Spring 5-15-2009

Contribution of Candida albicans to the Synthesis of the Volatile Organic Compound Ethanol in Putrefied Bodies

Chelsey Mozingo
Coastal Carolina University

Contribution of Candida Albicans in the Synthesis Of the Volatile Organic Compound Ethanol In Putrefied Bodies

By

Chelsey Mozingo

Biology

Submitted in Partial Fullfillment of the Requirements for the

Degree of Bachelor of Sciences in the Honors Program at

Coastal Carolina University

May 2009

Abstract:

This experiment sought to determine if *Candida albicans* contributed to the production of the volatile organic compound ethanol in decomposing beef livers. The results this experiment proved to be inconclusive. This experiment showed no correlation between the amount the presence of *Candida albicans* and the production of the volatile organic compound ethanol based on the standard addition method. This may be indicative that VOC analysis is not an appropriate method for determining time of death based on a quantitative analysis of volatile organic compounds. However, the experimental design most likely needs be altered to accommodate a more accurate method of sample retrieval and analysis.

Introduction:

Volatile organic compounds (VOCs) are organic molecules that vaporize at room temperature. For years, VOCs have been used to analyze air quality (Nalli et al. 2006). More recently, they have been used in the biomedical field to analyze post-mortem tissue (Lewis et al. 2004), for breath analysis of patients to determine a medical diagnosis (Amann et al. 2004) and to find people trapped in collapsed buildings following earthquakes (Statheropoulos et al. (B) 2006). VOCs from decomposing bodies are analyzed by gas chromatography – mass spectrometry or high performance liquid chromatography and can contain over eighty recognizable compounds (O'Neal et al. 1996). There are eleven "common core" VOCs which include ethanol, 2-propanone, 2-butanone, octane, methyl benzene, methyl ethyl disulfide, dimethyl disulfide, dimethyl trisulfide, and *o*-, *m*-, and *p*-xylenes (Statheropoulos et al. 2007). It is believed that these

compounds can be used to determine time of death in bodies that have a progressed decomposition state.

VOCs are naturally produced by bacteria, fungi, and yeast through a series of oxidation-reduction reactions that are common in the environment. They are also produced through metabolic and biochemical reactions, including fermentation, that take place within living higher organisms (Nalli et al. 2006, Statheropoulos et al. (B) 2006). The breakdown of the four major biological macromolecules are thought to be the substrate for the production of post-mortem volatile organic compounds from human corpses (Statheropoulos et al. 2007).

VOCs, such as ethanol, have been suggested as biological indicators that can be used to identify the time of death in a decomposing body. The concentration of ethanol in postmortem tissue is thought to gradually increase in concentration from the time of death until the glucose within the sample has been converted into ethanol and therefore diminished (Lewis et al. 2004, Yajima et al. 2006).

Microorganisms are the source of VOC ethanol formation in post-mortem tissues (Corry 1978, Johnson et al. 2004). Yeast is thought to be a key component in the breakdown of glucose. More specifically, *Candida albicans,* a species of yeast found on the skin and in the mouths of human beings has been identified as the source in the production of ethanol in postmortem tissue and blood (Davis et al. 1972, Lewis et al. 2004, Yajima et al. 2006). However, it must be noted that the *Candida albicans* found in samples following the initiation of the putrefaction process could be microbial contamination and not necessarily the microorganisms found within the cadaver prior to decomposition (Johnson et al. 2004).

Candida albicans converts sugars, mainly glucose, into ethanol by first using the EMP (Embden-Meyerhof Pathway) glycolytic pathway to convert glucose into pyruvate which is then taken through the fermentation process to produce ethanol (Yajima et al. 2006). In this process, pyruvate is converted to acetaldehyde utilizing the enzyme pyruvate decarboxylase and then finally to ethanol by the enzyme alcohol dehydrogenase. (Boumba et al. 2008)

As a body decomposes, certain VOCs are produced in different concentrations and in different parts of the cadaver. As putrefaction progresses, the concentration of these VOCs increase and decrease according to the amount of substrate present (e.g. glucose) (Davis et al. 1972), the temperature under which postmortem VOCs are produced and the environment in which the body is found (e.g in the water, buried underground, soil salinity, soil moisture) (Wilson 2007). The lungs, pancreas, liver, brain, and muscle tissue all produce increasing amounts of VOCs during the putrefaction process assuming they remain intact and are not removed from the decomposing body (Davis et al. 1972).

Research is currently being carried out to determine if the chemical distribution and concentration of certain VOCs can be used to determine the time of death for decomposing cadavers. Statheropoulos et al. (2005, 2007) have shown that certain VOCs are present in postmortem tissue and in headspace air surrounding decomposing bodies that had been placed in air tight bags during the putrefactive process. However, the microbial source of the ethanol in postmortem headspace air has yet to be determined.

The purpose of this experiment is to test if *Candida albicans* could be a source of ethanol in putrefying livers (as a model for decomposing organs). Further, it will

determine if there is a correlation between VOC ethanol concentration and the presence of *Candida albicans* within the putrefactive liquid of air-tight containers. It is hypothesized that *Candida albicans* will produce ethanol during the decomposition process of yeast-inoculated beef liver and that can be quantified by high performance liquid chromatography (HPLC).

Materials/Methods:

Preparation of Samples

Beef liver was obtained from the locker plant in Conway, South Carolina. The liver was cut into six pieces of approximately equal mass. These pieces were then weighed, the masses recorded and the liver pieces were placed into labeled GladWare® tight-sealed containers. A pure culture of *Candida albicans* was purchased from Carolina Biological Supply Company. It was received in a sealed tube grown on Yeast Malt Agar and was stored at room temperature until use. Swabs were used to transfer approximately equal amounts of *Candida albicans* onto three of the liver samples. The controls were samples in which no yeast was added. The samples were observed for twenty one days noting appearance, color, and growth of any microbial colonies.

Collection of Putrefying Liquid Samples

Samples were collected nine times during the twenty one day sampling period by pipetting one milliliter of putrefied liquid surrounding the livers into a labeled 1.0 ml microcentrifuge tubes and placing them in the freezer (-20 C) to prevent in reactions of

the sample's components. On day 21, the livers were reweighed to determine if any mass was loss over the course of the experiment.

Determination of Unknown Ethanol Concentration

The ethanol content in the Control 2 Day 3 Sample was quantified using High Performance Liquid Chromatography (HPLC) and a Standard Addition Method. Serial dilutions were prepared for the Standard Addition Method of 0 M, 0.1 M, 0.2M, 0.3 M, 0.4 M known ethanol content. Standard Addition samples were further prepared for the HPLC by using a Polytetrafluoroethylene (PTFE) Acrodisk 0.45 μm syringe filter with one milliliter of deionized water. The samples were analyzed using a Shimadzu-UFLC High Performance Liquid Chromatography with a Shim-pack XR-ODS column. Samples were run for fifteen minutes using 85% Acetonitrile and 15% deionized water. There were five minute runs between the sample runs in order to ensure that everything had come off the column.

Each sample was run three times. Peak area versus concentration of ethanol standard additions were plotted in order to extrapolate the concentration of ethanol in the original sample. The reason that only the Control 2 Day 3 sample was run will be discussed later.

Results:

Initial and Final Mass

An observation of the mass loss by the samples was considered to be an indication that decomposition of the liver tissue had occurred. Initial and final masses recorded between

the control samples and the samples containing the *Candida* showed significant differences in masses as confirmed by a t-test (control: $t = 20.17$, $df = 2$, $p = 0.0024$; variable: $t = 7.29$, $df = 2$, $p = 0.018$) (see Table 1). This suggests that some type of decomposition occurred during the sampling period though this can only be speculated because the dry sample weights were not taken. However, there was not a significant difference in the rate of decomposition based on the final masses of the control and *Candia* samples ($t = 2.78$, $df = 4$, $p = 0.61$). This suggests that *Candida albicans* did not to expedite the putrefactive process.

Microbial Competition

On Day 5, mold started to form on the surfaces of the livers in both the control and the *Candida* samples. After 23 days, the appearance of livers had changed substantially from onset of the experiment. The microbial growth present in the control samples had a grayish cauliflower appearance that turned black in some areas. The samples in which the *Candida* was present showed a green slimy film over the surface of the livers (Fig. 1). This is indicative of some type of microbial competition that may have taken place during the putrefying process of the liver.

Chromatographs

Chromatographs resulting from the HPLC runs showed large peaks that eluted from the column at approximately 0.64 minutes in all of the standard addition samples (Fig. 2- Fig. 6). These samples were compared against an ethanol standard in order to

properly identify the compound (Fig. 7) based on retention time. The drop that is seen in this chromatograph is normal when a standard of pure ethanol is run.

Quantifying the Unknown Ethanol Concentration

Areas under the peak for each standard addition sample were integrated in order to quantify the amount of ethanol present in each sample (Fig. 3). Concentration of known ethanol verses peak area was plotted. A linear regression was performed in order to extrapolate the concentration of ethanol in the unknown sample. With an r value of 0.581 and an R^2 value of 0.338, there is a very small linear correlation between the points with only 33.8% of the points being explained by the linear regression.

Discussion

There was a significant decrease in the mass of the liver samples between the initial and final recordings. These findings are indicative that some type of decomposition took place. However, these results cannot be confirmed due to the fact that dry weights were not taken. Thus, the water loss from the livers is not accounted for and could have contributed to the weight loss during the sampling period.

There was not a significant mass loss between the control livers and the *Candida* samples. This indicates that though decomposition took place, *Candida albicans* did not result in any noteworthy weight loss. However, this could have resulted from water loss rather than the decomposition of the liver. This experiment purely speculates that the weight loss during the sampling period was attributed to putrefaction rather than water loss based on the fact that microorganisms were present on the samples and have been

proven to play a significant role in tissue decomposition (Corry 1978, Davis et al. 1972, Lewis et al. 2004, Petkovic et al. 2005). Overall the control and *Candida* livers decomposed at similar rates based on the t-test performed.

The appearance of the livers during the sampling period is indicative of some type of microbial competition based on the differences of appearances between the control and the *Candida* samples (see Fig. 1). This microbial competition might have influenced the production of ethanol (perhaps VOCs, in general) in the control samples and the samples exposed to the *Candida albicans*. It is possible that *C. albicans* could have been out-competed by some other microorganism. It can only be assumed that the green slimy substance on the surface of the *Candida* samples was, in fact, *Candida*. Thus, the production of ethanol by *C. albicans* could have been affected. Furthermore, it is not known what microorganisms were present on the control and *Candida* samples, it can only be speculated. More accurate assumptions could have been made if the species of the microorganisms had been identified. Future efforts could attempt to isolate the microorganisms present.

The HPLC analysis of the Control 2 Day 3 sample showed inconsistencies in the peak area of the chromatographs used to determine ethanol content. These inconsistencies seem to indicate that perhaps the peaks contained other compounds beside ethanol or the samples were too volatile and reacted before samples were run. Though samples were frozen from the time they were collected to the time they were tested, samples had to be prepared and sat at room temperature during the HPLC runs. After these inconsistencies were noted, subsequent samples were not run due to the fact that the samples had been compromised, most likely by temperature. Perhaps a different

column would have been able to separate the compounds more efficiently. However, it seems more likely that the volatility of the compounds during the HPLC runs caused the inconsistent peak areas.

When plotting known molar concentration of ethanol verses peak area (Fig. 8), the points did not produce the strong linear relationship that is expected from the Standard Addition Method. A linear regression produced a low R-squared value of 0.3377. With the results given, there does not seem to be enough evidence to make an accurate assumption about the quantity of ethanol based on the chromatographs produced. This is why subsequent samples were not run. If the experiment were to be repeated, a more efficient way of preparing and running the samples would need to be implemented.

This experiment sought to determine if there could be an established trend associated with the presence of *Candida albicans* and the production of VOC ethanol. VOC analysis is an innovative method that is thought to become a quantitative way of establishing time of death in corpses where the state of decomposition is so advanced that an autopsy cannot be performed (Statheropoulos et al. 2005). From the results produced by this experiment, no definite conclusions can be made about *Candida albicans* contribution to the production of VOC ethanol. Perhaps the VOCs produced during the sampling period were too volatile and reacted with one another while the samples were being analyzed by the HPLC, despite the fact that samples were frozen from the time the samples were taken.

Due to the fact that the standard addition method implicated for analyzing ethanol content in the Control 2 Day 3 sample produced insignificant results, a linear regression

with a very small correlation, subsequent samples were not analyzed or quantified. These findings could suggest that VOC analysis is not an effective way of determining time of death, unless a more efficient way of sample collection is established.

Many different variables play in the putrefaction process. Places like the Body Farm at the University of Tennessee use qualitative observations to estimate time of death in various situations under varying conditions (Forensic Anthropology Center… [updated 2009]). Similarly, this experiment sought to determine a quantitative way evaluating time of death. However, this experiment only focused the effect of one microorganism and its effect on the production of the VOC ethanol. Numerous microorganisms make up the natural flora of the human body and are actively involved in the production of many of the VOCs (Corry 1978, Petkovic 2005, Wilson 2007). The fact remains that temperature, humidity, soil content, and many other factors play a key role in decomposition. In order to implicate VOC analysis as an effective way of determining time of death, every factor would have to be analyzed. Perhaps quantitative analysis of VOCs is too complex to implicate in real world situations.

Literature Cited:

Amann, A., Poupart, G., Telser, S., Ledochowski, M., Schmid, A., Mechtcheriakov, S. 2004. Applications of breath gas analysis in medicine. International Journal of Mass Spectrometry 239: 227-233.

Boumba, V. A., Ziavrou, K. S., Vougiouklakis, T. 2008. Biochemical pathways generating post-mortem volatile compounds co-detected during forensic ethanol analyses. Forensic Science International 174: 133-151.

Corry, J. E. L. 1978. A review: Possible sources of ethanol ante- and post-mortem: its relationship to the biochemistry and microbiology of decomposition. Journal of Applied Bacteriology 44: 1-56.

Davis, G., L.; Leffert, R. L.; Rantanen, N. W. Putrefactive ethanol sources in postmortem tissues of conventional and germ-free mice. 1972. Archeological Pathology 94.

Forensic Anthropology Center [Internet]. [updated 2009 May 7]. Knoxville (TN): University of Tennessee, Department of Anthropology; [cited 7 May 2009]. Available from: http://web.utk.edu/~fac/

Johnson, R. D., Lewis, R. J., Angier, M. K., Nicole, T. V. 2004. The formation of ethanol in postmortem tissues. Civil Aerospace Medical Institute, U.S. Department of Transportation, Federal Aviation Administration.

Lewis, R. J., Johnson, R. D., Angier, M. K., Vu, N. T. 2004. Ethanol formation in unadulterated postmortem tissues. Forensic Science International 146: 17-24.

Nalli, S., Owen, J. H., Grochowalski, A. R., Cooper, D. G., Nicell, J. A. 2006. Origin of 2-ethylhexanol as a VOC. Environmental Pollution 140: 181-185.

O'Neal, C. L, Wolf II, C. E., Levine, B., Kinsman, G., Poklis, A. 1996. Gas chromatographic procedures for determination of ethanol in postmortem blood using t-butanol and methyl ethyl ketone as internal standards. Forensic Science International 83: 31-38.

Petkovic, S. M., Simic, M. A., Vujic, D. N. 2005. Postmortem production of ethanol in different tissues under controlled experimental conditions. Journal of Forensic Sciences 50: 204-208.

Statheropoulos, M., Spiliopoulou, C., Agapiou, A. 2005. A study of volatile organic compounds evolved from the decaying human body. Forensic Science International 153: 147-155.

Statheropoulos, M., Agapiou, A., Georgiadou, A. 2006. Analysis of expired air of fasting male monks at Mount Athos. Journal of Chromatography B 832: 274-279.

Statheropoulos, M., Mikedi, K., Agapiou, A., Georgiadou, A., Karma, S. 2006. Discriminant analysis of volatile organic compounds data related to a new location method of entrapped people in collapsed buildings of an earthquake. Analytica Chimica Acta 566: 207-216.

Statheropoulos, M.; Agapiou, A.; Spiliopoulou, C.; Pallis, G.C.; Sianos, E. 2007. Environmental aspects of VOCs evolved in the early stages of human decomposition. Science of the Total Environment 385: 221-227.

Wilson, A. S., Janaway, R. C., Holland, A. D., Dodson, H. I., Baran, E., Pollard, A. M., Tobin, D. J. 2007. Modelling the buried human body environment in upland climes using three contrasting field sites. Forensic Science International 169: 6-18.

Yajima, D., Motani, H., Kamei, K., Sato, Y., Hayakawa, M. Iwase, H. 2006. Ethanol production by *Candida albicans* in postmortem human blood samples: effects of blood glucose level and dilution. Forensic Science International 164: 116-121.

Acknowledgements:

Initially, I would like to thank Dr. Mike Ferguson for his support and assistance. He was always to provide guidance and support whenever it was needed. I would also like to thank Dr. Brett Simpson for his assistance with the quantitative analysis of the samples and the HPLC. Lastly, I would like to thank Greer Baxter for her help in preparation and sample collection. Without their help, the completion of this project would not have been possible.

Table 1. Initial and final masses of control and variable samples.

	Initial Weight	Final Weight	Difference
C1	82.67	61.89	20.78
C2	102.05	84.26	17.79
C3	90.96	70.29	20.67
Average	91.89	72.15	
S1	79.55	61.32	18.23
S2	81.27	58.67	22.6
S3	111.53	97.6	13.93
Average	90.78	72.53	

This table displays the initial and final masses of the control and variable samples. This table also shows the mass loss during the sampling period. C1, C2, and C3 refer to the control samples while S1, S2, and S3 refer to those samples in which *Candida albicans* was present.

Fig. 1. Microbial competition on Day 23 livers.

The top three containers contain the control samples while the bottom three display the livers in which *Candida albicans* was present. The control samples have a grayish growth that turned black in some area. The samples with *Candida albicans* had a slimy green growth that covered the surface of the livers. This figure suggests that there was some type of microbial competition present; however, the identity of these microbes is unknown.

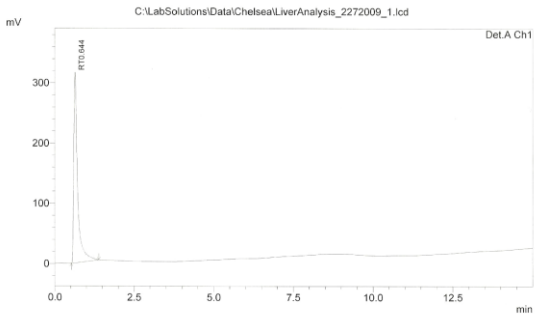

Fig. 2 – *Chromatograph of sample with no known amount of ethanol present*

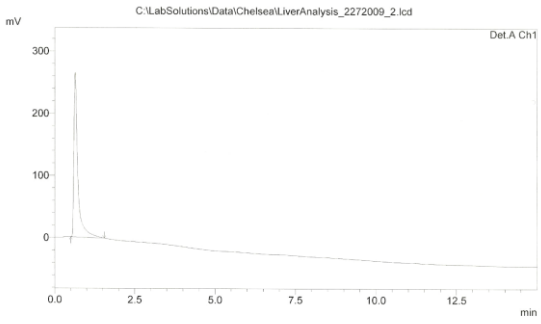

Fig. 3 – *Chromatograph of 0.1 M ethanol*

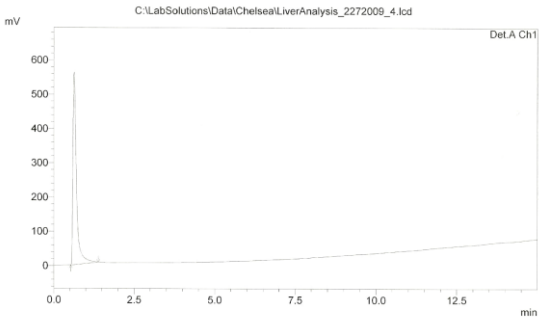

Fig. 4 – *Chromatographs of 0.2 M ethanol*

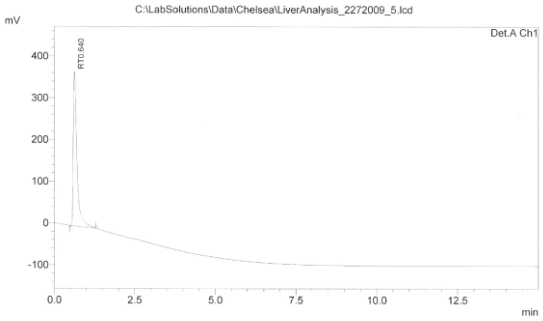

Fig. 5 – *Chromatographs of 0.3 M ethanol*

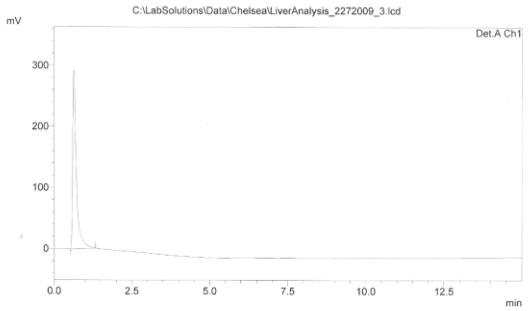

Fig. 6 – *Chromatographs of 0.4 M ethanol*

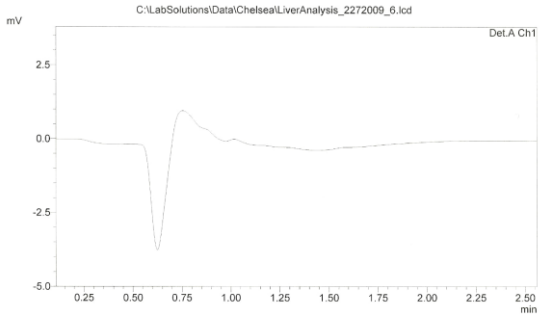

Fig. 7 – *Chromatographs of ethanol standard*

Fig. 2- Fig. 7. Chromatographs produced from the standard addition analysis of the Control 2 Day 3 liver.

These figures display the chromatographs obtained from the HPLC runs that analyzed the ethanol content of the Control 2 Day 3 sample using the standard addition method.

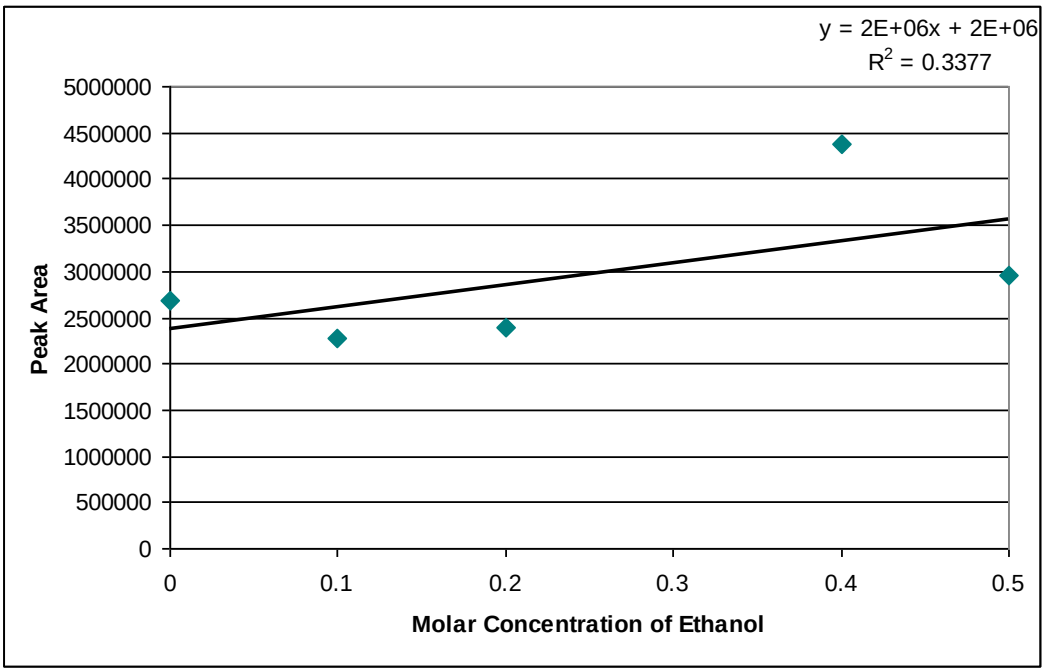

FIG. 8. Molar concentration verses peak area

This graph illustrates the relationship of molar concentration of known ethanol verses the peak area obtained from the integration of the HPLC chromatograph. By extrapolation, the amount of ethanol in the sample can be determined based on the construction of a linear regression displaying the relationship between known concentrations of ethanol and the peak area obtained by the standard addition method.