Chemical and Biochemical Means to Detect Alcohol

Determination of Ethanol Concentration in Fermented Beer Samples and Distilled Products

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Key Words:

Ethanol

Biofuel

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The fermentation of sugars to alcohol is used for a number of different applications, most notably the production of alcoholic beverages. The beer and wine industry ferment extracts of barley and grapes respectively to produce numerous alcoholic beverages, while distilleries further concentrate ethanol using the evaporation and condensation differences of ethanol and water. Ethanol can be biochemically quantitated from a diverse matrix, such as fermenting beer, using alcohol oxidase enzyme, while relatively pure ethanol can be determined chemically using the oxidizer pyridinium chlorochromate (PCC). Here we describe the use of ethanol oxidase and PCC to monitor ethanol production during the fermentation of beer and from distilled products.

Introduction

The production of alcoholic beverages has been associated with mankind from the beginning of civilization. As a result they are a key element of many parts of our culture. One such alcoholic beverage, beer, is produced by the fermentation of starchy grains through a process known as brewing. This has existed since the 6th millennium BC, with recipes being found in some of the earliest Sumerian writings [1]. Brewing is the production of beer through steeping a starch source (usually cereal grains) in water and then fermenting the resultant mixture with yeast (Figure 1). The basic ingredients of beer are water, a starch source, brewer's yeast and a flavoring agent such as hops. All of these ingredients work in concert to provide the individual flavor of different beers. A number of secondary sources of starch, such as corn, rice, millet, sorghum, and cassava root, are also used because of availability, to impart specific flavors or for economic reasons. Water is the primary ingredient in beer and it suffices to say that the dissolved minerals in water have a great deal to do with the style of beer from different regions of the world. Malted or germinated barley is used because the process of germination induces the grain seed to express several key amylase enzymes, such as α-(1→4) endo glycosylase and α-(1→6) endoglycosylase, necessary for the digestion of starch [2]. Hops or more specifically the flower or cone of the hop vine are used a flavoring agent (taste and aroma).

Figure 1. Glass fermentation carboys. Small batch brewing methods often employ the use of glass containers with liquid air-lock systems.

Fermentation of released glucose is carried out by brewer's yeast (Saccharomyces cerevisiea, Saccharomyces uvarum, and Brettanomyces), which convert glucose to ethanol (Figure 2).

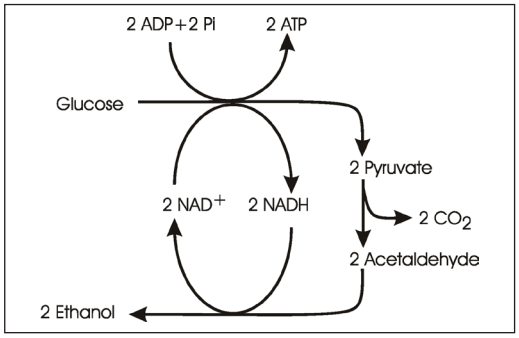

Figure 2. Schematic representation of the fermentation process.

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In order to monitor fermentation, as well as comply with federal and state labeling statues regarding alcohol, brewers need to quantitate ethanol concentration in fermented beer batches. In addition numerous vintners and distillers have similar requirements.

Distillation is a physical process (rather than a chemical process) that separates mixtures based on differences in the volatility of different components. Because ethanol has a lower vapor pressure than water, distilleries can concentrate ethanol produced by fermentation by heating the mixture. At a given temperature ethanol vapor will concentrate in the vapor relative to water. When the vapor is condensed back to liquid form, the effective ethanol concentration will have been increased (Figure 3). Using either a continuous distillation or several batch distillation processes, the ethanol concentration can be increased from the initial fermentation concentration of 5-10% to 95%. Distilled beverages are then aged as necessary to produce desired color and flavors, while bioethanol for industrial purposes can be used immediately.

Figure 3. Copper Distillation Kettles.

There are number of different methods to quantitate ethanol in samples. One of the most commonly used methods is densitometry [3]. First described by Joseph Gay-Lussac in 1824, this method takes advantage of the differences in density between water and ethanol. Using a calibrated hydrometer that has a % vol. scale, also known as an alcoholmeter, one can determine the percent alcohol in a given fluid. Since the 19th century a number of improvements have been developed in order to improve or automate the process. Ebulliometry or boiling point depression has been used to estimate the percentage of alcohol in beer or wine [4]. With increasing amounts of ethanol present the boiling point of the mixture will decrease. There is a linear relationship between ethanol concentration and the change is boiling temperature. Note that residual sugars greater than 4% will influence the value and need to be accounted for. HPLC has been utilized to monitor the fermentation process. This method has the advantage of being able to monitor not only the production of ethanol, but also the reaction substrates and byproducts [5]. FTIR [6], gas chromatography [7], and IR [8] technologies have also been used to detect and quantitate ethanol in samples. While FTIR requires a large investment in instrumentation, the use or less expensive IR technology has been demonstrated to be just as accurate [8]. The chemical methods employed have focused on oxidation of the lone hydroxyl group present on the ethanol molecule. This redox reaction forms an aldehyde or ketone, as well as a reduction of the oxidizing agent. The oxidizing agent pyridinium chlorochromate (PCC) oxidizes ethanol to form a ketone, while the reduction of PCC results in the formation of chromate, which has high molar absorptivity at 570 nm (Eq. 1). Biochemical or enzymatic methods utilize enzymes that specifically react with ethanol. By linking two or more reactions a measureable product can monitored.

Eq. 1. $2C_2H_5NHCrO_3Cl + 3\ CH_3CH_2OH \rightarrow 2C_2H_5NHCl + Cr_2O_3 + 3CH_3CHC{=}O + 3\ H_2O$

Materials and Methods

Pyridinium chlorochromate (cat # 190144), ethanol (cat# A962), methanol (cat# 34860), glycerol (cat# G5516) and 1-butanol (cat# B7906) were purchased from Sigma Aldrich (St. Louis, MO). Amplite™ fluorometric ethanol quantitation kits (cat# 40001) were obtained from AAT Bioquest (Sunnyvale, CA). Clear (3598) 96-well and solid black ½-area (3694) 96-well microplates were procured from Corning (Corning, NY). Barley grain, hops and yeast were purchased from a local homebrew beer supply store. Commercially available beverages were obtained at a local beverage distributor.

Fermentation:

Vienna style lager beer was produced by standard production procedures. Briefly, crushed malted barley was wetted and incubated at 77° C for approximately 60 minutes to convert grain starch to glucose by action of the endogenous α-(1→4) and α-(1→6) glycosylase enzymes present in the germinated barley. The sugar rich aqueous extract was isolated by flow through filtration and boiled for 60 minutes. Hop buds were added at intervals during the boil for flavor. After cooling, the unfermented wort was inoculated with Bavarian lager yeast (Wyeast strain 2206). The culture was sealed with an air lock and allowed to ferment at approximately 16° C. Aliquots (15 mL) were removed daily, centrifuged at 800x g and the supernatant stored at -20° C until assayed for ethanol and glucose content.

PCC Assay:

A 1 M working stock of pyridinium chlorochromate (PCC) was prepared fresh in deionized water. Samples and standards (100 µL) were pipetted and the reaction was initiated by the addition of 100 µL of working PCC reagent. The reaction was monitored using absorbance measurements at 570 nm.

Amplite™ Ethanol Assay:

Ethanol was measured using the Amplite™ fluorescent ethanol assay kit. The assay was performed according to the assay kit instructions. Briefly, a 250 X Amplite™ reagent stock solution was prepared by dissolving the contents of the pre-weighed vial provided in the assay kit with 40 µL of DMSO. A working enzyme solution (100X) was prepared by diluting the provided lyophilized vial with 1X assay buffer supplied in the kit. Working reaction mixture was prepared by mixing 20 µL Amplite™ reagent, 50 µL enzyme solution, and 4.97 mL of 1X assay buffer. Aliquots (50 µL) of each sample were added to the solid black ½ area microplate and the reactions were initiated by the addition of 50 µL of working reaction mixture. Reaction fluorescence was measured using a Synergy™ H4 Multi-Mode Microplate Reader with an excitation of 540 nm and an emission of 590 nm.

Amplex® Red Glucose Assay:

Glucose production was measured using the Amplex® Red fluorescent glucose assay kit. The assay was performed as previously described [2]. Reaction fluorescence was measured using a Synergy H4 multimode reader with an excitation of 540 nm and an emission of 590 nm.

Results

The reaction with purified ethanol and PCC results in a linear response (Figure 4). Because the reaction does not require any form of stop solution, it can be read continuously. The length of the reaction time can be varied depending on the expected concentrations of the unknown samples.

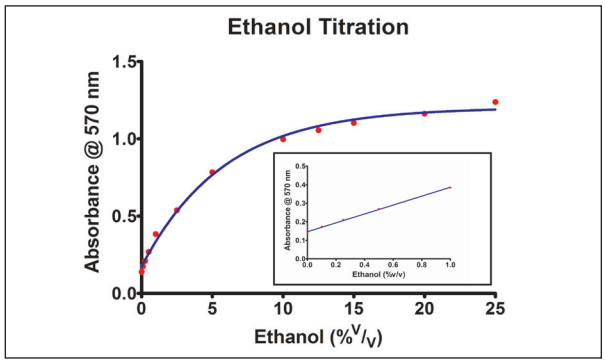

Figure 4. Ethanol titration with PCC reaction.

Pyridinium Chlorochromate is a strong oxidant that changes color as it reacts with ethanol [9, 10]. The formation of chromate results in a change from brilliant orange to darker shades of brown. The color change is demonstrated by the spectral scans of reacted and unreacted wells (Figure 5). Unreacted wells have a marked transition from significant absorbance below 550 nm, to virtually no absorbance above 550 nm. Reacted wells demonstrate absorbance up to 725 nm. A broad peak in absorbance is seen when the absorbance of unreacted well is subtracted from reacted (Figure 5).

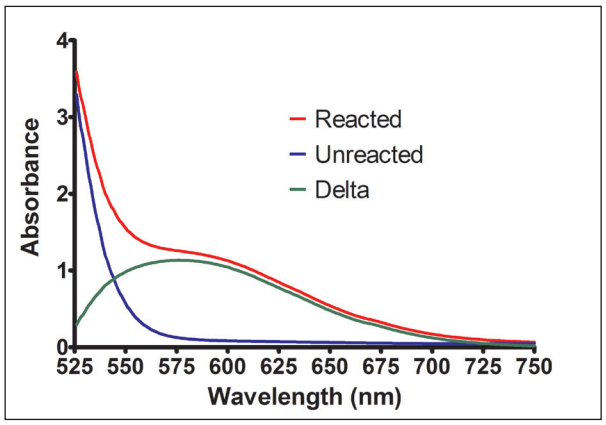

Figure 5. Spectral Scan of Reacted and Unreacted PCC Sample.

The increase in absorbance at 570 nm is not specific to ethanol. When other alcohols are reacted with pyridinium chlorochromate significant increases in the absorbance at 570 nm are also observed (Figure 6). Interestingly, each alcohol had different reactivity with respect to PCC.

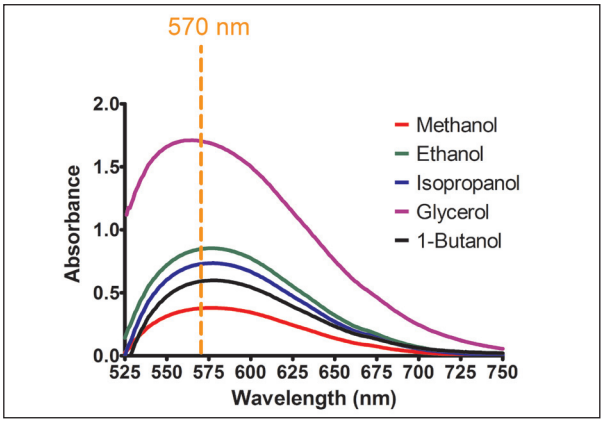

Figure 6. Spectral scan of PCC reaction with different alcohols. Equimolar amounts of different alcohols were reacted with PCC and their absorbance spectra compared.

When equimolar amounts of four different alcohols are reacted with PCC and compared, methanol results in the least increase in absorbance, while ethanol has the greatest change. The three carbon polyol glycerol, which has three hydroxyl groups, demonstrates significant more absorbance than isopropanol, which also has three carbon atoms, but only one hydroxyl group (Figure 7).

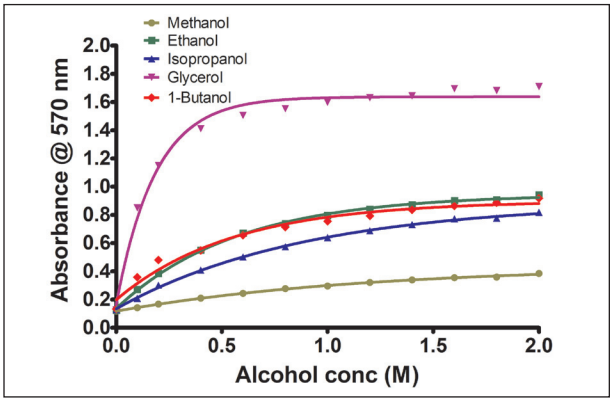

Figure 7. PCC reaction with different alcohols. Equimolar amounts of different alcohols were reacted with PCC and their absorbance compared.

As a result of the reactivity demonstrated with glycerol, the reactivity of the sugars galactose and glucose were investigated. Both sugar moieties, which contain numerous hydroxyl groups, reacted with PCC to a greater extent than ethanol on a molar basis. Despite only differences in isomerization between the two sugars, galactose is more reactive than glucose (Figure 8).

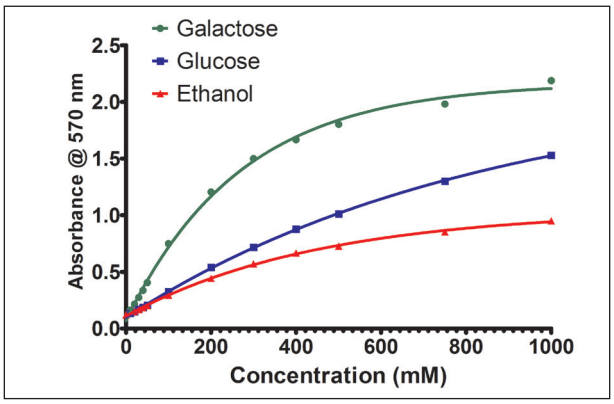

Figure 8. PCC reactivity with sugars.

Using the PCC assay several distilled commercial products were analyzed for ethanol content. As demonstrated in Table 1, there is good agreement between the determined ethanol concentration and what is reported by the manufacturer. The only exception is Southern Comfort, which had a determined content in excess of 50% v/v. The discrepancy can be explained by the sugar content of the spirit, which is reported to be 100 g/L. The other products are simply distilled spirits, which do not contain additives.

Product	% Ethanol Determined	% Ethanol Reported
Finlandia Vodka	44.50	40
Johnnie Walker Scotch Whiskey	41.82	40
Absolut Vodka	42.29	40
Southern Comfort	>50	35
Jameson's Irish Whiskey	41.04	40
Jack Daniels Bourbon	38.56	40
Seagram's 7 American Whiskey	41.58	40
50% Ethanol mixture	47.16	50

Table 1. Determined ethanol concentrations of commercially distilled spirits.

Numerous ethanol containing solutions also possess constituents that have hydroxyl groups that are reactive with PCC. In order to assess the ethanol content of these mixtures, assay methods that are more specific to ethanol are required. The Amplite™ ethanol assay uses the enzyme ethanol oxidase, which is specific for primary alcohols [11]. This assay kit has a linear response to ethanol (Figure 9).

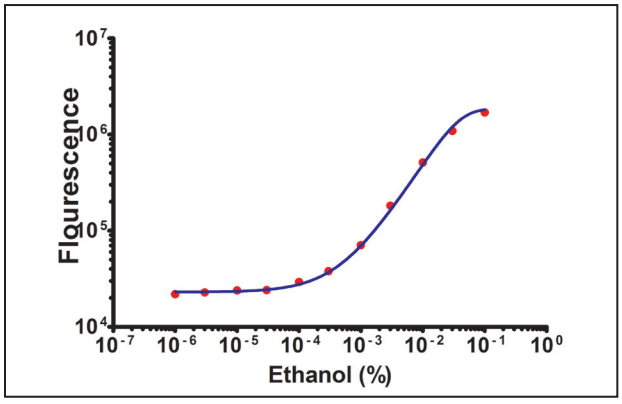

Figure 9. Ethanol titration with Amplite™ Assay.

Using the assay kit production of ethanol in fermenting beer was monitored. As demonstrated in Figure 10 the ethanol concentration of fermenting Vienna lager style beer increased steadily from 0 to approximately 5.5% over a period of 350 hours (14 days). This is in good agreement with ethanol calculations based on the change in specific gravity. The initial specific gravity of the unfermented beer was determined to be 1.055 g/mL, while the final gravity was 1.010 g/mL. Using the change in gravity one can estimate the concentration of ethanol produced to be 5.9% (See Eq. 2), where IG is the initial specific gravity, FG is the final specific gravity, 1.05 represents the mass in grams of ethanol produced per 1 gram of CO_2 generated and 0.79 is the specific gravity of ethanol.

Eq. 2 % ethanol = (((1.05*(IG-FG))/FG)/0.79

During the fermentation process glucose was consumed quite rapidly. Glucose is present in high concentration (approx 80 mM) in the media at the beginning of the fermentation process and is completely consumed in the first 50 hours of fermentation (Figure 10). This rate is much faster than the production of ethanol, suggesting that glucose transporters are quickly sequestering the glucose from the media. Only as the glucose is consumed for energy by the yeast is ethanol produced.

The ethanol concentration of several commercially available beer brands was determined. As demonstrated in Table 2, there is good agreement between the determined concentrations and the ethanol content reported by the manufacturers.

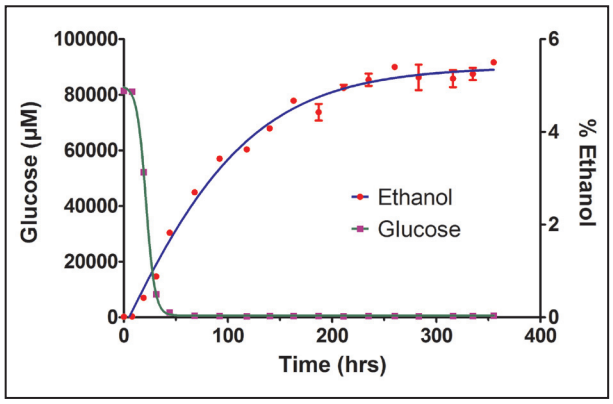

Figure 10. Vienna style lager fermentation.

Product	% Ethanol Determined	% Ethanol Reported
Gaffel Kolsch	6.45	4.8
Sam Smith Oatmeal Stout	3.22	5.0
DeKonnick	4.96	5.0
Scherlenkerla Helles	3.90	5.1
Paulaner Salvator Dopple Bock	5.53	7.9
Theakston Old Peculier	3.53	5.6
Sunner Kolsch	5.14	5.3
Weinhenstephan vitus Werenbock	7.41	7.7
St.Peter's IPA	4.24	4.9
Brooklyn Local #1 (Belgium Tripple)	10.14	9
Spaten Helles lager	5.84	5.2
Spaten Octoberfest	5.77	5.9
Spaten Optimator Dopplebock	7.17	7.6
Zywiec	5.82	5.6
Podge Belgium Imperial Stout	9.10	10.5
Beck's NA Bier Lager	0.34	<0.5

Table 2. Comparison of determined and reported commercial beer ethanol concentrations.

In general, the determined ethanol concentrations for dark colored beer styles tended to be slightly below that which was reported, while light colored beers were much closer. For example, the determined ethanol concentration for Theakston Old Peculier was 3.53%, while the concentration reported by the manufacturer was 5.6%. This beer has been described by its manufacturer as having a deep dark ruby color. Alternatively Sünner Kölsch, which has been described as hazy golden color, was measured to have 5.14 % ethanol verses a reported alcohol content of 5.3%.

Discussion

Ethanol is used for a myriad of different applications. Vast quantities of ethanol are produced as an additive for gasoline as a means to increase oxygen content of the fuel. More recently ethanol has been thought of as a means to replace fossil fuel derived energy for transportation. Perhaps more importantly is the presence of ethanol in alcoholic beverages such as beer, wine and distilled products, such as whiskey, and vodka.

These data demonstrate the utility of two different means to determine ethanol concentrations from aqueous mixtures. The oxidation of alcohols by PCC is a fast inexpensive method to determine ethanol concentrations in relatively pure samples such as distilled products. It's reactivity to sugars makes it inappropriate for use in fermenting samples. Its working concentration range (0.5-25%v/v) is amenable to many distilled products with minimal sample dilution. Besides distilled products, this reaction is utilized by many portable breathalyzers for the determination of blood alcohol concentrations. Because the exhaled breath ethanol concentration is proportional to that in the blood, and exhaled breath is relatively free of reactive contaminants, the determination from this non-invasive test can reliably be used by law enforcement agencies to test motorists suspected of driving under the influence.

Quantitation of ethanol in mixed matrices, which is the norm for fermenting products, requires greater specificity than oxidation with PCC. Reactions that are linked to the generation of H_2O_2 by alcohol oxidase are specific to primary alcohols despite the presence of reducing sugars such as glucose. The Amplite™ ethanol assay was very sensitive and capable of making ethanol determinations in fermenting samples. While it was not tested, it stands to reason that this method can be used to test fermenting wine samples as well. The use of hops as a flavoring agent instills beer with antioxidants, which can interfere to some extent with the reaction. For maximal accuracy it is advised to use an equivalent dilution of unfermented wort when making dilutions for the standard curve.

Alcohol oxidase enzyme based assays are very sensitive, capable of detecting concentrations as low as 0.003% v/v ethanol concentrations. As such many potential problems caused by contaminates can be reduced or eliminated through sample dilution. As stated previously there are numerous ways to quantitate ethanol from samples. The methods described here have the advantage of being rapid, inexpensive, and requiring only modest instrumentation. The ability to run these assays in microplate format allows for numerous samples to be run simultaneously.

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