of 0.50

to 3.0 µM under optimum conditions determined earlier (Figure 4). When paraformaldehyde

was analyzed (Figure 4), peak area response of the paraformaldehyde did not decrease upon

addition of enzyme compared to the signal with no enzyme, which would have been indicative of

conversion to the acid.

Enzyme Amount Comparison

The optimum enzyme amount was defined as that required to achieve maximum percent

conversion of alcohol to aldehyde in the shortest amount of time. This was tested by using 50,

100, and 200 µL of 2 units mL^{-1} alcohol oxidase, yielding 0.095, 0.18, and 0.33 units mL^{-1} respectively when added to 1 mL of sample. Samples were reacted under optimum conditions (pH 8.5, 40 ± 0.1˚C, n=3). Optimum enzyme amount was 100 µL, resulting in a 91.4% conversion of methanol with a RSD of 1%, 43% conversion of ethanol with a RSD of 4%, and a 10% conversion of propanol with a RSD of 5%. However, greater percent conversions were observed for ethanol and propanol when reacted with double the amount of enzyme (200 µL) for 60 minutes. Percent conversions of ethanol and propanol were 92.3% and 24.2% with RSDs of 1% and 2% respectively. Studies are still being conducted for ethanol and propanol at longer enzyme reaction times with 200 µL addition of enzyme. Methanol was the primary component being analyzed in rain samples therefore 100 µL enzyme was used to validate the method. Results of this study for methanol, ethanol, and propanol can be seen in Figures 5-7 below. Enzyme blank signal increased with increasing enzyme concentration due to presence of methanol in the enzyme. The decrease in percent conversion for 100 µL addition of enzyme to methanol after 40 minutes, and for 200 µL addition of enzyme to methanol after 20 minutes, was presumably due to oxidation to formic acid.
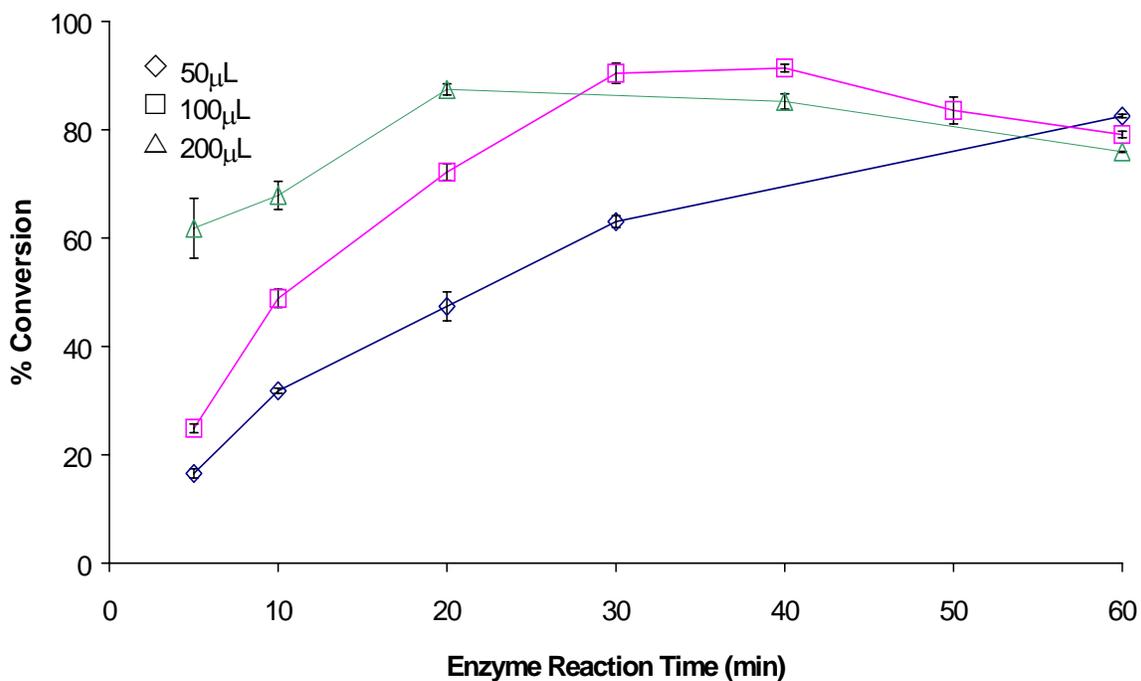
Figure 5: Percent conversion of methanol versus enzyme reaction time using 50, 100, and 200 µL of enzyme at pH 8.5 and 40 ± 0.1°C (n=3).

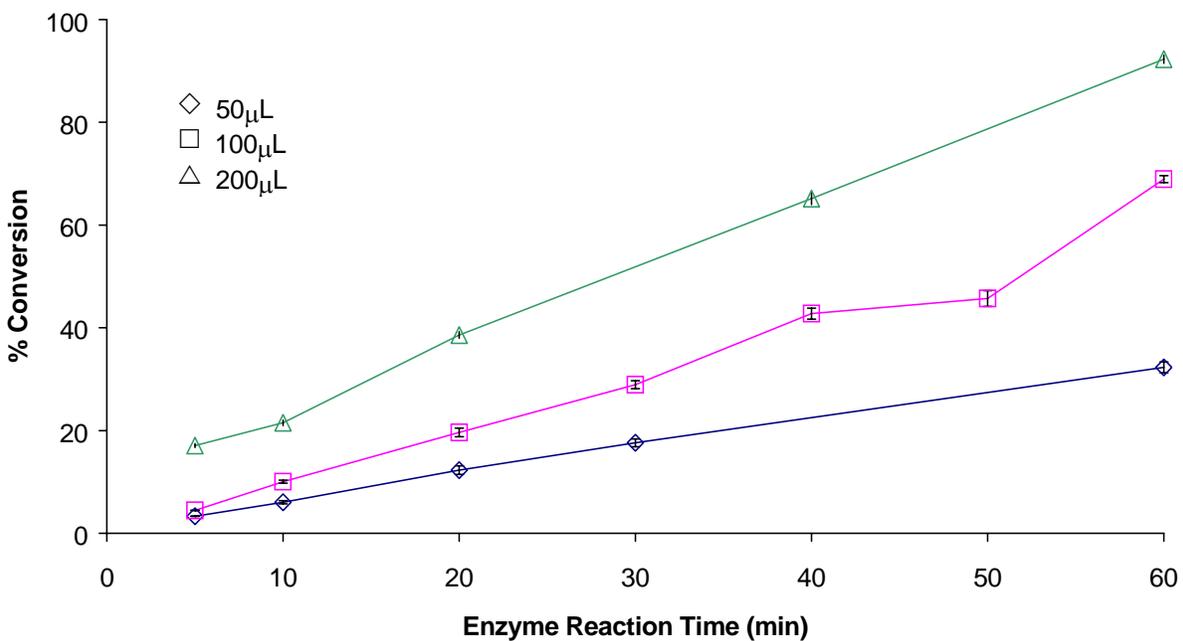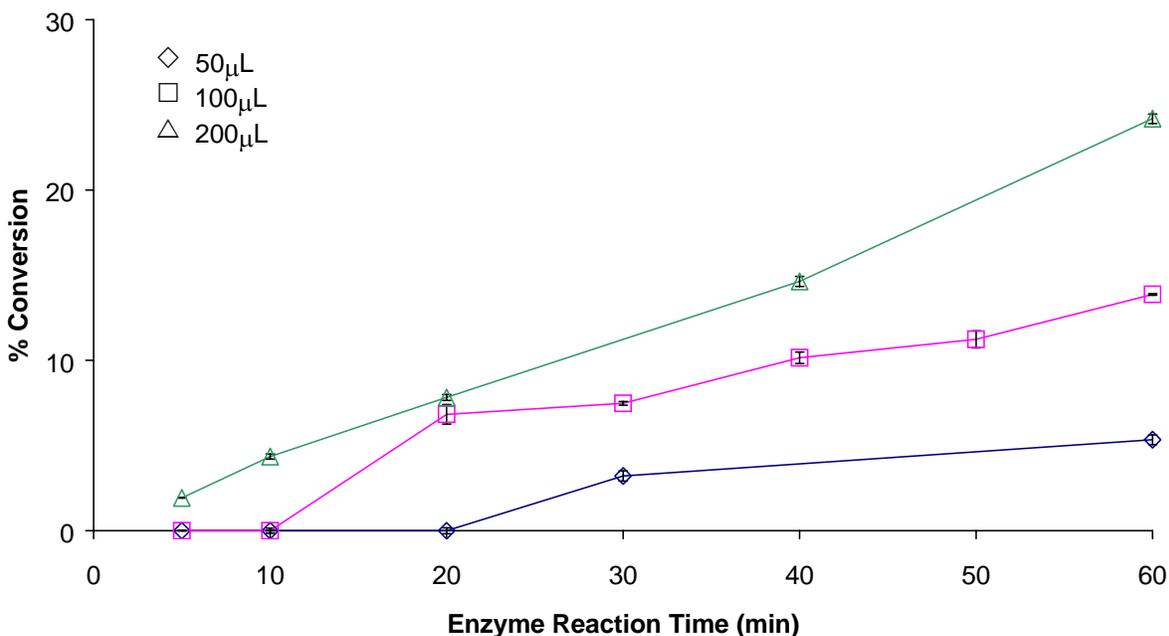Figure 6: Percent conversion of ethanol versus enzyme reaction time using 50, 100, and 200 µL of enzyme at pH 8.5 and 40 ± 0.1°C (n=3).

Figure 7: Percent conversion of propanol versus enzyme reaction time using 50, 100, and 200 µL of enzyme at pH 8.5 and $40 \pm 0.1°C$ (n=3).

Percent Conversions of Alcohols

Percent conversions for methanol, ethanol, and propanol are statistically equivalent at the 99.9% confidence interval over a range of 0.25-5.00 µM, when analyzed for 40 minutes at $40 \pm 0.1°C$ and enzyme reaction pH 8.0 (Figure 8). Using the student's t-test [9], the slopes of each of the regression lines are not significantly different from zero at the 99.9% confidence interval, suggesting that percent conversions of methanol, ethanol, and propanol are statistically the same over the concentration range. Data shows a 90.1% conversion with an average RSD of 2% for methanol, 40% for ethanol with an average RSD of 3%, and a 7% conversion for propanol with an average RSD of 3%.
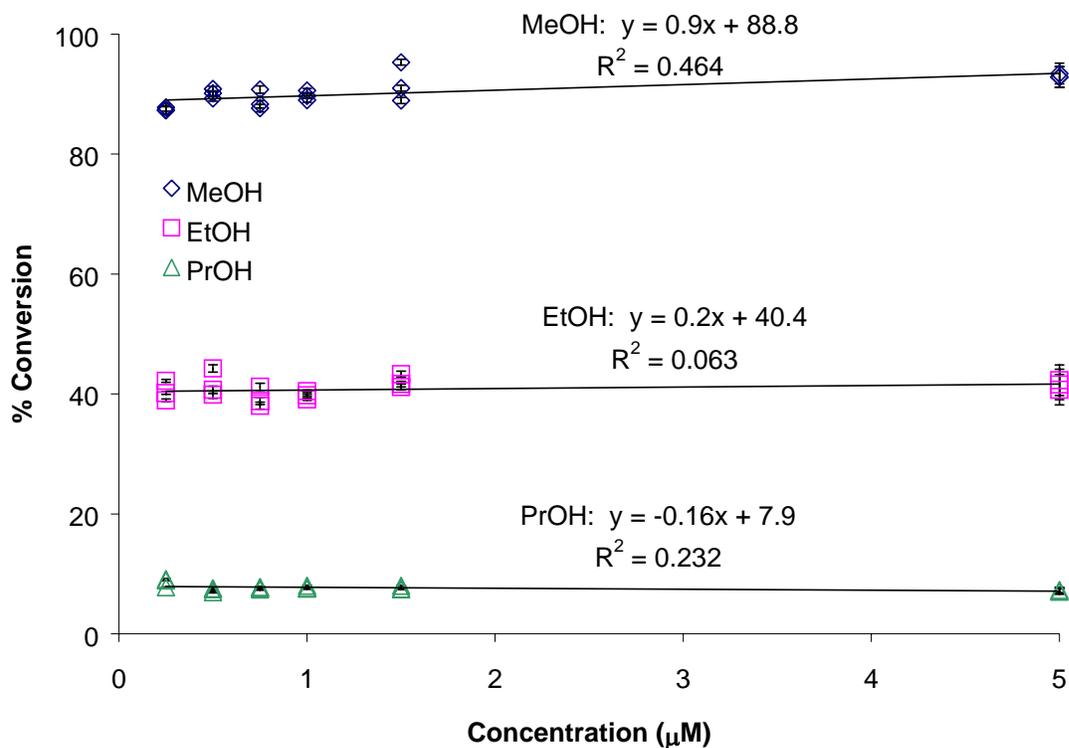
Figure 8: Percent conversions for methanol, ethanol, and propanol over a concentration range of 0.25-5.00 µM reacted at pH 8.0 and 40 ± 0.1˚C for 40 minutes (n=3).

Calibration Curves

Calibration curves (Figures 9-11) were constructed for the C_1-C_3 aldehydes and alcohols (pH 8.0, 40 ± 0.1˚C, 40 minute reaction time, n=3). By comparing slopes of alcohols to aldehydes percent conversions can be determined of alcohols since curves were linear over calibration range. Percent conversion of methanol was 93.5%, ethanol 41.6%, and propanol 7.1% with RSD values of 2%, 3%, and 3% respectively. This is important because unknown alcohol concentrations can be determined within the calibration range since percent conversions of each alcohol are known to be constant.
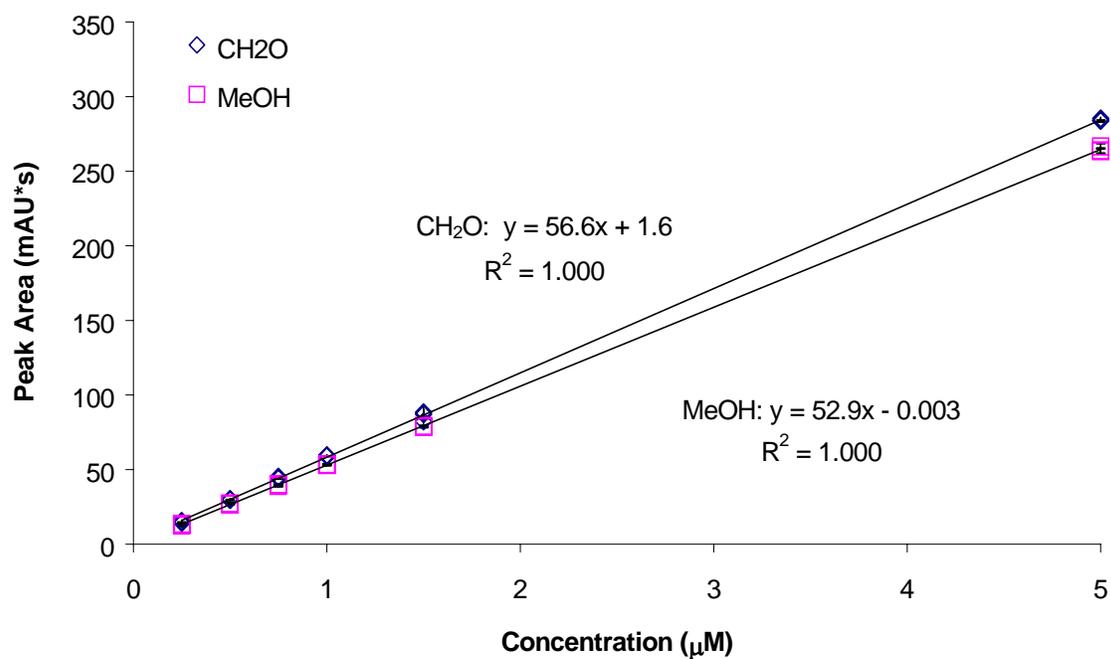
Figure 9: Calibration curves for formaldehyde and methanol over a concentration range of 0.25 to 5.00 μM (pH 8.0, 40 ± 0.1°C, 40 minute reaction time, n=3).
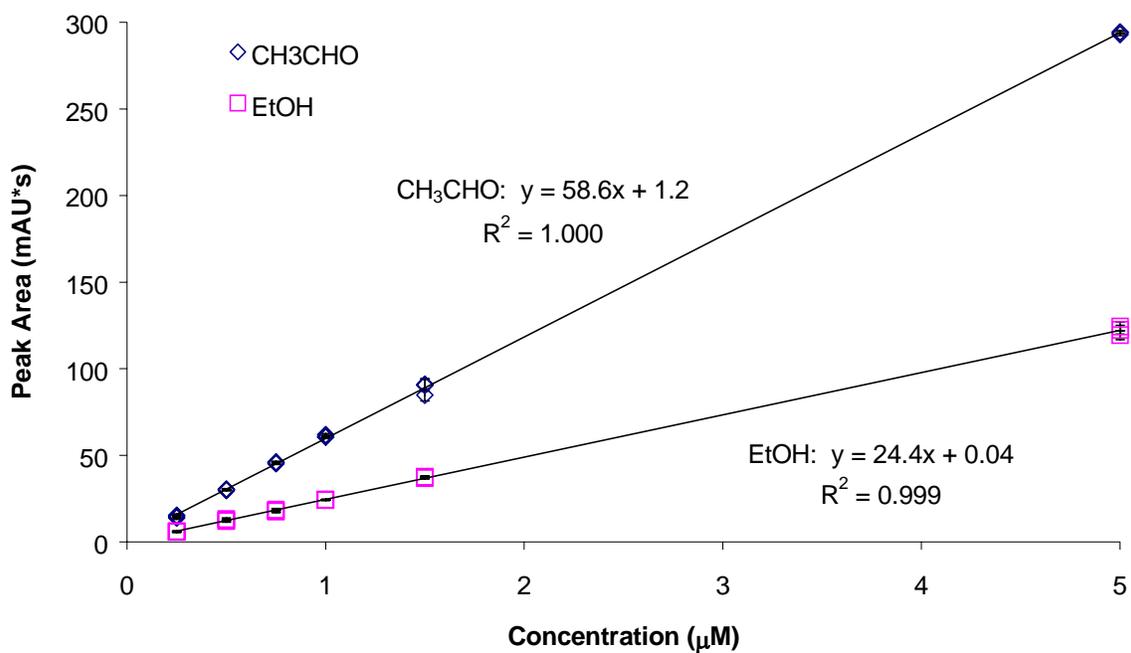

Figure 10: Calibration curves for acetaldehyde and ethanol over a concentration range of 0.25 to 5.00 μM (pH 8.0, 40 ± 0.1°C, 40 minute reaction time, n=3).
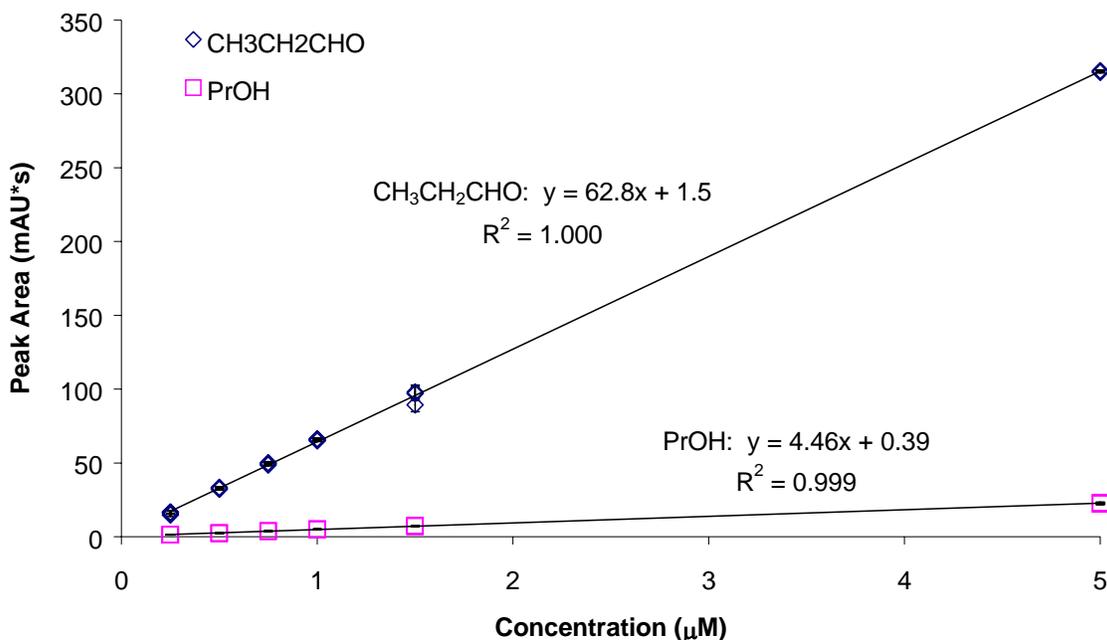
Figure 11: Calibration curves for propionaldehyde and propanol over a concentration range of 0.25 to 5.00 µM (pH 8.0, 40 ± 0.1˚C, 40 minute reaction time, n=3).

Limit of Detection

Experiments were done at pH 8.5 and 40 ± 0.1˚C, for 40 minutes on thirty-nine enzyme

blanks (38 for propanol). Enzyme blanks contained 1 mL of 1 mM potassium phosphate buffer,

100 µL of enzyme (2 units mL^{-1}), and 10 µL of DNPH. Enzyme blanks contained 0.44 ± 0.01

µM methanol and 0.050 ± 0.004 µM ethanol (Figure 12). Enzyme blank concentrations were

based on peak areas (100 µL sample loop) for 1 µM known alcohol standards. The propanol

blank, 0.15 ± 0.04 µM, was based on integration of instrument noise. Limit of detection was

defined as three times the standard deviation of the enzyme blank divided by the slope of the

alcohol calibration curve. Limits of detection were 0.03 µM for methanol, 0.02µM for ethanol,
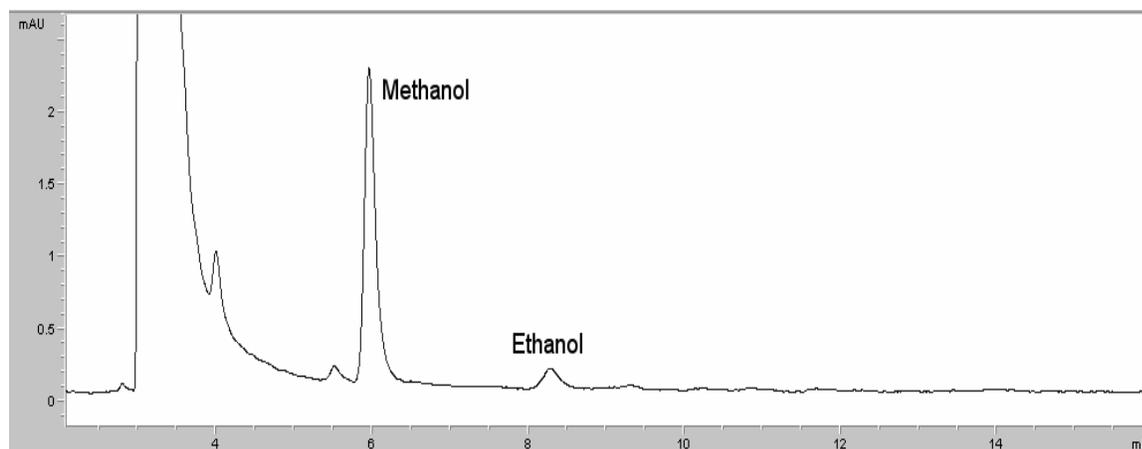
and 0.14 µM for propanol.

Figure 12: HPLC chromatogram of an enzyme blank showing methanol and ethanol peaks (pH 8.5, 40 ± 0.1˚C, 40 minutes, n=39). Propanol was not detected in the enzyme blank.

Precision

Precision of standard alcohol samples was determined based on triplicate analysis of methanol, ethanol, and propanol over concentration range of 0.25-5.00 μM reacted at pH 8.5, 40 ± 0.1˚C, for 40 minutes. The average relative standard deviation (RSD) for each of the alcohols was 2% for methanol, 3% for ethanol, and 3% for propanol over the concentration range. Precision in rain samples was determined on an event basis for methanol and ethanol. Each sample was analyzed in sets of five under the above conditions and percent relative standard deviations calculated (Table 1). Relative standard deviations were not reported for propanol because such low concentrations of propanol were present. Conditions are still being optimized for propanol.

Event Number	570A	573	574
Methanol	4%	3%	1%
Ethanol	8%	8 %	6%

Table 1: Percent relative standard deviations for methanol and ethanol for each rain event analyzed at pH 8.5, 40 ± 0.1˚C, 40 minutes (n=5).

Accuracy

Accuracy of the method was determined by addition of 1 µM methanol, ethanol, and propanol to rain samples. Experiments were done at pH 8.5, 40 ± 0.1˚C, for 40 minutes (n=3). The concentration of methanol and ethanol in rain samples was subtracted from the total alcohol concentration (including spike) in each event. Alcohol concentrations in spiked samples were determined by comparison to1 µM known alcohol standards taking percent conversions into account (Table 2).

Event Number	570A	573	574
Methanol	1.05 ± 0.04 µM	1.01 ± 0.03 µM	0.97 ± 0.02 µM
Ethanol	0.90 ± 0.03 µM	0.91 ± 0.02 µM	0.92 ± 0.02 µM

Table 2: Recovery in rain samples of 1 µM alcohol spike analyzed at pH 8.5, 40 ± 0.1˚C, 40 minutes (n=3).

Alcohol Concentrations in Rain

Information from rain events (570A, 573, and 574) such as storm type, rain amount, pH, hydrogen peroxide concentration, and dissolved organic carbon (DOC) concentrations are presented in Table 3.

Rain Event Number	570A	573	574
Storm Type	Coastal/terrestrial	Marine	Coastal/terrestrial
Amount	84.07 mm	13.97 mm	25.40 mm
pH	5.010	4.205	4.868
$[H^+]$	9.772×10^{-6} M	6.237×10^{-5} M	1.357×10^{-5} M
H_2O_2	1.72 µM	20.81 µM	2.20 µM
DOC	6.16 µM	68.79 µM	34.44 µM

Table 3: Data collected from rain events 570A, 573, and 574.

Alcohol concentrations in rain were dependent on the type of storm and calculated based on average peak areas of 1µM alcohols. Peak areas from alcohol standards (Table 4) were used to

calculate concentrations of alcohol in rain instead of calibration curves because standard
concentrations were in the linear region of calibration curves, were similar to peak areas found in
rain, and showed high precision.

| 1.00 µM Alcohol	Experiment	Peak Areas (mAU*s)	Standard Deviation
Methanol	1 µM Standard	53.034 53.447 53.111	0.220
	Limit of Detection	54.776 55.232 55.490	0.362
	Rain 570A	53.958 52.341 53.736	0.877
	Rain 573	50.125 52.141 51.791	1.077
	Rain 574	52.085 53.392 52.276	0.706
Ethanol	1 µM Standard	24.204 24.180 24.484	0.169
	Limit of Detection	30.926 30.005 30.467	0.461
	Rain 570A	32.173 31.424 32.443	0.528
	Rain 573	28.232 28.499 31.314	1.708
	Rain 574	30.529 31.136 30.556	0.343

Table 4: Peak areas for 1.00 µM standards of methanol and ethanol on an experimental basis (40 ± 0.1˚C, 40 minutes, pH 9.0, n=3).

Experiments were run at pH 8.5 and $40 \pm 0.1°C$ for 40 minutes (n=5). Data was collected and is presented in Table 4; percent conversions were taken into account when calculating concentrations. The methanol concentration in event 570A was low because the enzyme blank was high (1.12 ± 0.04 μM). Chromatograms for each of the events and the enzyme blank (Event 570A) are presented in Figure 13. The origin of the storm also has an impact on the type and number of other compounds present between the reagent and methanol peaks. Propanol concentrations were not reported because conditions are still being optimized. Percentage of carbon contributed by methanol and ethanol combined was calculated (Table 5) using dissolved organic carbon (DOC) concentrations (Table 3) for the individual rain events.

Event Number	570A	573	574
Storm Type	Coastal/terrestrial	Marine	Coastal/terrestrial
% Carbon from alcohols (C_1-C_2 combined)	3.1%	0.4%	3.5%
Methanol	0.08 ± 0.05 μM	0.13 ± 0.03 μM	0.91 ± 0.02 μM
Ethanol	0.08 ± 0.01 μM	0.11 ± 0.02 μM	0.23 ± 0.02 μM

Table 5: Alcohol concentrations (μM) and types of storms for rain events 570A, 573, and 574 at pH 8.5 and $40 \pm 0.1°C$ for 40 minutes (n=5).

Spike recovery was used for preliminary identification of some peaks eluting between the reagent and methanol peaks (Figure 14). Standards (1 μM) of the compounds were analyzed to determine retention times using reversed phase HPLC (optimum HPLC conditions). Rain samples were then spiked with the desired compounds and reacted with DNPH for 30 minutes. These peaks were identified as succinic semialdehyde (~3.6 minutes), nitrite (~4.0 minutes), a 5-carbon oxo-acid (~4.5 minutes), glyoxal (~5.4 minutes), and pyruvic aldehyde (~5.7 minutes-just prior to the methanol peak). Further analysis to confirm these compounds and their concentrations is underway.
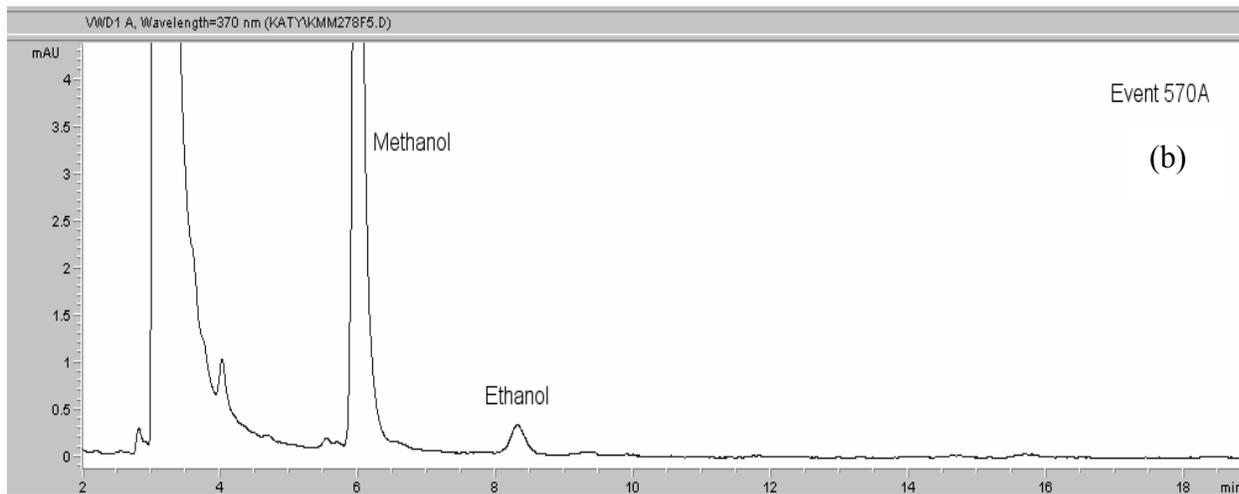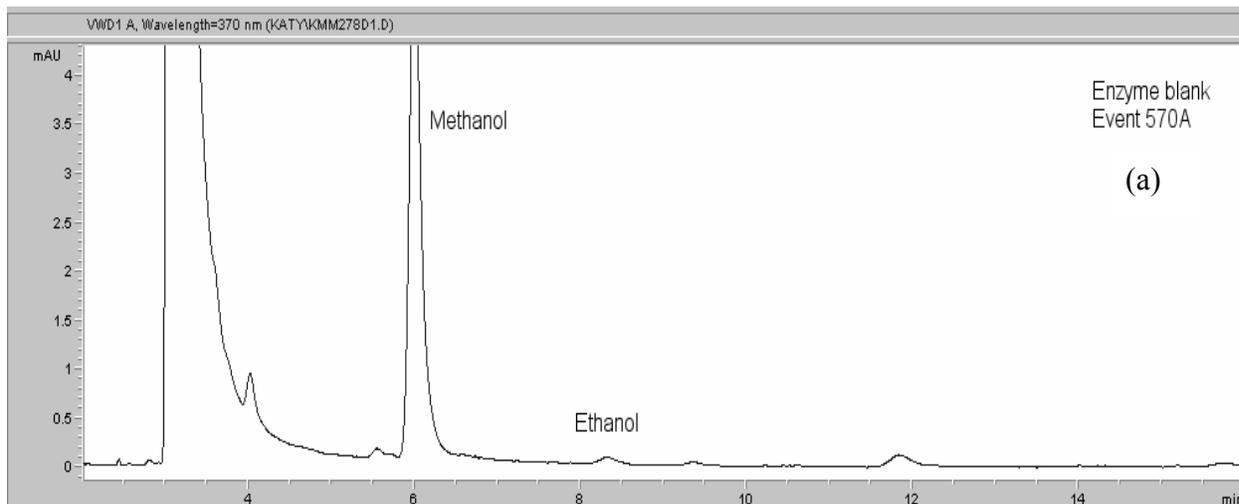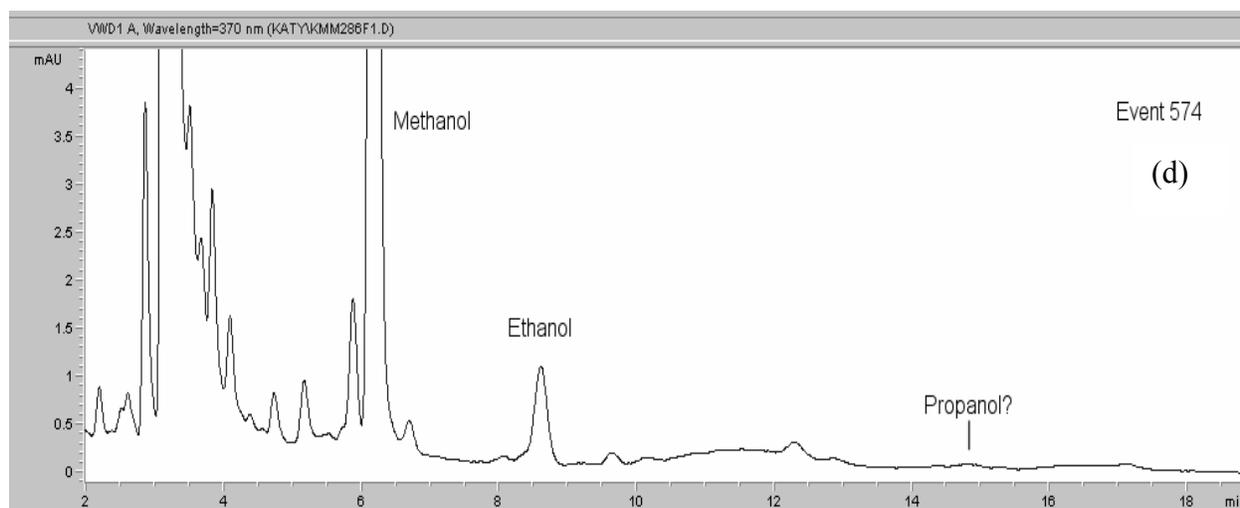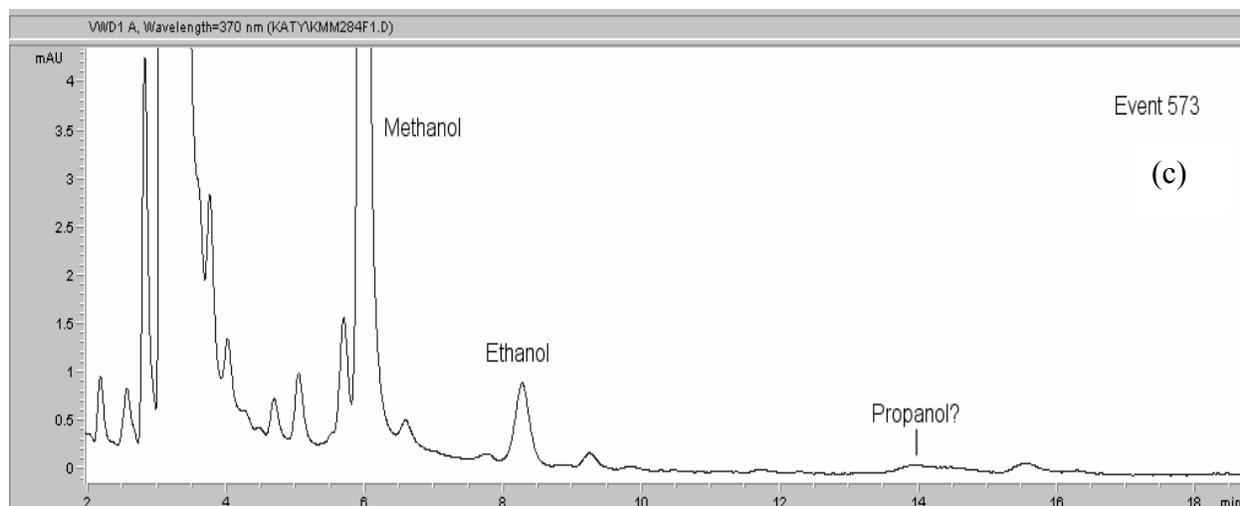
Figure 13: HPLC chromatogram from rain events (a) Enzyme blank 570A, (b) event 570A, (c) event 573, and (d) event 574 showing methanol, ethanol, and propanol peaks (pH 8.5, 40 ± 0.1˚C, 40 minutes, n=5).
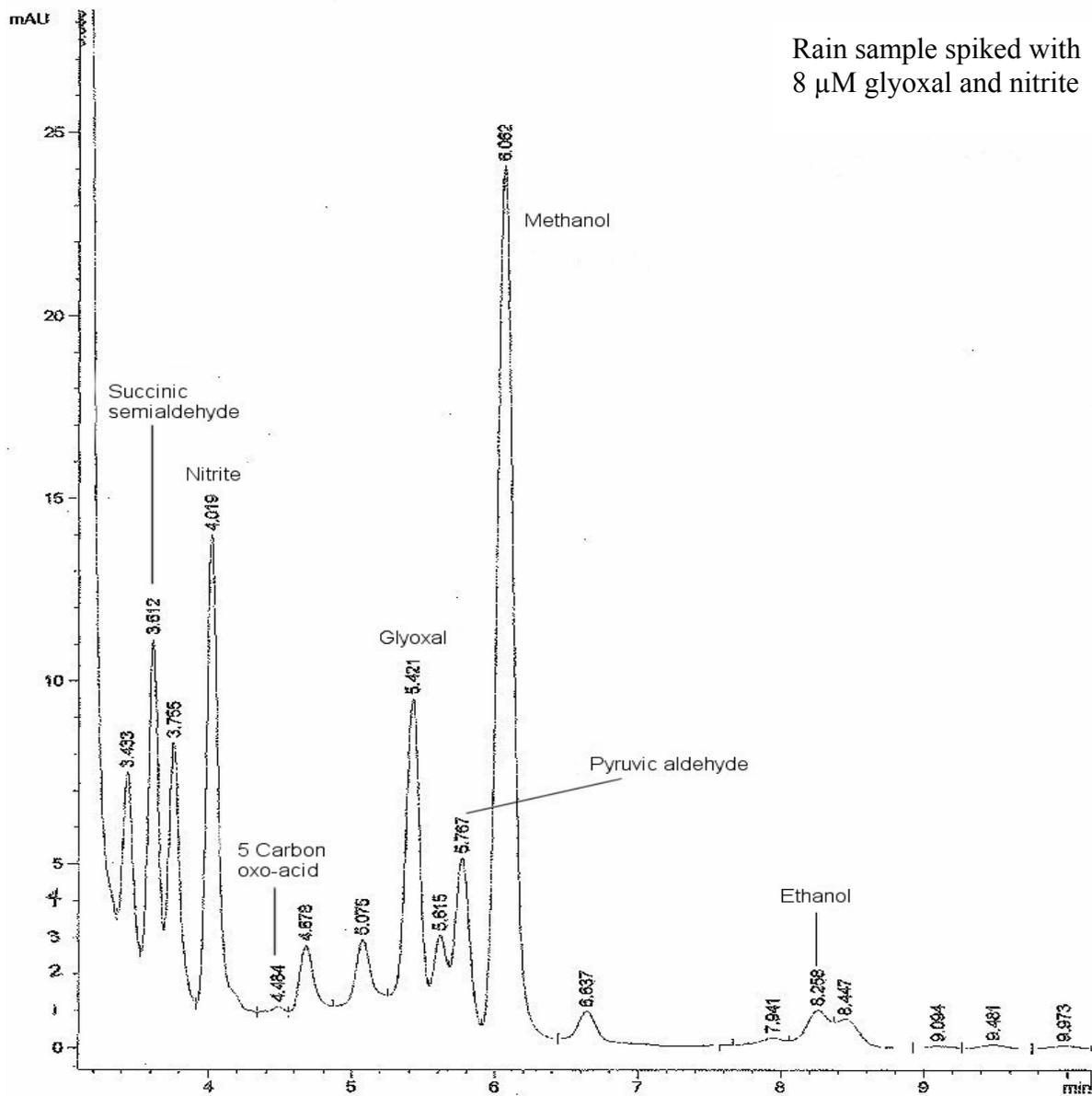
Figure 14: HPLC chromatogram showing preliminary identification of other compounds present in rain by spike recovery. Sample spiked with 8 µM glyoxal and nitrite.

CONCLUSIONS

The first method for the detection of submicromolar concentrations of aliphatic alcohols in aqueous rainwater samples has been developed. Optimum conditions for the reaction of alcohols with alcohol oxidase were pH 8.5, $40 \pm 0.1^{\circ}C$, 100 µL enzyme addition, and a reaction time of 40 minutes. Under these conditions, consistent percent conversions were seen for each of the alcohols over a concentration range of 0.25-5.00 µM. No conversion of methanol/formaldehyde to formic acid was seen under the above conditions. Using this method, low limits of detection were achieved; 0.03 µM for methanol, 0.02µM for ethanol, and 0.14 µM for propanol. Precision of the alcohol standards was high with relative standard deviations of 2% for methanol, and 3% for ethanol, and 3% for propanol.

This method has been successfully applied to rainwater samples. Alcohols in rain were dependant on storm origin and varied in concentration from about 0.08-0.91 µM for methanol and 0.08-0.23 µM for ethanol. Precision in rain samples was dependent on the type of event and ranged from 1-4% for methanol and 6-8% for ethanol. Conditions for propanol are still being optimized. Accuracy was determined by spike recovery of 1 µM alcohols in rainwater samples reacted with enzyme. Methanol recoveries were 1.05 ± 0.04 µM (event 570A), 1.01 ± 0.03 µM (event 573), and 0.97 ± 0.02 µM (event 574) in rain events. Ethanol recoveries were 0.90 ± 0.03 µM (event 570A), 0.91 ± 0.02 µM (event 573), and 0.92 ± 0.02 µM (event 574) in alcohol spiked rain samples.

Methanol and ethanol accounted for about 0.4% to 3.5% of the dissolved organic carbon in rainwater samples depending on storm type and origin. The presence and concentration of other compounds in rainwater eluting between the reagent and methanol peaks, were also

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dependent on the type of storm. Further studies include: further HPLC method development;

optimization of conditions for analysis of propanol; quantification of alcohols in rainwater over

several seasons; identification and quantification of other species present in rainwater; and

application of this method to seawater samples.

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