Quantification of Esters Produced with Kveik Under Varying Fermentation Conditions Using HS-SPME/GC-MS

by

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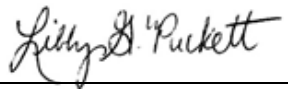

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Abstract

Kveik is a strain of ale yeast, *Saccharomyces cerevisiae,* isolated from Scandinavian farmhouse breweries, that is new to the U.S. market and can ferment at high temperatures without the overproduction of volatile aromatic esters. This has the potential to greatly decrease production times, thereby increasing throughput in the brewery. Little research has been done to quantify the effects that varying fermentation conditions, such as temperature and starting gravity (density of the solution due primarily to dissolved malt sugars prior to fermentation), has on the production of esters when using Kveik for fermentation. For typical ale yeast, fermentation temperatures range from 18-20 ℃ and anything above will produce undesirable esters. In excess, these compounds are responsible for undesirable flavors and aromas. Using head space solid phase microextraction gas chromatography with mass spectrometry (HS-SPME/GC-MS), ester production from four Kveik strains fermenting worts at 1.040, 1.060, and 1.080 original gravities at 25 ℃ and 37 ℃ was quantified. An increase in fermentation temperature was shown to decrease ester production for all yeast while changing the starting gravity had no predictable effect. The results from this study can be used by brewers to predict the results of altering fermentation temperature more accurately when using Kveik yeast.

Acknowledgements

I would like to thank Dr. Brett Taubman and Dan Parker for helping me throughout this entire process. Both played a tremendous role in teaching me the biological and fermentation processes applicable to this project. Both also pushed me to think more critically about aspects of the project and come to conclusions on my own rather than handing me the answers, a skill that will help me greatly down the road.

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Introduction

Kveik

Kveik is a yeast that has been passed down through many generations of farmers in the Scandinavian peninsula, specifically Norway. Kveik is made up of multiple *Saccharomyces cerevisiae* strains which have been domesticated since roughly 1600 A.D.[1] Through sensory analysis it has been stated that Kveik is capable of fermenting between 28-40 ℃ without the production of excess aromatic esters, unlike traditional brewer's yeast which will readily ferment at those temperatures, but will produce off-flavors from excess ester production.[1] This range contains the optimum temperature for growth of *S. cerevisiae* (29-35 ℃) which allows Kveik to reproduce at a rapid rate.[2, 3] While the reason for Kveik's ability to ferment at higher temperatures has not been studied in great detail, it is hypothesized that the yeast adapted over many generations to withstand the high temperature solutions into which the yeast were pitched.[1] Many farmers would inoculate the malt sugar solution referred to as wort with Kveik around 28 ℃ and they would also reuse the strains from batch to batch, thereby selecting for yeast capable of fermenting at high temperatures.[1, 4] Fermenting at higher temperature allows the brewer to complete the primary fermentation in 24-48 hours whereas common ale yeast can take up to 12 hours just to begin the fermentation process and 3-5 days to complete it. This ability to produce lower concentrations of ester compounds arises from the process by which esters are formed in the yeast cell.

Ester Production

During fermentation of beer or wine, *S. cerevisiae* produces two types of esters: acetate esters and medium chain fatty acid (MCFA) ethyl esters.[5] These esters are responsible for the many important aromas found in fermented beverages and are detectable at

concentrations in the low parts per million (ppm). The production of these esters is highly dependent on the brewing conditions of the fermented beverage and can easily turn an otherwise good product into one with off-flavors and aromas. Production of esters begins with acetyl coenzyme A (acetyl-CoA) in the cell.[6] Acetyl-CoA is used for three different purposes: cell growth through the citric acid cycle (III in figure 1), formation of lipids (II_a and II_b in figure 1), and the formation of acetate esters (I in figure 1).

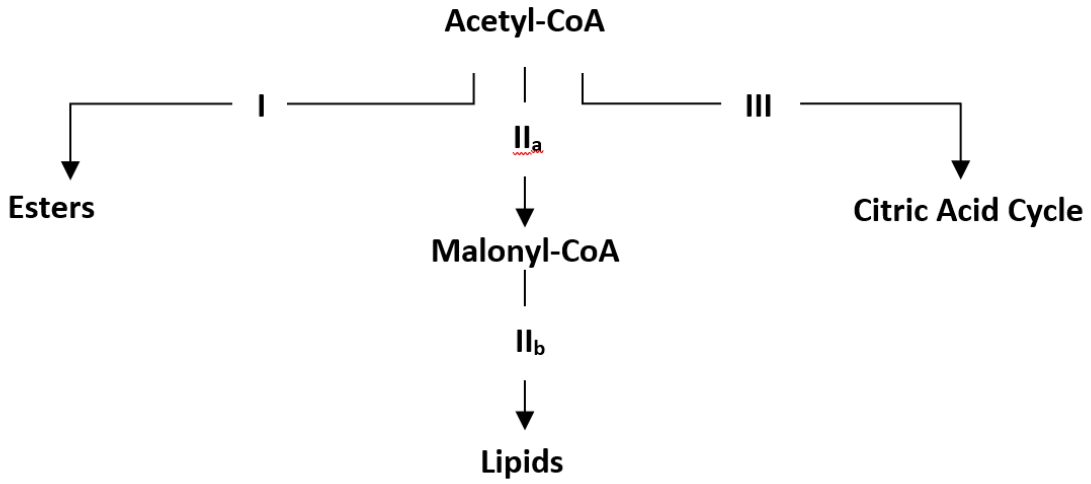

Figure 1. Simplified reaction scheme for uses of acetyl-CoA in yeast cells adapted from Nordström[6]

During acetate ester formation acetyl-CoA and an alcohol (ethanol or higher alcohols) are combined using a condensation reaction that is catalyzed by alcohol O-acetyltransferases (AATs).[7, 8] There are two main AATs found is *S. cerevisiae*, Atf1 and Atf2 with Atf1 being the most important for ester production.[7, 8] Reduction in Atf1 as well as reduction is acetyl-CoA can lead to a reduction in the formation of acetate esters.[6-8]

The formation of ethyl esters, made from MCFAs, is like that of acetate esters, but does not directly require acetyl-CoA. Whereas acetate esters require acetyl-CoA for the acetate portion of their structures, ethyl esters require fatty acyl groups. These acyl groups

are produced through a decarboxylation reaction with malonyl-CoA and a fatty acid.[6] Malonyl-CoA is produced from acetyl-CoA during reaction II_a in figure 1. The enzyme acetyl-CoA carboxylase regulates fatty acid biosynthesis from the fatty acid synthase (FAS) complex.[8] As long-chain fatty acids are produced, the efficiency of acetyl-CoA decarboxylase is inhibited and MCFAs are released instead.[7] These fatty acids formed are what get decarboxylated in the step above to form the acyl-CoAs.[6] In this study, the acyl groups are specifically hexanoyl, octanoyl, and nonanoyl which are obtained from hexanoyl-CoA, octanoyl-CoA, and nonanoyl-CoA respectively. Similar to how acetate ester formation is catalyzed by Atf1 and Atf2, ethyl ester production is catalyzed by the ethanol O-acetyltransferases Eeb1 and Eht1 with Eeb1 being the main transferase.[7, 8] These enzymes catalyze the formation of ethyl esters from the acyl-CoAs and ethanol similar to the process mentioned for the acetate esters.

Acetyl-CoA is seen to play a key role in the formation of both acetate esters and ethyl esters. Availability of acetyl-CoA is reduced during the growth phase for the yeast as it is being used for the citric acid cycle and to produce fatty acids and sterols for new cells. This reduction of availability will lead to a reduction in esters being formed by yeast. At higher temperatures, yeast ferments and assimilates sugars at a higher rate leading to a higher rate of growth observed.[3] This optimum growth rate allows for acetyl-CoA to be kept unavailable for its use in ester formation.

Esters are lipid soluble and will permeate through the lipid membrane of a yeast cell into the medium of fermentation.[5, 7, 9] Acetate esters diffuse rapidly and completely into the fermenting medium, while the permeability of ethyl esters decreases with increasing chain length.[5, 7] This is due to increased size decreasing the permeability. Intuitively, as

temperatures increase, permeability of these ethyl esters can be expected to increase as well. With increasing temperature also comes an increase in the expression of Atf1, Atf2, and Eht1[10] which has been stated previously to increase ester formation in yeast.[6-8] The increase in ester production due to the expression of certain acetyltransferases counteracts the effect of reduction of available acetyl-CoA and ultimately plays a larger role in affecting ester concentrations. This increase in permeability and ester production from acetyltransferases has led brewers to ferment beer at lower temperatures. Normal fermenting temperatures for beer fall in the range of 18-20 ℃ depending on the style of beer. This low temperature helps to reduce the amount of ester compounds that permeate the yeast membrane; however, it also reduces the growth rate of the yeast.[2, 3] The optimum temperature for cell growth with *S. cerevisiae* is between 29-35 ℃ which indicates that conditions under which many beers are suboptimal.[2, 3] This is important because fermentation time for brewers is greatly increased at lower temperatures and is why Kveik yeast (Kveik) is something of great interest to the brewing industry.

HS-SPME/GC-MS

By using HS-SPME/GC-MS, ester compounds can be quantified and compared to literature olfactory threshold data. In addition, comparisons between the two temperatures as well as among gravities was made for each analyte of interest. SPME is an extraction technique that allows for simple and fast extraction of volatiles from the headspace of a vial or directly from the aqueous solution. For volatiles, extraction from the headspace is possible and thus reduces the need for sample filtration or preparation.[11] Until the discovery of SPME in 1990, extraction of organic compounds from a sample was either done through static headspace analysis, the use of organic solvents (which were often toxic), or through solid phase extraction (SPE) which was often slow and required preparation of samples.[11] Solvent

extraction and SPE often increased extraction time required valuable benchtop space resulting in labor increases for analysis of compounds.[12] Static headspace analysis results in no concentration of analytes and thus lower detection limits.[11] SPME solves all the above mentioned issues though the use of a fused silica fiber coated with a stationary phase that can be introduced to the headspace of a sample vial.[11, 13] For volatile analysis, the analytes of interest are adsorbed onto the fiber and thus concentrated. To analyze the adsorbed compounds the fiber is heated in the GC injection port where the analytes are desorbed onto the column.[11, 13] This process requires no filtering or preparation of the sample and can thus be automated for time-efficient analysis.

Most literature since 1990 involving the analysis of volatiles in beer utilize a HS-SPME method due to its simplicity and efficiency as well as its reproducibility of results. For ester quantification, it has become especially important, as quantification using other methods is typically expensive, time consuming, or does not have a broad range of detection.

Project Goal

In a previous study, the ester concentrations of varying Kveik strains were analyzed, but at one temperature with no varying parameters.[1, 14] These results were obtained at a fermentation temperature of 30 ℃ and there are no current studies that quantify ester concentrations at lower or higher temperatures nor ones that investigate the impacts from varying parameters such as original gravity and temperature. To better understand how fermentation temperature and the original gravity of the wort affect ester production, benchtop fermentations were conducted in this study. Small scale fermentations (250 mL) allow for quick set up as well as consistency in variations of parameters. This also allows for multiple specific gravities to be studied for multiple yeasts at varying temperatures in a quick

manner. Data for only two temperatures (25 ℃ and 37℃) were obtained and analyzed for eight common esters found in beer. Esters outlined in this study were ethyl acetate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl nonanoate, ethyl butyrate, isobutyl acetate, and 2-phenylethyl acetate the structures of which are shown in figure 2. Four of the compounds, ethyl acetate, isoamyl acetate, isobutyl acetate, and 2-phenylethyl acetate are acetate esters while the others are ethyl esters.

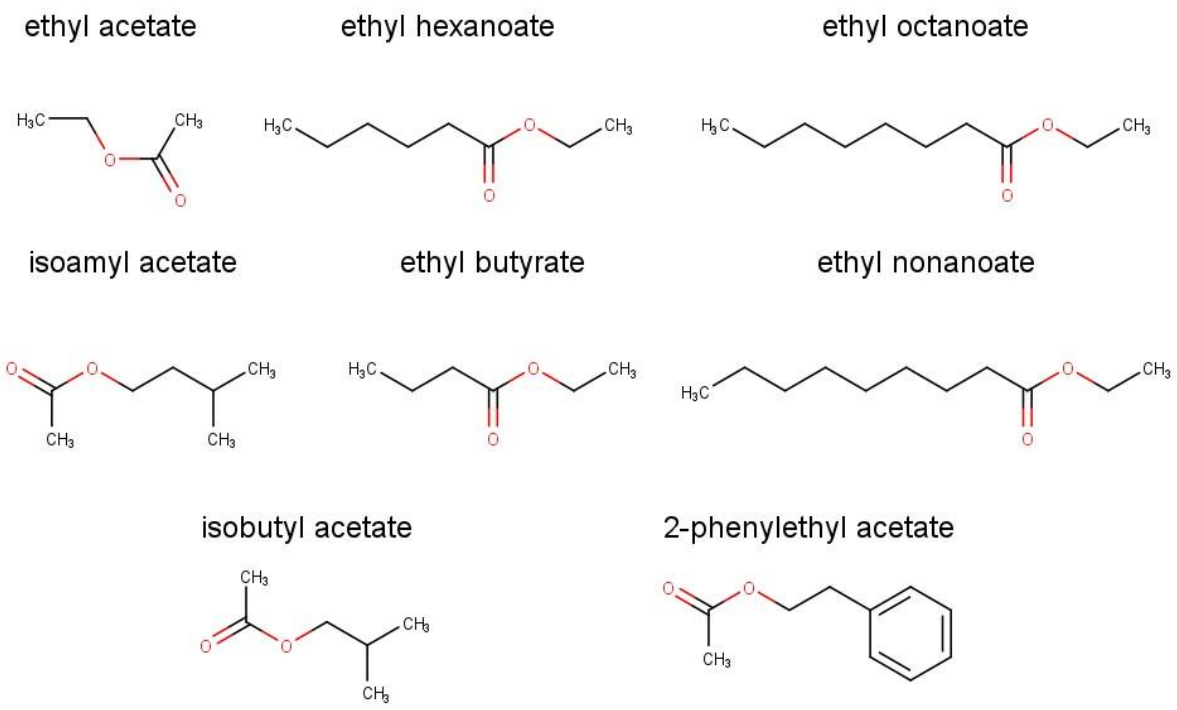

Figure 2. Chemical structures of the analytes of interest

Materials and Methods

Chemicals

Reagent grade esters were purchased from various sources. Ethyl acetate (141-78-6), isobutyl acetate (110-19-0), ethyl hexanoate (123-66-0), 2-phenyethyl acetate (103-45-7), and 1-butanol (71-36-3) were all purchase from Tokyo Chemical Industry (TCI). Ethyl octanoate (106-32-1) and ethyl nonanoate (123-29-5) were both purchased for Alfa Aesar.

Isoamyl acetate (123-92-2), ethyl butyrate (105-54-4), and acetone (67-64-1) which was used to clean glassware were purchased from Acros Organics. Ethanol, 200 proof (64-17-5) was purchased from Pharmco and Dried Malt Extract (DME, Briess Golden Light) was purchased from Northern Brewer.

Glassware and Micropipettes

All glassware used for quantification purposes was grade A. Micropipettes used were from Eppendorf. The 5-mL and 200-µL pipettes had a tolerance of 0.005 mL and 0.2 µL, respectively. Mason Jar canning jars were used for fermentation and propagation of yeast.

Equipment

The GC used was an Agilent technologies 7890B paired with an Agilent technologies 5977B MS. For auto sampling purposes, the Pal RSI 85 was used with the SPME arm and tool attached. Two separate autoclaves were used depending on the size of the material needing to be autoclaved. The smaller of the two was a Tuttnauer 2540M and the larger was Yamato SM820. Two separate incubators were used for the different trials. For the 25 ℃ trial, a Napco model 5410 incubator was used and for the 37 ℃ trial, a VWR Forced Air Microbiological incubator was used. A New Brunswick Excella E24 incubator shaker was used to shake propagations of yeast.

Preparation of Yeast Cultures

Three of the four strains of Kveik, Jovaru Lithuanian OYL-033 (OYL-033), Hornindal OYL-091 (Hornindal), and Hothead Ale OYL-057 (Hothead) were all purchased from Omega Yeasts and the fourth, POG A37 (POG) was purchased from Imperial Yeast. All yeasts were bought as liquid yeast and were transferred to DME media plates. For each trial, yeast was propagated from the plates to a final volume of around 600 mL. To begin the propagation, twenty, 50-mL conical tubes were filled with 40 mL of DME at a specific

gravity of 1.050 with 50 ppm calcium as $CaCl_2$ and 1g/US barrel (bbl) of Servomyces yeast nutrient (Lallemand Brewing). The tubes were autoclaved with the wort solution to ensure proper sterilization and minimize contamination through transfer. Each of the yeast strains were propagated from plates into five conical tubes and allowed to propagate for 3-4 days. After the initial propagation period, the contents from the five tubes of each yeast were transferred to a canning jar containing 350-mL of the same DME solution described above for a total volume of 550-mL. This was allowed to propagate for five days at 25 °C on a shaker plate to ensure healthy yeast growth.

Fermentation Solution Preparation

The fermentation solutions were prepped using DME to achieve original gravities of 1.040, 1.060, and 1.080. Calcium and Servomyces were added in the same amounts as mentioned above for the starter solutions. Original gravities were measured using a Mettler Toledo densitometer with a tolerance of ± 0.0001 and are presented in table 1.

Table 1. Starting gravity readings of fermentation solutions

Trial #	**Original Gravity**		
1 (25 °C)	1.040_1	1.059_6	1.080_1
2 (37 °C)	1.040_0	1.060_2	1.079_7

Each solution was split into four 800-mL canning jars for a total volume of 250 mL. These solutions were autoclaved at 121 °C for 45 minutes to sterilize.

Fermentation

The fermentation solutions outlined above were inoculated with the yeast cultures on day five of their propagation. To keep the total volume as consistent and close to 250 mL as possible, the yeast cultures were allowed to flocculate out of solution at room temperature for an hour and decanted to create roughly 150 mL of yeast slurry. This concentrated the yeast

solution and allowed for a smaller volume to be added when inoculating the fermentations. Yeast cells were counted, and yeast viability determined using a hemacytometer and the volume needed for each inoculation was calculated using a 500,000 cells/mL/°Plato pitch rate. Prior to pitching the yeast, each jar was opened, and 100% pure oxygen gas (Andy Oxy Co. Inc.) was introduced to approximately 10 ppm dissolved oxygen using an oxygen stone. After oxygenation, yeast was pitched according to table 2.

Table 2. Volume of yeast slurry pitched

		Total Volume Added (mL)		
Trial #	**Strain**	**1.040 OG**	**1.060 OG**	**1.080 OG**
Trial 1 25 °C	Hothead	3.935	5.800[a]	7.610[a]
	Hornindal	4.965	7.330[a]	9.610[a]
	OYL-033	2.190	3.225	4.230
	POG	2.090	3.085	4.045
Trial 2 37 °C	Hothead	2.050	3.025	3.970
	Hornindal	3.415	5.040[a]	6.600[a]
	OYL-033	2.750	4.060	5.320[a]
	POG	2.055	3.030	3.975

[a] **Indicates volume added by two doses of half the total volume**

Volume of yeast slurry was varied to achieve a uniform pitch rate. A 5-mL micropipette with sterilized tips was used to measure and introduce yeast into each solution. Some total volumes needed were greater than 5 mL and thus two doses of half the total volume needed was added as indicated in table 2. By decanting the supernatant, the total volume added was able to be kept under 10 mL for all yeast strains and original gravities. This kept the total volume of fermented solution close to 250 mL and did not affect the original gravity to an appreciable extent. Considering that the yeast cells were propagated using DME with the same initial amount of calcium and Servomyces, it was assumed that the original gravity and concentration of above additives did not vary to an appreciable extent upon addition of yeast.

For each trial, the solutions were capped with a silicone fermentation cap with a nipple to allow for the escape of gas, but no reentry of gases. Each solution was placed in an incubator for seven days held at a constant temperature. Trial 1 was held at 25 ℃ and trial 2 was held at 37 ℃. On day seven, the final gravities of each solution were measured as reported in table 3.

Table 3. Final gravity readings for each trial

		Final Gravity		
Trial #	**Strain**	**1.040**	**1.060**	**1.080**
Trial 1 25 ℃	Hothead	1.006_5	1.010_5	1.016_5
	Hornindal	1.007_5	1.011_1	1.017_0
	OYL-033	1.004_8	1.008_8	1.028_0[a]
	POG	1.007_2	1.011_6	1.019_3
Trial 2 37 ℃	Hothead	1.006_9	1.011_4	1.037_6[a]
	Hornindal	1.007_3	1.011_6	1.016_5
	OYL-033	1.004_1	1.007_3	1.016_2
	POG	1.007_1	1.012_0	1.018_9

[a] Did not ferment to completion

After seven days, each solution had fermented until completion except for under two conditions. It was not determined why each of the two fermentations did not proceed to completion, but each point will be analyzed and discussed as if it had. Immediately after obtaining a final gravity reading, 5.00 mL of each solution was transferred to a 20 mL headspace vial for analysis.

Standard Solution Preparation

To begin the process of making standard solutions literature concentration ranges were obtained and are shown in table 4. Data for ethyl acetate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, and 2-phenylethyl acetate were obtained from a study published by Verstrepen Et al..[15] Data for isobutyl acetate and ethyl butyrate were obtained as a calibration curve range made during a study done by Jelen Et al.[16] and the concentration for ethyl nonanoate was obtained from a study by Niu Et al.[17]

Table 4. Concentration ranges for analytes of interest

Compound	Concentration Range (ppm)
Ethyl acetate	8-32
Isoamyl acetate	0.3-3.8
Ethyl hexanoate	0.05-0.3
Ethyl octanoate	0.04-0.53
Ethyl nonanoate	3.15[a]
2-phenylethyl acetate	0.1-0.73
Isobutyl acetate	0.05-10.12
Ethyl butyrate	0.04-8.64

[a] Avg. range could not be found so a range around the olfactory threshold value was estimated

Single Component Standard Preparation

A 5% ethanol solution was used for dilution in all standard solutions. The ethanol solution was prepared by diluting 50 mL of 100% ethanol to 1 L with deionized water. To obtain a calibration curve with ranges close to that of literature values, a modified version of the ASBC Beer-48 method was used.[18] Single component standard solutions were made for each analyte of interest by diluting set volumes of each analyte into their own volumetric flask. For ethyl acetate and isoamyl acetate, a volume of 100 µL of reagent solution was diluted to 100 mL using the 5% ethanol solution. For all other compounds, 25 µL of the reagent solution was diluted to 50 mL using the 5% ethanol solution. The concentrations of these eight single components standards are shown in table 5.

Multicomponent Standard Preparation

Following the section of the ASBC Beer-48 method regarding calibration solutions[18], a multi component working calibration solution was prepared. To a 100-mL volumetric flask, volumes of the single component standards were added as follows and diluted with the 5% ethanol solution: 10 mL of ethyl acetate, 5 mL of isobutyl acetate and ethyl butyrate, and 1 mL of the remaining esters. The concentrations of each analyte in the working calibration solution are shown in table 5.

Table 5. Concentration of stock solutions

Analyte	Single Component Standard Concentration (ppm)	Working Calibration Solution Concentrations (ppm)
Ethyl hexanoate	435	4.35
Isobutyl acetate	435	21.8
Isoamyl acetate	876	8.76
Ethyl butyrate	437	21.8
Ethyl octanoate	436	4.36
Ethyl nonanoate	433	4.33
Ethyl acetate	900	90.0
2-phenyl-ethyl acetate	520	5.20

Calibration Solution Preparation

Calibration solutions were made following the ASBC Beer-48 method.[18] To separate 50-mL volumetric flasks, volumes of 20, 10, 5, 1, and 0.5 mL of the working calibration solution were diluted with the 5% ethanol solution. The concentration of analytes in the above calibration solutions are shown in table 6 as calibration solutions 7, 6, 5, 4, and 3, respectively.

To increase the range of concentrations for isoamyl acetate, ethyl hexanoate, ethyl octanoate and ethyl nonanoate, single component calibration solutions were made using the single component standard solutions outlined above.

Single component calibration solutions for isoamyl acetate, ethyl hexanoate, and ethyl nonanoate were made following the same steps. To a 50-mL volumetric flask, 250 µL of the single component standard solution was diluted with the 5% ethanol solution. To individual 25-mL volumetric flasks, 50 and 150 µL of the previous solution was diluted with the 5% ethanol solution. Concentrations for the single component calibration solutions are shown in table 6 as calibration solutions 1 and 2 respectively.

For ethyl octanoate, 250 µL of the single component standard solution was diluted with the 5% ethanol solution in a 50-mL volumetric flask. To a 100-mL volumetric flask, 1 mL of the previous solution was diluted with the 5% ethanol solution. The concentration of this solution is shown in table 6 as calibration solution 2. To a 25-mL volumetric flask, 15 mL of calibration solution 2 was diluted using the 5% ethanol solution. The concentration of this solution is shown in table 6 as calibration solution 1.

Table 6. Concentrations of analytes in calibration solutions

	Concentration of Analytes (ppm)						
Analyte	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Ethyl hexanoate	0.00435	0.0131	0.0435	0.0870	0.435	0.87	1.74
Isoamyl acetate	0.00876	0.0263	0.0876	0.175	0.876	1.752	3.504
Ethyl octanoate	2.62	4.36	0.0436	0.0872	0.436	0.872	1.74
Ethyl nonanoate	0.00433	0.0130	0.0433	0.0866	0.433	0.866	1.732
Ethyl acetate			0.900	1.80	9.00	18.0	36.0
Isobutyl acetate			0.218	0.435	2.18	4.35	8.70
Ethyl butyrate			0.218	0.437	2.18	4.37	8.74
2-phenyl-ethyl acetate			0.0520	0.104	0.520	1.04	2.08

Table 6 shows the concentration of calibration standards that were used to make the calibration curves during the analysis portion of this study. Calibration solutions 1 and 2 are single component solutions while calibration solutions 3-7 are multicomponent solutions.

For the internal standard, 50 µL of 1-butanol was diluted in a 50-mL volumetric flask with 100% ethanol. This internal standard was made following the ASBC Beer-48 method.[18] The final concentration was 810.0 ppm.

Analysis

Analysis of both standards and fermentation samples was performed using head space solid phase microextraction with gas chromatography and mass spectrometry (HS-

SPME/GC-MS). A Stabilwax (Restek) column, a cross-bound carbowax polyethylene glycol column (60m, 0.32 mmID, 1 μm df), was used for separation of analytes. Parameters for the GC followed a method performed for the optimization of beer volatile analysis.[19] For the carrier gas, helium was used with a flow rate of 1 mL/min. The oven temperature settings began at 40 ℃ for 5 min, and then were ramped to 240 ℃ at 4 ℃/min where the temperature was held for 15 minutes. The total runtime was 80 minutes, but the GC cycle time was set to 85 minutes to account for time needed to cool.[19] The MS transfer line, quadrupole, and ion source were set to 270, 150, and 230 ℃ respectively per the method.[19]

A PAL RSI 85 autosampler was used to run all samples and standards. Settings for auto sampling were obtained from the same method as the GC parameters.[19] Incubation, with shaking, was done at 44.8 ℃ for 10 minutes. Conditioning of the fiber was performed in the headspace vial at the same temperature for 46.8 minutes with shaking. The sample was desorbed into the inlet of the GC for 1.5 minutes at 270 ℃ using splitless injection mode.

To a 20 mL headspace vial, 5.00 mL of either sample or standard was added along with 1.50 g of NaCl and 100 μL of the internal standard solution. Triplicate vials were made for each sample and standard. Quantification of peak areas was done using the current version of the Agilent MSD ChemStation computer software.

Calibration Curve

The ions quantified for each analyte along with their respective retention times are shown in table 8. A ratio of the peak area of the analyte to the peak area of the internal standard was calculated. This ratio was plotted vs the concentration of the analyte to produce a calibration curve. A sample table for ethyl acetate and curve are presented as table 7 and figure 3, respectively.

Table 7. Concentration and responses from ethyl acetate used to create the calibration curve for ethyl acetate

Concentration (ppm)	Response 1	Response 2	Response 3	Avg. Response
0.9		0.080	0.433	0.256
1.8	0.172	0.836	0.916	0.641
9	4.102	4.541	4.400	4.347
18	8.462	9.493	9.101	9.019
36	15.847	18.644	17.807	17.433

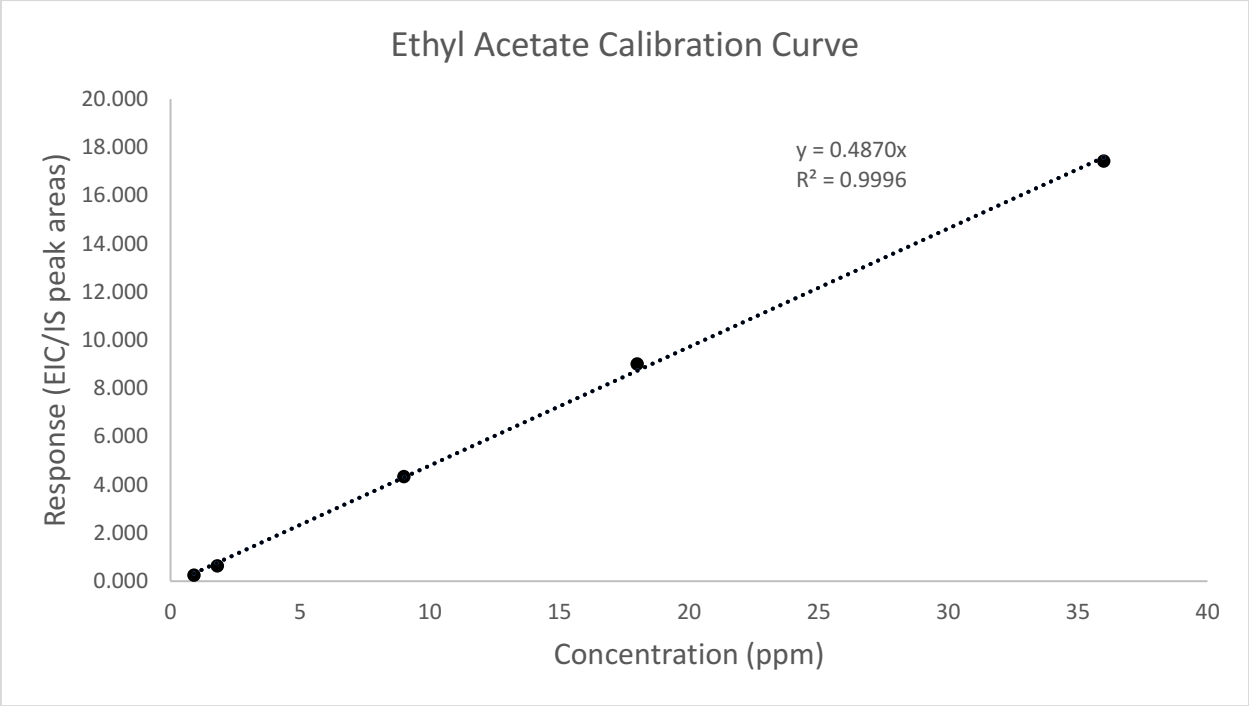

Figure 3. Calibration curve for ethyl acetate

Calibration curves were made for each analyte which are presented as supplementary figures and tables 1-5. For all calibration curves, only five concentrations were used. Upon initial analysis of data for isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl nonanoate it was determined that too many derived concentrations from samples fell outside of the calibration range. This was the reason for the addition of calibration solutions 1 and 2 outlined in the calibration solution preparation section. Upon further analysis with the new

range of concentrations, it was determined that the error in the derived concentrations for these four compounds was too large and thus two calibration standards were removed on either the high end or the low end. This forced sample concentrations to be closer to the mean concentration of the respective calibration curve and thus reduced calculated error. Linear equations and R^2 values for all calibration curves are presented in table 8. In all cases, forcing the line through the origin increased the R^2 value and thus the slope is the only information from the equation of the line presented.

Table 8. Analyte ions and retention times

Analyte	Ion	RT (min)	Slope of Line	R^2
Ethyl acetate	43	12.20	0.487 (±0.005)	0.9996
Isobutyl acetate	56	17.16	2.58 (±0.09)	0.9953
Ethyl butyrate	71	18.17	4.3 (±0.2)	0.9954
Isoamyl acetate	70	21.84	14.9 (±0.2)	0.9996
Ethyl hexanoate	88	26.30	45.2 (±0.5)	0.9996
Ethyl octanoate	127	33.64	10.4 (±0.5)	0.9913
Ethyl nonanoate	101	37.09	10.8 (±0.2)	0.9983
2-phenylethyl acetate	104	46.37	103 (±4)	0.9946
1-butanol (ISTD)[a]	31	22.40		

[a] ISTD: no calibration curve made

Data Analysis

Derived concentrations of esters were compared to literature threshold data using the standard deviation calculated using equation 1. The terms s_y and m are the standard deviation in y and the slope of the calibration curve obtained using the LINEST function in excel. The terms k, n, and m_y represent the number of sample measurements, the number of measurements used to make the curve, and the average of sample measurements, respectively. The x_s term in equation 1 represents the sum of the squared deviations of the concentrations, while the $\bar{y}$ term is the mean of the average responses.

$$\mu_x = \frac{s_y}{m} * \sqrt{\frac{1}{k} + \frac{1}{n} + \frac{\left(m_y - \overline{y}\right)^2}{m^2 * x_s}} \quad (1)$$

Comparison between temperatures was done through a t test for comparison of means at the 95% confidence level. To determine whether equal variance in mean values could be used, a F-test was performed on the standard deviation of the compared values. In all cases, the variances could be pooled and thus equation 2 was used to calculate a t-score.

$$t = \frac{|\overline{x_1} - \overline{x_2}|}{s_{pool}} * \sqrt{\frac{x_1 x_2}{x_1 + x_2}} \quad (2)$$

The same test was used for comparison of ester production between different original gravities for each yeast. For both the comparison between temperatures and comparison between gravities, a t-score greater than the tabulated t-value indicated that there was a statistically significant difference in the two compared mean values. A simple comparison of means could then be used to determine if differences in values were statistically significant at the 95% confidence level.

Results and Discussion

Upon analysis of sample data, it was determined that isobutyl acetate and ethyl butyrate were below the limit of quantitation for all yeast strains at all gravities and both temperatures. For that reason, all sample data analyzed excludes those two compounds. While running the second trial (37 °C), the SPME fiber broke after running exactly half the samples. The fiber broke clean and did not puncture any of the septa for the remaining vials. Each of the vials were incubated and then left to sit over the weekend at room temperature. While some volatiles may have escaped around the seal during this time, it was assumed that

the internal standard escaped at a consistent rate and therefore, therefore, corrected for any reduction in ester concentrations.

Table 9 shows derived concentrations for the six analytes with quantifiable responses. Some concentrations were outside of the calibration curve and thus could not be accurately quantified.

Table 9. Concentrations of esters produced during fermentation

Analyte	Gravity[c]	Hornindal	Hothead	OYL-033	POG
25 °C					
Ethyl acetate	**1.040**	16.7 ±0.3	18.7 ±0.3	25.3 ±0.3	17.2 ±0.3
	1.060	21.9 ±0.3	24.5 ±0.3	33.6 ±0.4	20.3 ±0.3
	1.080	26.7 ±0.3	26.4 ±0.3	33.5 ±0.4	23.7 ±0.3
Isoamyl acetate	**1.040**	0.210 ±0.006	0.264 ±0.006	0.395 ±0.006	0.205 ±0.006
	1.060	0.221 ±0.006	0.285 ±0.006	0.489 ±0.007	0.199 ±0.007
	1.080	0.206 ±0.006	LOQ [a]	0.434 ±0.008	0.196 ±0.006
Ethyl hexanoate	**1.040**	0.198 ±0.003	0.167 ±0.003	0.0271 ±0.004	0.165 ±0.003
	1.060	0.183 ±0.003	0.189 ±0.003	0.348 ±0.004	0.178 ±0.004
	1.080	0.171 ±0.003	0.167 ±0.003	0.310 ±0.004	0.166 ±0.003
Ethyl octanoate	**1.040**	LOQ [b]	2.3 ±0.2	3.0 ±0.2	3.0 ±0.2
	1.060	LOQ [b]	3.0 ±0.2	3.1 ±0.2	2.8 ±0.2
	1.080	3.3 ±0.2	2.7 ±0.2	3.1 ±0.2	2.5 ±0.2
Ethyl nonanoate	**1.040**	0.109 ±0.006	0.043 ±0.006	0.047 ±0.006	0.056 ±0.006
	1.060	0.95 ±0.006	0.043 ±0.006	0.035 ±0.006	0.041 ±0.007
	1.080	0.55 ±0.006	0.030 ±0.006	0.038 ±0.007	0.025 ±0.006
2-phenylethyl acetate	**1.040**	0.28 ±0.06	0.27 ±0.06	0.59 ±0.06	0.18 ±0.06
	1.060	0.33 ±0.06	0.33 ±0.06	0.58 ±0.06	0.018 ±0.07
	1.080	0.25 ±0.06	0.24 ±0.06	0.41 ±0.07	0.019 ±0.06
37 °C					
Ethyl acetate	**1.040**	6.7 ±0.3	5.3 ±0.3	15.8 ±0.3	4.5 ±0.3
	1.060	7.0 ±0.3	7.0 ±0.3	14. ±0.3	4.7 ±0.3
	1.080	6.3 ±0.3	LOQ [a]	14.8 ±0.3	4.2 ±0.3
Isoamyl acetate	**1.040**	0.055 ±0.007	0.031 ±0.007	0.093 ±0.007	LOQ [a]
	1.060	0.029 ±0.008	0.028 ±0.007	0.107 ±0.006	LOQ [a]
	1.080	LOQ [a]	LOQ [a]	0.106 ±0.006	LOQ [a]
Ethyl hexanoate	**1.040**	0.018 ±0.004	0.026 ±0.003	0.026 ±0.004	0.026 ±0.003
	1.060	0.050 ±0.004	0.030 ±0.003	0.060 ±0.003	0.031 ±0.003
	1.080	0.034 ±0.004	0.009 ±0.003	0.058 ±0.003	0.028 ±0.003
Ethyl octanoate	**1.040**	1.9 ±0.2	0.8 ±0.2	0.8 ±0.2	1.0 ±0.2
	1.060	2.2 ±0.2	1.2 ±0.2	0.9 ±0.2	1.2 ±0.2
	1.080	1.6 ±0.2	LOQ [a]	0.9 ±0.2	0.9 ±0.2
Ethyl nonanoate	**1.040**	0.029 ±0.007	LOQ [a]	LOQ [a]	0.012 ±0.006
	1.060	0.037 ±0.007	0.015 ±0.006	LOQ [a]	0.016 ±0.006
	1.080	0.030 ±0.007	LOQ [a]	LOQ [a]	LOQ [a]
2-phenylethyl acetate	**1.040**	0.16 ±0.07	0.20 ±0.06	0.32 ±0.07	LOQ [a]
	1.060	0.19 ±0.07	0.21 ±0.06	0.35 ±0.06	LOQ [a]
	1.080	0.17 ±0.07	LOQ [a]	0.38 ±0.06	LOQ [a]

[a] Concentration range falls below calibration curve
[b] Concentration range falls above calibration curve
[c] Original Gravity

Olfactory Threshold Comparison

Table 10 includes olfactory threshold concentrations in beer for the six analytes investigated in this study. Data for ethyl nonanoate in beer could not be found and thus the threshold value used was from an analysis of baijiu, a distilled spirit made from sorghum or rice. Threshold concentrations are typically found as a range due to differences between the sensitivity of panel members.[20, 21] Meilgaard reported that the most sensitive 10% of participants on a panel have thresholds 16-20 times lower than those of the least sensitive 10%.[20] This variation can also be accentuated by overlapping flavor profiles. Meilgaard studied the effects of flavor differences perceived in beer based on different chemical compositions and found that compounds with similar sensory characteristics (i.e. fruity, solvent-like, or metallic) had additive effects and thus led to lower thresholds being observed.[20] Compounds with different sensory characteristics showed partial independence, meaning that their coexistence in a beer did not affect the threshold concentration observed by panel members.[20]

Table 10. Literature olfactory threshold values

Compound	Threshold (ppm)	Odor Description
Ethyl acetate [a]	21-30	Fruity, solvent-like [a] Pineapple [b]
Isoamyl acetate [a]	0.6-1.2	Banana, pear [a/b]
Ethyl hexanoate [a]	0.17-0.21	Apple, aniseed [a]
Ethyl octanoate [a]	0.3-0.9	Apple [a] Fruity,fat[b]
2-pheyl-ethyl acetate [a]	3.8	Roses, honey, sweet [a/b]
Ethyl nonanoate[b]	3.15	Fruity [b]

[a] Data obtained from Verstrepen Et al.[15]
[b] Data obtained from Niu Et al.[17]

Comparison to the literature values was done to within one standard deviation of the derived concentrations of esters. At both 25 ℃ and 37 ℃ the ester concentrations for ethyl nonanoate and 2-phenylethyl acetate were below the threshold values. Tables 11 and 12

outline where the ester concentrations fall in comparison to literature values for the 25 ℃ ferment and 37 ℃ ferment, respectively. Comparison terms for both tables are as follows: "Above" for concentrations where the standard deviation interval was above the literature range, "Below" for where the interval was below the lower limit, and "Within" for when the interval range fell within the literature range. Two distinctions were made for when the standard deviation interval was not entirely in the literature range. The term "Within^{L}" was used for when the interval contained the lower limit of the literature range, and "Within^{H}" was used for when the interval contained the upper limit of the literature range. As stated above, thresholds vary from person to person, so these distinctions may provide slightly greater insight into whether the aroma will be detectable.

Table 11. Comparison of ester concentrations to literature values at 25 ℃

Yeast Strain	Gravity	Ethyl Acetate	Isoamyl Acetate	Ethyl Hexanoate	Ethyl Octanoate	Ethyl Nonanoate	2-phenylethyl Acetate
Hornindal	1.040	Below	Below	Within	Above	Below	Below
	1.060	Within	Below	Within	Above	Below	Below
	1.080	Within	Below	Within^{L}	Above	Below	Below
Hothead	1.040	Below	Below	Below	Above	Below	Below
	1.060	Within	Below	Within	Above	Below	Below
	1.080	Within	Below	Within^{L}	Above	Below	Below
OYL-033	1.040	Within	Below	Above	Above	Below	Below
	1.060	Above	Below	Above	Above	Below	Below
	1.080	Above	Below	Above	Above	Below	Below
POG	1.040	Below	Below	Below	Above	Below	Below
	1.060	Below	Below	Within	Above	Below	Below
	1.080	Within	Below	Below	Above	Below	Below

^{L} Confidence interval contains lower limit of threshold range, but is also below the range
^{H} Confidence interval contains higher limit of threshold range, but is also above the range

During the 25 ℃ ferment, ethyl octanoate was produced in concentrations above the literature threshold range for all yeast strains at all gravities. Isoamyl acetate was produced below the threshold range for all yeast strains at all gravities. Ethyl hexanoate, at all gravities was produced within the range for Hornindal, and above the range for OYL-033. The Hothead strain produced ethyl hexanoate below the range during the 1.040 trial and within

the range for the other two gravities. The POG strain produced ethyl hexanoate below the range for both the 1.040 and 1.080 gravities and within the range for the 1.060. Ethyl acetate was produced below the literature range at the 1.040 starting gravity for the Hornindal and Hothead strains while the same strains produced ethyl acetate within the range for the 1.060 and 1.080 gravities. The POG strain produced ethyl acetate below the range for both the 1.040 and 1.060 gravities while producing it within the range at the 1.080 starting gravity. For the OYL-033 strain, ethyl acetate was produced within the literature range for the 1.040 gravity, and above for the range for the other two gravities.

An increase in temperature generally results in an increase in ester production, but the effect that temperature has on certain esters is more pronounced than others. One study shows that increasing temperatures above 20 °C has a larger effect on ethyl octanoate than smaller chain esters such as ethyl hexanoate.[7] This is a likely reason for the excess amounts of ethyl octanoate produced by the Kveik strains at 25 °C. Organoleptic characterizations of each yeast according to their respective websites state that the yeasts are more fruit forward with their aromas. This was observed by the concentrations of ethyl acetate, ethyl hexanoate, and ethyl octanoate being produced within or above the literature threshold concentrations under most parameters of the 25 °C ferment.

Table 12. Comparison of ester concentrations to literature values at 37 ℃

Yeast Strain	Gravity	Ethyl Acetate	Isoamyl Acetate	Ethyl Hexanoate	Ethyl Octanoate	Ethyl Nonanoate	2-phenylethyl Acetate
Hornindal	1.040	Below	Below	Below	Above	Below	Below
	1.060	Below	Below	Below	Above	Below	Below
	1.080	Below	LOD*	Below	Above	Below	Below
Hothead	1.040	Below	Below	Below	Within^{H}	LOD*	Below
	1.060	Below	Below	Below	Above	Below	Below
	1.080	LOD*	LOD*	Below	LOD*	LOD*	LOD*
OYL-033	1.040	Below	Below	Below	Within^{H}	LOD*	Below
	1.060	Below	Below	Below	Within^{H}	LOD*	Below
	1.080	Below	Below	Below	Within^{H}	LOD*	Below
POG	1.040	Below	LOD*	Below	Within^{H}	Below	LOD*
	1.060	Below	LOD*	Below	Above	Below	LOD*
	1.080	Below	LOD*	Below	Within^{H}	LOD*	LOD*

^{L} Confidence interval contains lower limit of threshold range, but is also below the range
^{H} Confidence interval contains higher limit of threshold range, but is also above the range
LOD* Below Limit of Detection

Ethyl octanoate was the only ester produced within or above the literature range for the 37 ℃ ferment. All other esters were produced below their respective literature ranges and for some the concentrations were below the limit of detection. For the Hornindal strain, ethyl octanoate was produced above the literature range while for the OYL-033 strain it was produced within the range, but on the high side. For the POG strain, ethyl octanoate was produced within the range on the high side at the 1.040 and 1.080 gravities while it was produced above the range for the 1.060 gravity. The Hothead strain followed the same trend as the POG for the 1.040 and 1.060 gravities but produced ethyl octanoate below the limit of detection during the 1.080 ferment which was likely due to the incomplete fermentation with these parameters as shown by the elevated final gravity.

Comparison Between Temperatures

Ester production during the 37 ℃ ferment did not follow the trend that increasing temperature increases ester production. For all gravities and yeast strains (excluding OYL-033 at 1.080 for 2-phenylethyl acetate) ester production was statistically greater during the 25 ℃ ferment than the 37 ℃ ferment. The lack of significant difference between mean

concentration values for 2-phenylethyl acetate at the above-mentioned parameters was likely due to OYL-033 not fermenting to completion during the 25 °C trial at 1.080.

A likely reason that esters were produced in lower concentrations at the elevated temperature is the thermotolerance of Kveik.[1] While the optimum temperature for yeast growth peaks around 35 °C[2, 3], it has also been shown that recoverable yeast populations begin to rapidly decline once this peak population is met.[22] This decline is due to the breakdown of the lipid membrane.[2, 3] As temperatures increase the lipid membrane becomes more fluid and its ability to protect vital proteins is decreased.[23-25] In contrast to typical brewer's yeast, Kveik has been shown to express genes that are linked to thermotolerance, the result of which is stabilization of the lipid membrane.[1] *S. cerevisiae* has been shown to increase thermotolerance after thermal shock has been applied to yeast cells.[4] This study showed an increase in thermotolerance just 30 minutes after initial heat shock was applied.[4] It can be assumed that application of heat shock to many generations of cells would lead to an even greater thermotolerance. Certain lineages of Kveik have been pitched into high temperature wort for hundreds of years and thus their thermotolerance has increased drastically compared to that of common ale yeast.[1] This increased stability and thermotolerance has shown a positive effect on the reproduction rate of Kveik at temperatures exceeding 37 °C.[1] The formation of a stable membrane allows for Kveik populations to remain at elevated temperature without the rapid decline in viability. As shown by Nordström, acetyl-CoA plays a vital role in both the production of esters as well as in cell growth.[6] While acetyl-CoA is used for cell growth, it becomes less available for use in the production of esters. Since Kveik has shown the ability to grow more readily at higher temperatures, acetyl-CoA is likely less readily available and thus ester production decreases.

Along with increased survival rate, increased lipid stability has also been linked to a decline in esters that are allowed to permeate into the medium.[26] By producing a more stable lipid membrane at higher temperatures, esters produced in Kveik would permeate into the fermentation medium less resulting in lower concentrations of esters being detected. The need for a stable lipid membrane would also require more lipid production in the cell thus making less acetyl-CoA available for ester synthesis. Verstrepen Et al. showed that an increase in fatty acids present resulted in a lower Atf1 and Atf2 activity.[15] Fatty acids are a big component of the lipid membrane and thus would decrease Atf1 and Atf2 function. This loss of function has been shown to decrease acetate ester production.[6-8, 15] The effect that the combination of increased membrane stability, increased cell growth, and increased lipid production has on ester production was seen by comparison of ester production in the 25 and 37 ℃ trials in this study. From this study it was determined that the production of esters can be reduced through an increase in fermentation temperature, which is ideal for brewers looking to reduce fermentation time while keeping the aromatic activity under control.

Comparison Between Original Gravities

Through comparison of ester production between original gravities, only one trend was consistent for all yeast strains at a given temperature. During the 37 ℃ ferment if there was a statistically significant difference in ester production between the 1.060 and 1.080 gravities, the 1.060 ferment produced higher concentrations of esters than the 1.080. This trend stood true for all esters and all strains of yeast during the 37 ℃ ferment. During the 25 ℃ ferment the same trend was seen for all esters except for ethyl acetate. For these trials there was no consistency as to how ester production was affected during the 1.040 ferment.

Typically, an increase in gravity results in an increase in ester production[15, 27], but the opposite was observed under the above temperature. This is likely due to the same reason that increasing fermentation temperature had a reverse impact on ester production than what is typically seen. Anderson and Kirsop noted that as yeast growth rates per unit of sugar decreased during fermentation of higher gravity wort, the ester production began to increase.[27] This was attributed to acetyl-CoA being more available for the production of esters as is it was used less for growth of cells. The increase in fermentation temperature increased the ability for the Kveik cells to grow, and thus it is likely that Kveik would more readily use the excess sugar in a high gravity ferment to grow. This would lead to a reduction in the available acetyl-CoA for ester production and would result the differences in ester production seen in this study. Traditionally Kveik was pitched into wort with sugar concentrations near 1.080 which may have led to the yeast being more suitable for growth at this specific gravity.[1] By increasing the sugar concentration, there are more nutrients to sustain a larger population of yeast meaning that the rate of growth per unit of available sugar wouldn't decrease as much as with traditional yeast.

Conclusion

Through analysis and comparison of the ester production during fermentation using Kveik it was determined that increasing the temperature from 25 to 37 ℃ resulted in a decrease in ester production for all six esters outlined in this study. The thermotolerance and the ability to continuously synthesize lipids is likely the main driving force for this reduction in ester production. During fermentation at both temperatures, ethyl octanoate was the only ester produced at or above the olfactory threshold range. At the lower temperature, ethyl acetate and ethyl hexanoate were produced within the olfactory threshold range for some yeasts, but upon increasing the fermentation temperature they fell below the range. The effect

that the original gravity had on ester production was inconsistent. The only exception to this was that at 37 ℃ the middle gravity consistently produced higher concentrations of esters than the highest gravity. In future experiments fermentations should be carried out at multiple temperatures between the range of 15 and 40 ℃ to more accurately determine the optimal fermentation temperature for each Kveik strain. Pitch rate was kept consistent during this study but should be varied in future experiments to determine its effect on ester production. This study has shown that Kveik can be a useful yeast to brew beer with if a fast fermentation is desired with low ester concentrations.

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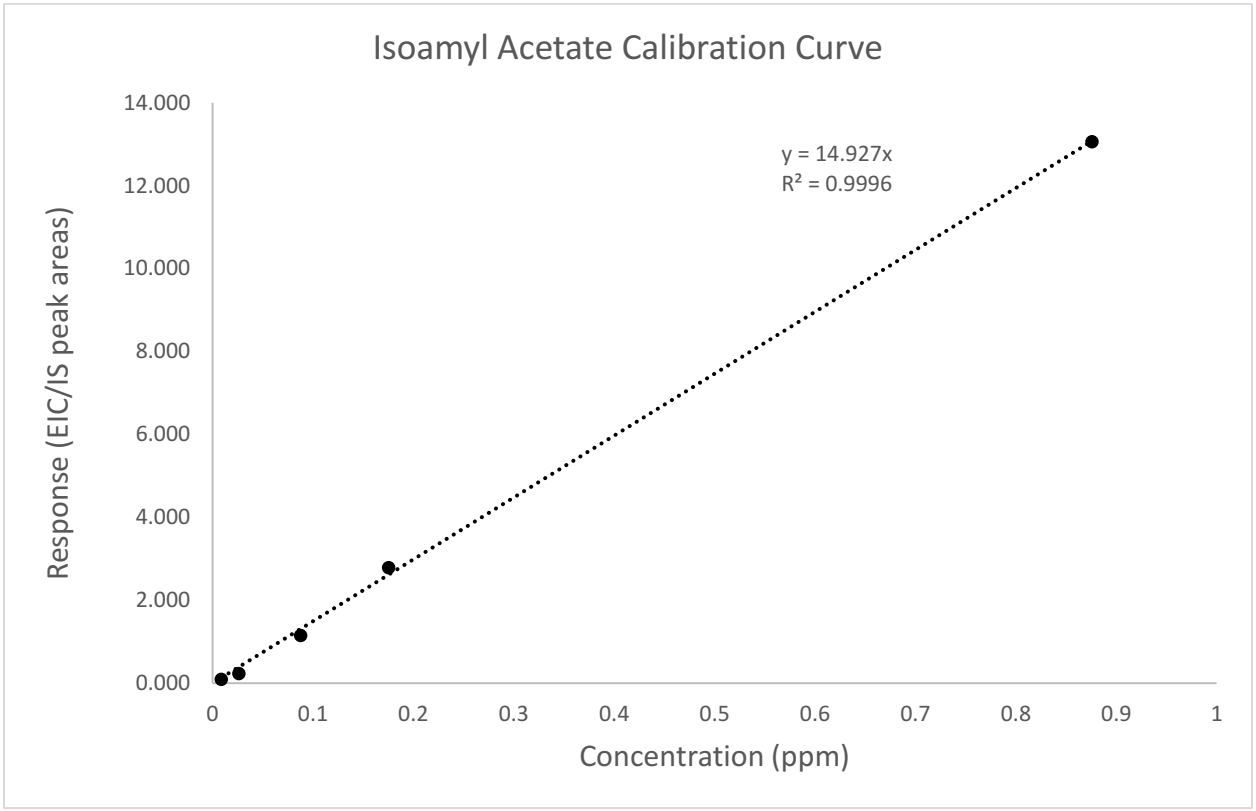
Supplementary Figures and Tables

Supplementary Table 1. Concentration and responses from isoamyl acetate used to create the calibration curve for isoamyl acetate

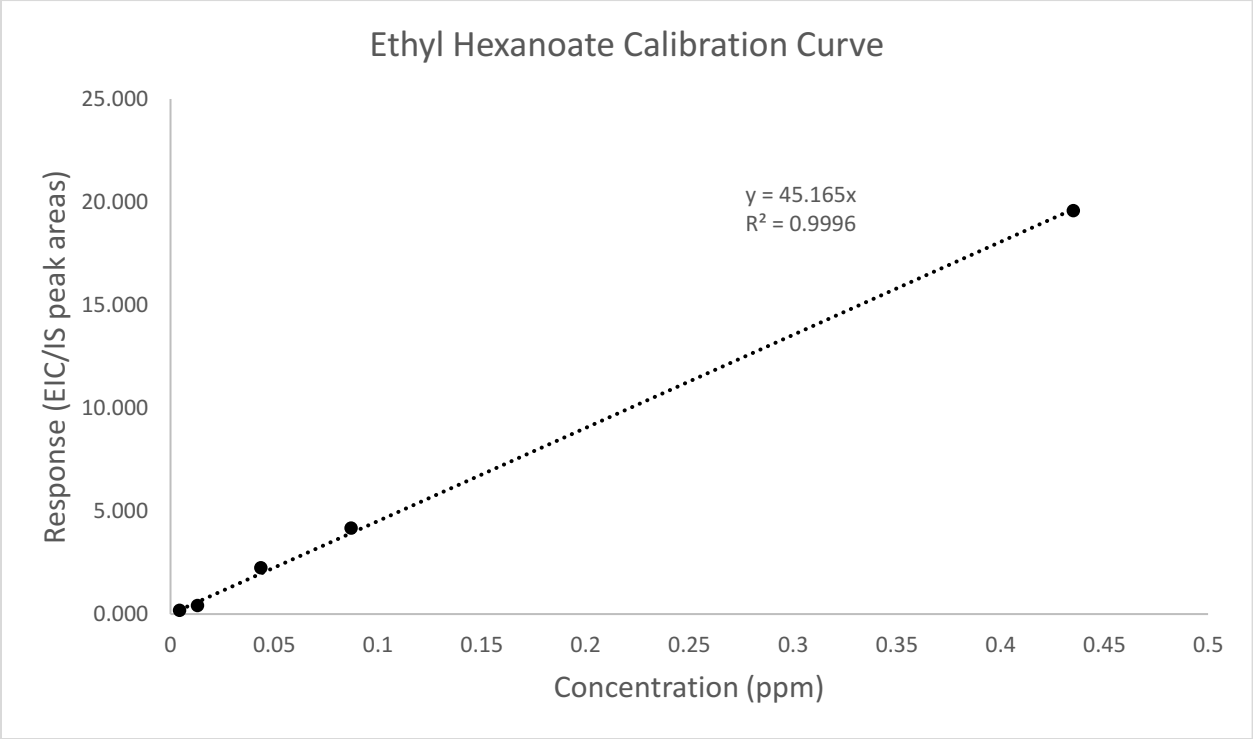
Concentration (ppm)	Response 1	Response 2	Response 3	Avg. Response
0.00876	0.107	0.090	0.079	0.092
0.02628	0.244	0.223	0.240	0.236
0.0876	0.470	1.500	1.485	1.152
0.1752	2.830	2.460	3.071	2.787
0.876	11.796	14.505	12.886	13.062



Supplementary Figure 1. Calibration curve for isoamyl acetate

Supplementary Table 2. Concentration and responses from ethyl hexanoate used to create the calibration curve for ethyl hexanoate

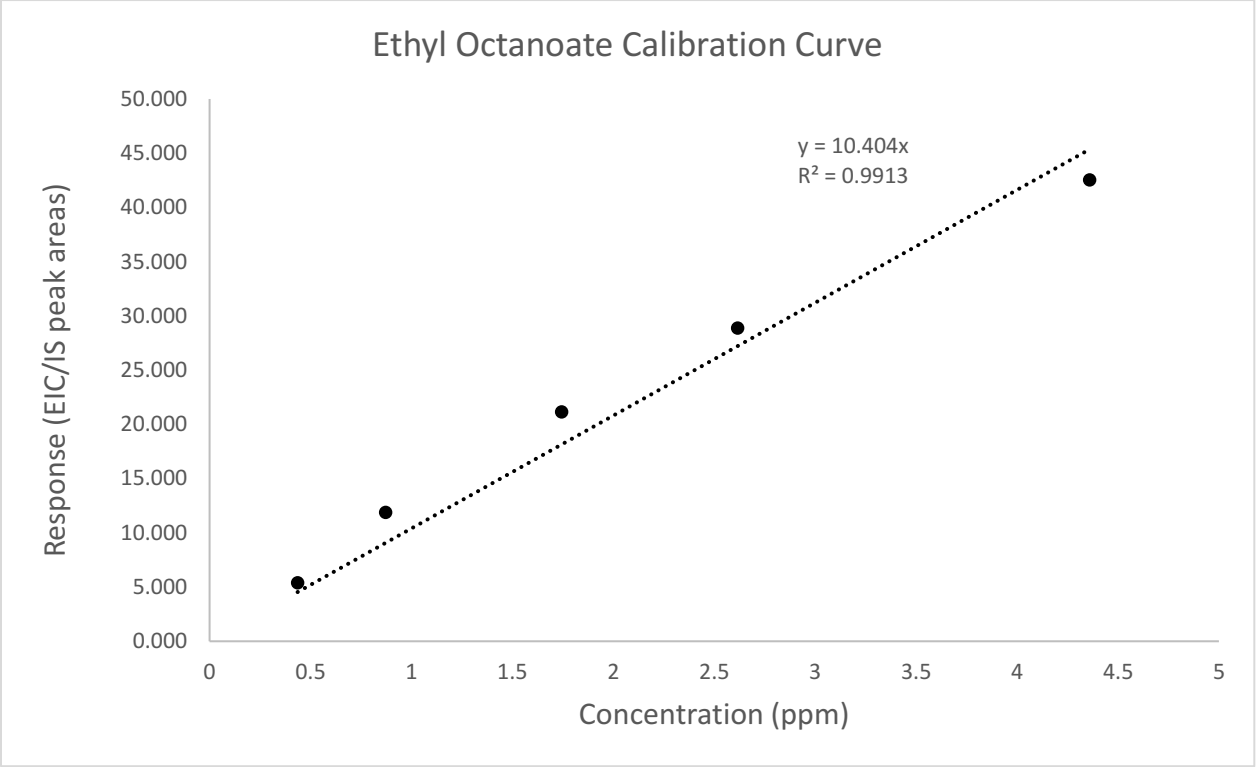
Concentration (ppm)	Response 1	Response 2	Response 3	Avg. Response
0.00435	0.201	0.168	0.142	0.170
0.01305	0.428	0.392	0.418	0.413
0.0435		2.220	2.265	2.243
0.087	4.495	3.469	4.558	4.174
0.435	17.726	22.478	18.522	19.575



Supplementary Figure 2. Calibration curve for ethyl hexanoate

Supplementary Table 3. Concentration and responses from ethyl octanoate used to create the calibration curve for ethyl octanoate

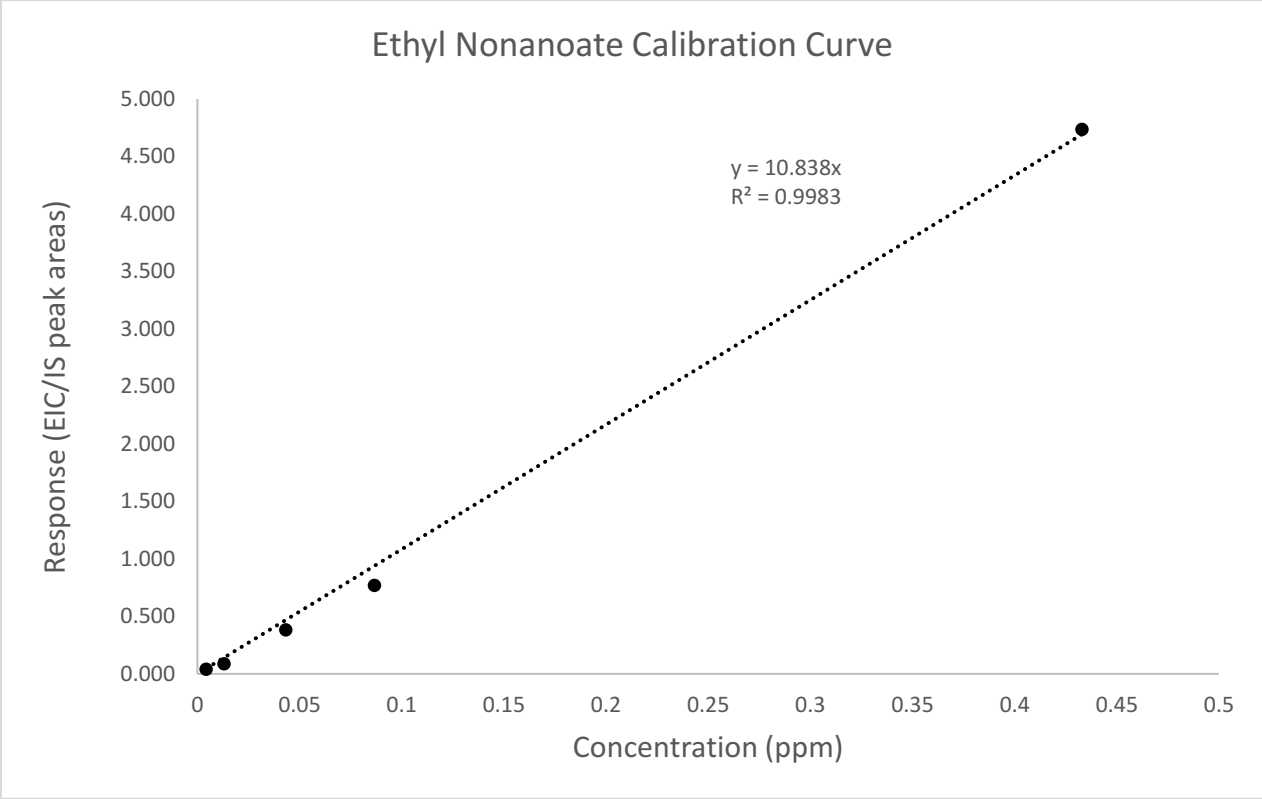
Concentration (ppm)	Response 1	Response 2	Response 3	Avg. Response
2.616	26.570	30.568	29.470	28.869
4.36	40.658	43.761	43.170	42.530
0.436	5.377	6.077	4.715	5.390
0.872	11.918	12.665	11.054	11.879
1.744	21.215	22.903	19.277	21.132



Supplementary Figure 3. Calibration curve for ethyl octanoate

Supplementary Table 4. Concentration and responses from ethyl nonanoate used to create the calibration curve for ethyl nonanoate

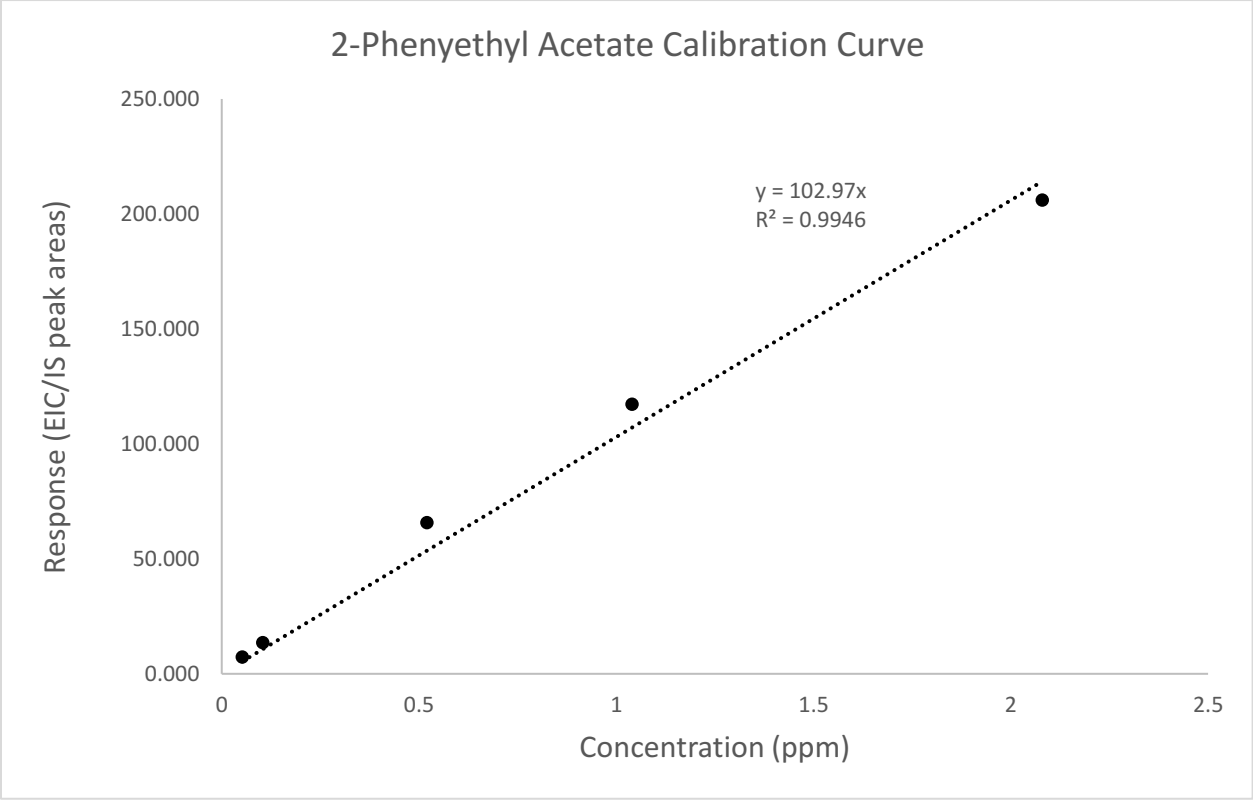
Concentration (ppm)	Response 1	Response 2	Response 3	Avg. Response
0.00433	0.050	0.035	0.036	0.040
0.01299	0.090	0.087	0.087	0.088
0.0433		0.374	0.395	0.384
0.0866	0.907	0.645	0.759	0.770
0.433	4.971	5.333	3.907	4.737



Supplementary Figure 4. Calibration curve for ethyl nonanoate

Supplementary Table 5. Concentration and responses from 2-phenyethyl acetate used to create the calibration curve for 2-phenylethyl acetate

Concentration (ppm)	Response 1	Response 2	Response 3	Avg. Response
0.052		7.418	6.919	7.169
0.104	10.811	14.731	14.776	13.440
0.52	58.238	64.462	74.478	65.726
1.04	100.266	122.523	128.603	117.131
2.08	177.122	229.902	210.791	205.938



Supplementary Figure 5. Calibration curve for 2-phenylethyl acetate