




RESEARCH ARTICLE

Online monitoring of higher alcohols and esters throughout beer fermentation by commercial *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* yeast

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Abstract

Higher alcohols and esters are among the predominant classes of volatile organic compounds (VOCs) that influence the quality of beer. The concentrations of these compounds are determined through a specific yeast strain selection and fermentation conditions. The effect of yeast strains on the formation of higher alcohols and esters throughout fermentations (at 20°C) was investigated. Flavour-relevant esters (ethyl acetate, isoamyl acetate, ethyl hexanoate and ethyl octanoate) and higher alcohols (isoamyl alcohol, isobutyl alcohol and phenylethyl alcohol) were monitored throughout the fermentation using proton transfer reaction–time of flight–mass spectrometry (PTR-ToF-MS) coupled with an automated sampling system for continuous measurements. Compound identification was confirmed by analysis of samples using gas chromatography–mass spectrometry (GC-MS). Results demonstrated the specific time points where variation in higher alcohol and ester generation between yeast strains occurred. In particular, the concentrations of isoamyl acetate, ethyl octanoate and isoamyl alcohol between yeast strains were significantly different over the first 2 days of fermentation; whereas, after Day 3, no significant differences were observed. The two *Saccharomyces pastorianus* strains produced comparable concentrations of the key higher alcohols and esters. However, the key higher alcohol and ester concentrations varied greatly between the two *S. cerevisiae* strains. The use of PTR-ToF-MS to rapidly measure multiple yeast strains provides new insights on fermentation for brewers to modify the sensory profile and optimise quality.

KEYWORDS

brewing, higher alcohols and esters, proton transfer reaction–time of flight–mass spectrometry, yeast

Abbreviations: ANOVA, analysis of variance; GC-FID, gas chromatography–flame ionization detector; GC-MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; PCA, principal component analysis; ppb_v, parts per billion by volume; PTFE, polytetrafluoroethylene; PTR-ToF-MS, proton transfer reaction–time of flight–mass spectrometry; SBSE, stir bar sorptive extraction; SPME, solid phase microextraction; VOCs, volatile organic compounds.

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1 | INTRODUCTION

The aroma of beer is derived from the raw materials (malt, hops and yeast) and from numerous metabolic and chemical reactions that occur throughout fermentation, which increase the volatile composition complexity. Understanding how yeast produce key secondary metabolites, in particular higher alcohols and esters, is essential to control and optimize flavour.¹ As most compounds are present in alcoholic beverages in a concentration close to their odour-detection threshold, small changes can have a large sensory impact on the final product.²

Brewer's yeasts are conventionally part of the genus *Saccharomyces*, where the two most commonly used yeast species are *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* (a hybrid of *S. cerevisiae* and *S. eubayanus*).^{3,4} *S. cerevisiae* are typically used for the production of top fermenting ale beers, and *S. pastorianus* are typically used for the production of bottom fermenting lager beer.⁵ Each of these *Saccharomyces* species has numerous variants or strains, which have different fermentation characteristics (e.g., terminal gravity, flocculation and alcohol tolerance) and impart different flavour characters, which determines whether the particular yeast is suitable for a given beer style.

A phenomenon of interest to brewing scientists are the differences between yeast strains that results in different concentrations of fermentation secondary metabolites and the resultant impact on beer flavour.¹ Ethanol and carbon dioxide are the most abundant volatile products of yeast metabolism. However, the predominant classes of volatile compounds that influence the quality of beer are higher alcohols, esters, aldehydes and ketones, which originate from yeast metabolism during primary fermentation.^{1,6} The concentrations of esters and higher alcohols in beer have been shown to differ dramatically with varying yeast stains. While the lower fermentation temperatures typical of lager production can have an impact on the formation of these compounds, it has been established that lager yeast strains inherently produce lower levels of esters and higher alcohols compared with ale yeast strains.⁷ Small changes in concentration of these secondary metabolites have a large effect on the sensory quality of

beer. Better understanding of the timing of their formation during fermentation will allow brewers to better control and predict the final flavour in beer. The final concentrations (at the end of fermentation) of these compounds are well documented⁸ (Table 1). However, the dynamics of the biosynthetic reactions that form these compounds over the course of fermentation remains less understood.

The most well-described flavour-active esters in beer are ethyl acetate (solvent, fruity-like aroma), ethyl hexanoate (sweet, fruity, pineapple aroma), ethyl octanoate (apple-like aroma), isoamyl acetate (fruity, banana aroma), isobutyl acetate and phenylethyl acetate (honey, fruity, roses, flowery aroma).¹⁵ The primary higher alcohols found in beer are isoamyl alcohol, isobutyl alcohol and phenylethyl alcohol. Higher alcohols contribute to beer flavour by intensifying alcoholic perception and imparting a warm mouthfeel.¹ Higher alcohols also provide the alcohol moiety required for the subsequent synthesis of esters.¹⁶ The physicochemical characteristics and proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS) fragmentation patterns of key aroma compounds in beer is illustrated in Table 2.

Aroma-active esters are formed intracellularly by yeast cells during active growth. Esters such as ethyl hexanoate and butanoate are produced from the ethanolysis of acyl CoA that is formed during the synthesis or degradation of fatty acids. Esters such as isoamyl and phenethyl acetate are a product of the reaction of higher alcohols with acetyl CoA. Higher alcohols are formed by yeast through a catabolic pathway that involves the breakdown of amino acids, specifically branched-chain amino acids such as leucine, isoleucine and valine. These amino acids are first converted into their corresponding α -keto acids, which are then further metabolized by yeast to produce higher alcohols. Another metabolic pathway for the formation of higher alcohols is the anabolic pathway. This pathway involves the synthesis of higher alcohols from simple precursors such as pyruvate, which is produced during glycolysis. Pyruvate can be converted into various higher alcohols such as isobutanol and isopentanol via a series of enzymatic reactions.^{18–21} An overview of the metabolic pathway of flavour active compounds known in beer is shown in Figure 1.²²

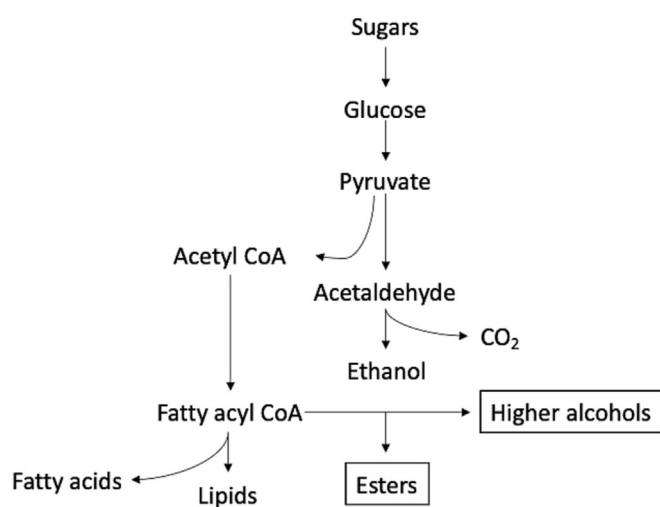
TABLE 1 Key beer flavour compounds and concentration adapted from literature.

Compound	Molecular weight (g/mol)	Concentration found in beer (ppm)	References
Esters			
Ethyl acetate	88.11	15.3–16.8	9–11
Phenylethyl acetate	164.20	0.1–0.73	2,10–12
Isoamyl acetate	130.19	0.078–0.489; 1.2	2,9,10
Isobutyl acetate	116.16	0.03–1.2	11
Ethyl hexanoate	144.21	0.081–0.411	9,12
Ethyl octanoate	172.26	0.04–0.53	11,12
Higher alcohol			
Isoamyl alcohol	88.15	8.73–44	12,13
Isobutyl alcohol	74.12	6.6; 58.9	12,13
Phenethyl alcohol	122.16	4–102	14

TABLE 2 Physiochemical characteristics and PTR-ToF-MS fragmentation patterns of key aroma compounds in beer.

Compound name	CAS N°	Physiochemical characteristics			Sensory description	Target mass	
		Chemical class	Chemical formula	Molecular weight (g/Mol)		Ion	m/z
Ethyl acetate	141-78-6	Acetate Ester	C ₄ H ₈ O ₂	88.110	Pineapple ¹⁷	(C ₄ H ₈ O ₂)H +	89.0589
Isoamyl acetate	123-92-2	Acetate Ester	C ₇ H ₁₄ O ₂	130.19	Banana ¹⁷	(C ₇ H ₁₄ O ₂)H +	131.1065
Ethyl hexanoate	123-66-0	Fatty Acid Ester	C ₈ H ₁₆ O ₂	144.21	Apple ¹⁷	(C ₈ H ₁₆ O ₂)H +	145.1213
Ethyl octanoate	106-32-1	Fatty Acid Ester	C ₈ H ₂₀ O ₂	172.26	Fruit, fat ¹⁷	(C ₈ H ₂₀ O ₂)H +	173.1529
Isoamyl alcohol	123-51-3	Higher alcohol	C ₅ H ₁₂ O	88.148	Alcoholic, banana ¹²	(C ₅ H ₁₂ O)H +	71.0850
Isobutyl alcohol	78-83-1	Higher alcohol	C ₄ H ₁₀ O	74.12	Solvent, sweet ¹²	(C ₄ H ₁₀ O)H +	58.0735
Phenylethyl alcohol	60-12-8	Higher alcohol	C ₈ H ₁₀ O	122.16	Roses ¹²	(C ₈ H ₁₀ O)H +	105.0689

Abbreviation: PTR-ToF-MS, proton transfer reaction-time of flight-mass spectrometry.

**FIGURE 1** Overview of the metabolic pathway of flavour active compounds known in beer.²²

The most common method used to analyse volatile organic compounds (VOCs) in beer is gas chromatography-mass spectrometry (GC-MS) coupled with an extraction method such as solid phase microextraction (SPME) or stir bar sorptive extraction (SBSE).²³⁻²⁶ Traditionally, liquid-liquid solvent extraction has been used to monitor VOCs during fermentation.^{27,28} However, due to the time involved in GC-MS extraction and analysis, few time points during fermentation are normally analysed. Hence, these methods are not optimal to monitor real-time changes in an active system, which leads to an incomplete picture and missing data.²⁴ PTR-ToF-MS has previously been used to track the release of VOCs during beer fermentation²⁹ and characterize the gas phase VOCs in complex food matrices such as apples, blueberries, bread, cheese, coffee, milk, olives, saffron, tea and wine.³⁰⁻⁴⁰

PTR-ToF-MS is a direct injection mass spectrometric technique that has been widely used for the rapid detection of VOCs.⁴¹ This non-invasive technique is based on the hydronium ion (H₃O⁺) transfer reaction and allows for the real time detection of VOCs with higher proton affinities than water, even at concentrations as low as part per billion by volume (ppbv).⁴² PTR-ToF-MS is an efficient technique for the measurement of aroma release studies in food matrices undergoing time-dependent transformations, for example, the aroma formation during fermentation in wine⁴³ and beer.²⁹ Richter et al produced the first study following the progression of alcoholic fermentation of beer using PTR-ToF-MS.²⁹ The production of VOCs from different combinations of yeasts was measured, demonstrating that PTR-ToF-MS was suitable to monitor the changing VOCs during fermentation in real time. The aim of the present study was to illustrate the ability of PTR-ToF-MS to follow the production of higher alcohols and esters throughout beer fermentation. A second objective was to quantify the formation of important higher alcohols and esters in beer produced by different *S. cerevisiae* and *S. pastorianus* yeast strains. A limitation of PTR-ToF-MS is the difficulty of distinguishing between isomeric compounds (compounds with the same molecular formula). Incorporating GC-MS measurements provides a method of compound identification and allows isomeric compounds to be distinguished (e.g. amyl acetate and isoamyl acetate).⁴⁴

2 | MATERIALS AND METHODS

2.1 | Yeast strains

Four yeast strains were selected for the investigation, with two *S. cerevisiae* (SafAle US-05, SafAle WB-06) and two *S. pastorianus* strains (SafLager W-34/70 and SafLager S-23) (Fermentis, Lesaffre,

TABLE 3 Yeast characteristics and pitching rate.

Name	Yeast species	Apparent attenuation (%)	Recommended rehydration temperature (°C)	Recommended fermentation temperature (°C)	Recommended pitch rate (cells/mL)
SafAle US-05	<i>Saccharomyces cerevisiae</i>	81	25–29	18–28	1×10^7
SafAle WB-06	<i>Saccharomyces cerevisiae</i> var. <i>diastaticus</i>	86	25–29	18–28	1×10^7
SafLager W-34/70	<i>Saccharomyces pastorianus</i>	83	15–25	12–15	1×10^7
SafLager S-23	<i>Saccharomyces pastorianus</i>	82	15–25	12–15	1×10^7

France). Rehydration was carried out according to the suppliers' instructions. To obtain a target pitching rate of 1×10^7 cells/mL, 0.5 g of yeast was added to 100 mL of boiled then cooled wort (12°P, pH 5.2), incubated at 25°C for 30 min and then pitched into the samples. Table 3 displays information about each yeast strain and their optimum fermentation temperature range.

2.2 | Wort preparation and fermentation conditions

Model wort was prepared by dissolving 260 g of spray dried malt extract (Briess Golden light) in 2 L of deionised water (18 MΩ cm) with 166 mg of calcium chloride (CaCl₂).⁴⁵ In place of bittering hops, 76.7 mg of iso-α-acids (ICS–I4 Iso Standard) was added, providing an International Bitterness Unit (IBU) of 20. The wort was heated to 90°C using a water bath (temperature held for 10 min), then decreased to 20°C using an ice bath. The main analytical characteristics of the wort were: pH 5.2, with a specific gravity of 12.0°P.

The rehydrated yeast solution was used to inoculate the vials, with 0.6 mL added to each sample (total sample volume was 3 mL) in order to achieve a consistent starting culture. This amount of yeast was in line with the recommendations of manufacturers and best brewing practices. Each yeast strain was fermented independently in triplicate with 3 mL wort in 20 mL headspace vials sealed with a polytetrafluoroethylene (PTFE) lined silicon septa (18 mm Magnetic Cap with Blue PTFE/White Silicone 1.5 mm [0.060"]). Fermentations were carried out at 20°C. Three replicates were produced solely for PTR–ToF–MS measurements. An additional five replicates were produced and measured both with PTR–ToF–MS and GC–MS. These samples were measured throughout the fermentation with PTR–ToF–MS; however, one sample each day was taken to be measured with GC–MS, with the sample then returned to the autosampler for further analysis (each of these samples were only measured once with GC–MS). Finally, one replicate was produced solely for GC–MS measurements.

2.3 | PTR–ToF–MS measurement

The 3 mL samples containing yeast and model wort were sealed and placed into an autosampler (MPS MultiPurposeSampler, Gerstel, Germany) especially adapted for PTR–ToF–MS,⁴⁶ with the position of each sample within a replicate randomised to limit systematic and analytical errors. Throughout fermentation, the headspace of each vial was measured every 6 h over 5 days, with the first set of measurements completed within 2 h of yeast inoculation.

Headspace measurements were performed with a commercial PTR–ToF–MS 8000 apparatus from Ionicon Analytik GmbH (Innsbruck, Austria), in a standard configuration (V mode). The ionization conditions in the drift tube were: drift tube temperature of 110°C, drift pressure of 2.80-mbar and drift voltage of 500-V. This led to an E/N ratio of 130 Townsend (1 Td = 10–17 cm²/V/s). The autosampler moved the sample from the incubation tray to the temperature-controlled purging site, connected to the PTR–ToF–MS inlet. Dynamic headspace analysis took place for 60 s with the headspace gas replaced with nitrogen gas and an acquisition rate of one mass spectrum per second between *m/z* 15 and 349. Due to the high ethanol concentration, argon was added to the inlet system at a flowrate of 120 sccm (standard cubic centimetres per minute) with the total flowrate of the system at 160 sccm. This reduced primary ion depletion and formation of ethanol clusters that may affect the final quantification of VOCs.⁴⁷ The argon flow rate was controlled by a multi-gas controller (MKS Instruments, Inc, Andover, MA, USA). After the measurement, the vial was moved back to the same position of the incubation tray, and the cycle was repeated on the following sample. During fermentation, the measurement was repeated every 6 h to monitor the fermentation process.

Deadtime correction, internal calibration of mass spectral data and peak extraction were performed according to previously described procedures.^{48,49} The peak intensity in ppb/v (parts per billion per volume) was estimated using the formula described in literature.⁵⁰ Concentrations of volatile compounds in ppb_v were determined as per Lindinger et al⁵⁰ using a reaction rate constant coefficient of $k = 2.10^{-9}$ cm³/s.

2.4 | HS-SPME/GC-MS analysis

VOCs were extracted using headspace solid phase microextraction (HS-SPME) with 2-cm fibre coated with 50/30- μm divinyl benzene/carboxen/poly-dimethylsiloxane (DVB/CAR/PDMS, Supelco, Bellefonte, PA, USA). The headspace was exposed to the fibre for 40 min. The compounds absorbed on the SPME fibre were desorbed at 250°C in the GC-MS injection port. The mass detector operated in electron ionization mode (EI, internal ionization source; 70 eV) with a scan range from m/z 33 to 350 (GC Clarus 500, PerkinElmer, Norwalk CT, USA). Analysis was carried out using Agilent Technologies 7820A/5977B Series MSD with an HP-INNOWax fused silica capillary column (30 m, 0.32-mm ID, 0.5- μm film thickness; Agilent Technologies, Palo Alto, CA, USA). The oven temperature was initially set at 40°C for 1 min, then increased to 220°C at 4°C/min and 250°C at 15°C/min and maintained for 2 min. Helium was used as a carrier gas with a flow rate of 1.5 mL/min. Compound identification was based on mass spectra matching with NIST14/Wiley98 libraries. Linear retention indices were calculated under the same chromatographic conditions after the injection of a C7-C30 n-alkane series (Supelco).

2.5 | Refractive index measurement

Original and final gravity were determined by refractive index measurement of the model wort and final ferment using a handheld refractometer (0-32°Bx; Fisher scientific, New Jersey, USA). The measured refractive index was converted directly into weight percent sucrose content (°Brix). The specific gravity was calculated using Equation (1), correcting for the effect of alcohol on the refractive index for measurements taken during fermentation.

$$\text{Specific gravity} = \left(\frac{\text{Brix}}{258.6 - \left(\frac{\text{Brix}}{258.2} \right) \times 227.1} \right) + 1. \quad (1)$$

The specific gravity was converted to alcohol by volume (ABV) using Equation (2).

$$\text{ABV} = \frac{1.05}{0.79} \left(\frac{\text{Original Gravity} - \text{Final Gravity}}{\text{Final Gravity}} \right) \times 100. \quad (2)$$

TABLE 4 Original and final gravity of the samples used in the brewing trial (mean \pm standard deviation of three replicates).

Name	Original gravity	Final gravity	ABV %
SafAle US-05	1.053 \pm 0.001	1.025 \pm 0.006	3.74
SafAle WB-06	1.053 \pm 0.001	1.025 \pm 0.006	3.77
SafLager W-34/70	1.053 \pm 0.001	1.026 \pm 0.006	3.58
SafLager S-23	1.054 \pm 0.001	1.026 \pm 0.006	3.65
Blank (model wort no yeast)	1.053 \pm 0.001	1.053 \pm 0.001	0.04

2.6 | Statistical analysis

A two-way analysis of variance (ANOVA) (yeast strain and fermentation time, $p < 0.001$) was used to determine the m/z that were significantly higher than the background. Where monoisotopic mass peaks were saturated, its isotopologue was selected for further analysis. All other mass peaks belonging to ^{13}C , ^{18}O and ^{27}S isotopologues, water and ethanol clusters were excluded from the dataset. This procedure reduced the dataset to 102 mass peaks.

Of the 102 mass peaks, 68 were assigned a sum formula, which were identified as one or more compounds based on GC-MS identification and literature. Data was processed using MATLAB (MathWorks Inc., Natick, MA, USA) and R (R Foundation for Statistical Computing, Vienna, Austria). Multivariate statistical analysis was performed using R 3.2.0 internal statistical functions and external packages, specifically, ggplot2 and ANOVA. Significant differences ($p < 0.001$) between averages were evaluated. Principal component analysis (PCA) was performed using R package 'mixomics'⁵¹ on the log transformed and mean centred data. All the information concerning the chemical variables (usually highly correlated) were condensed into a few latent variables.

3 | RESULTS AND DISCUSSION

3.1 | Chemical properties during fermentation

The original gravity, final gravity and %ABV was measured to compare the extent of fermentation between yeast strains (Table 4). The % ABV of the *S. cerevisiae* strains SafAle US-05 and SafAle WB-06 were 3.74% and 3.77%, respectively. In contrast, the %ABV of *S. pastorianus* strains SafLager S-23 and SafLager W-34/70 were 3.58% and 3.65%.

3.2 | PCA

To identify the most important VOCs produced during fermentation by the four *S. cerevisiae* and *S. pastorianus* yeast strains, a PCA was performed (Figure 2). PCA reduces the dimensionality of the data, limiting information loss.⁵² The two first components

explained 84.57% of the variation in the data with 78.86% and 5.71% explained by PC1 and PC2, respectively. The scores plot (Figure 2) displays the VOCs evolution by the yeasts and identifies the point at which the VOCs produced from the yeasts differ. At the beginning of fermentation (starting near the top left), the samples were clustered together indicating similarity. As fermentation progresses, there is a separation between *S. cerevisiae* and *S. pastorianus*. SafLager W-34/70 and SafLager S-23 (*S. pastorianus*) are displayed by the blue and orange circles. The evolution is similar, starting near the bottom left and finishing near the top right. The evolution of SafAle US-05 (identified by the green circles) differs from the other yeasts, with lower positive loadings on PC1 and 2. SafAle WB-06, SafLager S-23 and SafLager W-34/70 finish fermentation together near the top middle, indicating similarities in the concentration of major compounds formed.

3.3 | Evolution of volatile compounds during fermentation

To understand the fermentation behaviour of the selected yeast strains, VOCs were monitored throughout the duration of fermentation. As the parent ethanol peak (m/z 47.049) and carbon dioxide peak (m/z 44.996) were saturated, the isotopologues m/z 48.053 and 45.997 were evaluated as proxies. There was no significant difference in the concentration of carbon dioxide between yeast strains at any time point of fermentation (Figure 3). On the other hand, a significant difference in the concentration of ethanol was observed at the end of Day 1 (second, third and fourth timepoint; $p < 0.001$), but with no significant differences observed thereafter. A study comparing the physiological activities of lager and ale yeast with different wort gravities reported a higher concentration of ethanol at the beginning of fermentation for all lager yeast and no difference at the end of

fermentation (compared to ale yeast), independent of gravity, which is consistent with the results of the present study.⁵³

The effect of different yeast strains on the formation of higher alcohols and esters throughout fermentations was investigated. Seven typical beer aroma compounds were chosen for this investigation, four esters (ethyl acetate, isoamyl acetate, ethyl hexanoate and ethyl

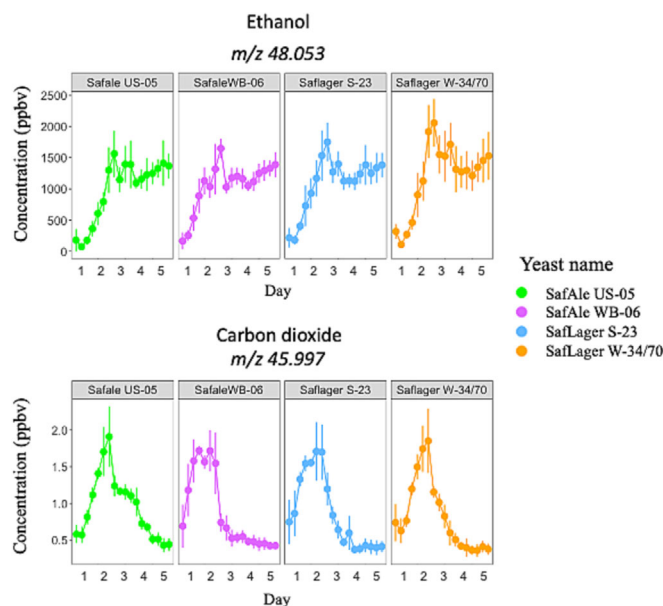


FIGURE 3 Mean concentration (ppbv) and standard deviation of ethanol (m/z 48.053) and carbon dioxide (m/z 45.997) during fermentation by commercially available yeast: *Saccharomyces cerevisiae* strain SafAle US-05, *S. cerevisiae* var *diastaticus* strain SafAle WB-06 and *S. pastorianus* strains SafLager S-23 and SafLager W-34/70 measured with proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS).

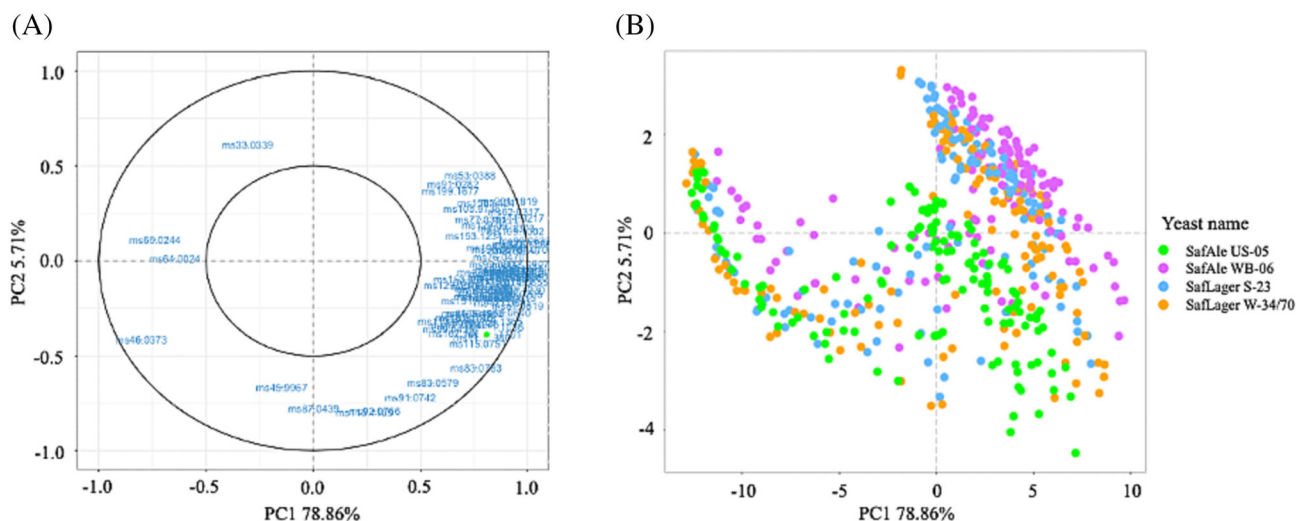


FIGURE 2 (A) Score and (B) loading plot of principal component analysis (PCA) of VOC produced during fermentation (5 days) by commercially available yeast: *Saccharomyces cerevisiae* strain SafAle US-05, *S. cerevisiae* var *diastaticus* strain SafAle WB-06 and *S. pastorianus* strains SafLager S-23 and SafLager W-34/70. Different colours (pink, orange, blue and green) indicated different yeast strains.

octanoate) and three higher alcohols (isoamyl alcohol, isobutyl alcohol and phenylethyl alcohol).

The concentrations of ethyl acetate (m/z 62.032), isoamyl acetate (m/z 131.106), ethyl hexanoate (m/z 145.121) and ethyl octanoate (m/z 173.152) throughout fermentation are displayed in Figure 4. The compounds were identified using the measured m/z combined with an elemental composition calculator corroborated with GC-MS results. Previous literature shows the most abundant ethyl acetate

peak is m/z 61 (originates directly from a McLafferty rearrangement) followed by the peak at m/z 43 (from a successive water loss).⁵⁴ The signal at m/z 61 was saturated, therefore, the isotopologue m/z 62.032 was evaluated. The masses of the esters selected in this work align with the results from Aprea et al, investigating esters in water and water/ethanol solutions with PTR-MS⁵⁴ (Table 5).

Similar formation patterns in the production of ethyl acetate and ethyl octanoate were observed during fermentation for the four yeast

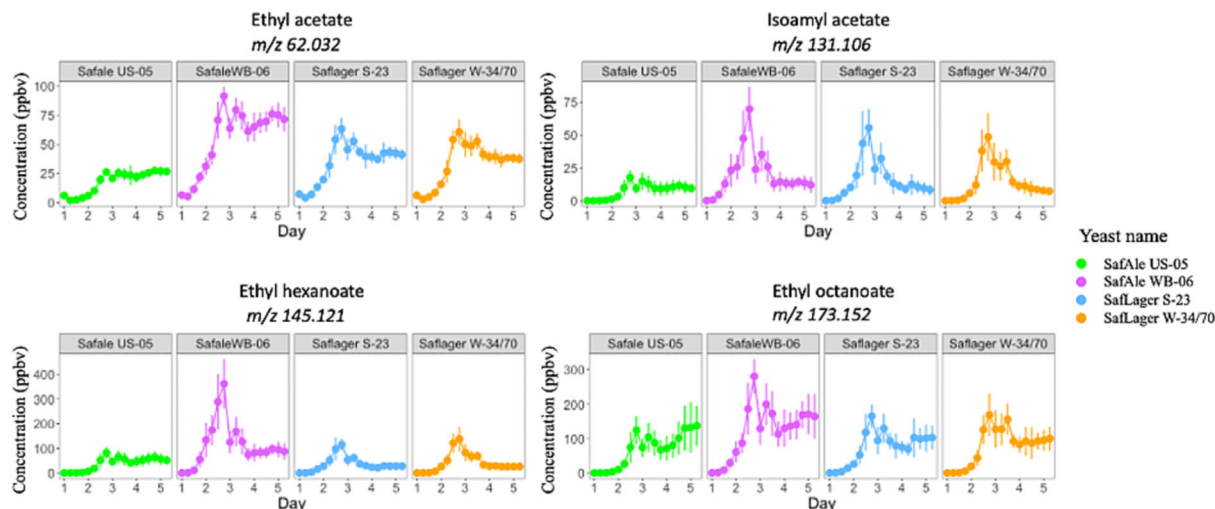


FIGURE 4 Mean concentration (ppbv) and standard deviation of esters: ethyl acetate (m/z 90.063), isoamyl acetate (m/z 131.106), ethyl hexanoate (m/z 145.121) and ethyl octanoate (m/z 173.152) during fermentation by commercially available yeast: *Saccharomyces cerevisiae* strain SafAle US-05, *S. cerevisiae* var *diastaticus* strain SafAle WB-06 and *S. pastorianus* strains SafLager S-23 and SafLager W-34/70 measured with proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS).

TABLE 5 Alcohols, higher alcohols and esters, their molecular weight (MW) and intensities of their major ions determined by proton transfer reaction-mass spectrometry. Fiches et al. has not been considered because of the very high ethanol content present in the Brandies.⁵⁶

Compound	MW (g/mol)	E/N (Td)	m/z	m/z	m/z	m/z	m/z	Reference	
Ethanol	46	120	47 (100)	45 (37)	-	-	-	55	
Ethyl acetate	88	120	61 (100)	43 (42)	89 (41)	-	-	54	
			145	89 (100)	177 (21)	135 (8)	-	-	
			454	61 (100)	89 (52)	-	-	-	
Isoamyl acetate	130	145	71 (100)	131 (40)	177 (4)	-	-	56	
			454	71 (100)	131 (20)	-	-	-	
Ethyl hexanoate	144	120	145 (100)	71 (7)	43 (5)	99 (5)	117 (4)	54	
			145	145 (100)	191 (4)	99 (2)	-	-	
			454	145 (100)	117 (99)	99 (28)	-	-	
Ethyl octanoate	172	120	173 (100)	57 (13)	127 (12)	145 (2)	-	54	
Isoamyl alcohol	88	120	43 (100)	71 (82)	41 (27)	70 (7)	72 (5)	-	
			145	71 (100)	135 (10)	-	-	-	56
			454	71 (100)	-	-	-	-	
Phenethyl alcohol	122	145	105 (100)	169 (2)	-	-	-	56	
			454	105 (100)	51 (73)	79 (35)	197 (13)	-	
Isobutyl alcohol	74	115	57 (100)	73 (7)	41 (3)	-	-	57	
			138	57 (100)	41 (47)	39 (19)	73 (19)	-	

strains. During the first 2 days, the concentrations increased and reached a maximum concentration at the end of Day 3, followed by a rapid decrease. Around Day 4, the concentration remained constant until the end of the fermentation when a second increase in headspace concentration occurred. There was a significant difference (ANOVA, $p < 0.001$) in the concentration of ethyl octanoate between yeast strains for the first 2 days of fermentation, with no significant difference detected thereafter.

Lower concentration of esters, ethyl acetate and isoamyl acetate, measured at the end of fermentation for beer produced by *S. cerevisiae* compared with *S. cerevisiae* var *diastaticus* yeast is consistent with previous findings.⁵⁸ This study identified the concentration of ethyl acetate throughout fermentation was significantly lower in the beer produced by *S. cerevisiae* strain SafAle US-05 than the beer produced by *S. cerevisiae* var *diastaticus* strain SafAle WB-06. At the beginning of the fermentation, the concentration of isoamyl acetate was significantly lower in the beer produced by *S. cerevisiae* strain SafAle US-05. At the end of fermentation, there was no significant difference between the yeast strains. Because of the continuous flushing of the headspace, the sum of isoamyl acetate released cannot be compared to a closed system. When visually inspecting the formation, it appears that the overall concentration produced (area under the curve) is the lowest in the beer produced by *S. cerevisiae* strain SafAle US-05. In *S. cerevisiae*, alcohol acetyltransferases I and II (AATase I and AATase II) enzymes, encoded by the genes ATF1 and ATF2, catalyse the synthesis of acetate esters from acetyl-CoA and numerous higher alcohols. Previous studies using yeast that overexpressed the ATF1 gene showed a 30-fold increase (compared with wild-type cells) in ethyl acetate production. Additionally, when the ATF1 and ATF2 were deleted, no acetate esters were observed.^{59,60} In *S. pastorianus*, ATF1, ATF2 and Lg-ATF1 genes have also been identified. Lg-ATF1 encodes Lg-AATase I, which is homologous to AATase I enzyme.⁶¹ The overexpression of Lg-ATF1 has shown a sevenfold increase in isoamyl acetate and a twofold increase in ethyl acetate concentrations.⁶⁰ The beers produced with *S. pastorianus* had higher concentrations of acetate esters than the beers produced with *S. cerevisiae*, because of the expression of Lg-ATF1. The activity of these enzymes are strain-specific.⁶² Therefore, a higher activity of AATase I and AATase I in *S. cerevisiae* var *diastaticus* (SafAle WB-06) could explain the higher concentration of ethyl acetate. A previous study investigating the difference in *S. cerevisiae* and *S. cerevisiae* var *diastaticus* fermentation on the VOCs of Italian Grape Ale found that *S. diastaticus* produced a higher concentrations of both ethyl acetate and isoamyl acetate when compared to *S. cerevisiae*.⁶³

Meier-Dörnberg et al demonstrated in two studies that the final concentrations of fermentation secondary metabolites differed greatly (specifically esters: isoamyl acetate and ethyl acetate) depending on the yeast species and strains.^{58,64} The first study compared eight *S. cerevisiae* with two *S. cerevisiae* var *diastaticus* strains^{58,64} and the second study compared eleven *S. cerevisiae* strains with two *S. pastorianus* strains.⁵⁸ For both of these studies by Meier-Dörnberg et al parameters such as wort composition (12.4°P wort), fermentation temperature (20°C for *S. cerevisiae* and

15°C for *S. pastorianus*) fermentation time (5 days) remained the same. Commercial yeast cultures *S. cerevisiae* var *diastaticus* strains DSM 70487, TUM 1-H-7, TUM PI BB 121 and TUM PI BA 124 produced beer with an average sum ester concentration of 63.23 ± 2.83 , 29.93 ± 1.83 , 25.67 ± 0.28 and 36.77 ± 1.56 mg/L, respectively. In comparison, the average ester concentration of *S. pastorianus* Frisinga-TUM 3470 and Securitas-TUM 193 was 20.40 ± 2.69 and 27.90 ± 1.94 mg/L and of *S. cerevisiae* LeoBavaricus-TUM 68, LunaBavaria-TUM 127 and Colonia-TUM 177 was 36.57 ± 3.43 , 40.93 ± 3.16 and 35.27 ± 1.83 mg/L.^{58,64} In general, the results showed that at the end of fermentation, the beer produced by *S. cerevisiae* var *diastaticus* had the highest concentration of esters; whereas, the lowest was detected in the beer produced by *S. pastorianus*. The results show a large variation in the generation of esters between yeast strains, with it being not as simple as using generalised guidelines of yeast species to predict ester concentration. The use of PTR-ToF-MS to rapidly measure multiple yeast strains is useful to identify the specific strain to use to obtain the desired ester and alcohol concentrations in the final beer. The benefits of measuring with PTR-ToF-MS are discussed in more detail in Section 2.4.

The evolution in the concentration of ethyl hexanoate and ethyl octanoate (m/z 145.121 and m/z 173.152, respectively) is displayed in Figure 4. The initial increase in the concentration of both esters appears 'similar' up to Day 2, after which there was a significant difference in their concentrations between the different yeast strains. For both ethyl hexanoate and ethyl octanoate, *S. cerevisiae* var *diastaticus* strain SafAle WB-06 had the highest final concentration. The concentration of ethyl hexanoate at the end of fermentation was 45.10 ± 1.77 , 77.31 ± 15.73 , 33.26 ± 14.27 and 26.12 ± 8.73 ppb_v for *S. cerevisiae* (SafAle US-05) *S. cerevisiae* var *diastaticus* (SafAle WB-06), *S. pastorianus* (SafLager W-34/70 and SafLager S-23), respectively. The concentration at the end of fermentation for ethyl octanoate was 118.13 ± 8.31 , 144.5 ± 23.47 , 122.89 ± 55.37 and 98.59 ± 30.97 ppb_v for SafAle US-05, SafAle WB-06, SafLager W-34/70 and SafLager S-23, respectively. *S. pastorianus* strain SafLager W-34/70 and SafLager S-23 had a similar concentration throughout fermentation. SafAle WB-06, SafLager W-34/70 and SafLager S-23 yeast strains had the highest concentration of ethyl hexanoate around Day 2 and then started to decrease before plateauing halfway through Day 3. In contrast, the concentration of ethyl hexanoate in the beer produced with *S. cerevisiae* strain US-05 peaked around Day 3 before decreasing until the end of fermentation.

The understanding of the genes involved in the synthesis of ethyl esters is limited, leading to a scarcity of literature on the topic in comparison to acetate esters. However, current research has shown that the formation of ethyl esters is dependent on the presence of acyl coenzyme A and ethanol, as well as the activity of enzymes involved in their synthesis and hydrolysis.^{8,65} A recent study investigating sake identified that the expression of certain yeast genes, such as the EEB1 and EHT, can affect the final concentration of ethyl esters produced during the fermentation. Variations in the expression of these

genes may result in different levels of ethyl esters produced by different yeast strains.⁵⁹

3.3.1 | Higher alcohols

Higher alcohols are important precursors of the more flavour-active esters; for this reason, their formation needs to be monitored, especially in the initial stages of fermentation. The use of PTR-ToF-MS is an effective way for brewers to control the progression of fermentation and allow for real-time manipulation to optimise the final product.

The concentration of isoamyl alcohol (m/z 71.086), phenylethyl alcohol (m/z 105.069) and isobutyl alcohol (m/z 58.074) throughout the fermentation of beer produced by *S. cerevisiae* strains SafAle US-05 and SafAle WB-06 and *S. pastorianus* strains SafLager S-23 and SafLager W-34/70 is displayed in Figure 5. Previous literature shows that the most abundant isobutyl alcohol peak is m/z 57; however, as this signal was saturated the isotopologue m/z 58.0735 was evaluated as a proxy.

A significant difference in the concentration of isoamyl and phenylethyl alcohol was detected in the beer produced by *S. cerevisiae* and *S. pastorianus* yeast at the start of fermentation (over the first 2 days). The concentration was initially higher in the SafAle WB-06 *S. cerevisiae* var. *diastaticus* samples. The production of higher alcohols during fermentation follows amino acid deamination and decarboxylation, known as the Ehrlich Pathway.²¹ Specifically, isoamyl alcohol is formed from the decarboxylation and reduction of α -ketoisocaproate. The synthesis of α -ketoisocaproate occurs primarily from glucose and secondly from the deamination of L-Leucine.⁶⁶ Feedback inhibition of the enzyme α -isopropylmalate synthase occurs in the presence of high L-Leucine concentration, which is accompanied by a decrease in the synthesis of isoamyl alcohol.^{67,68} As isoamyl acetate is formed from isoamyl alcohol, the increase in the concentration of isoamyl alcohol throughout fermentation is expected and displayed in the results of this study.⁶⁹

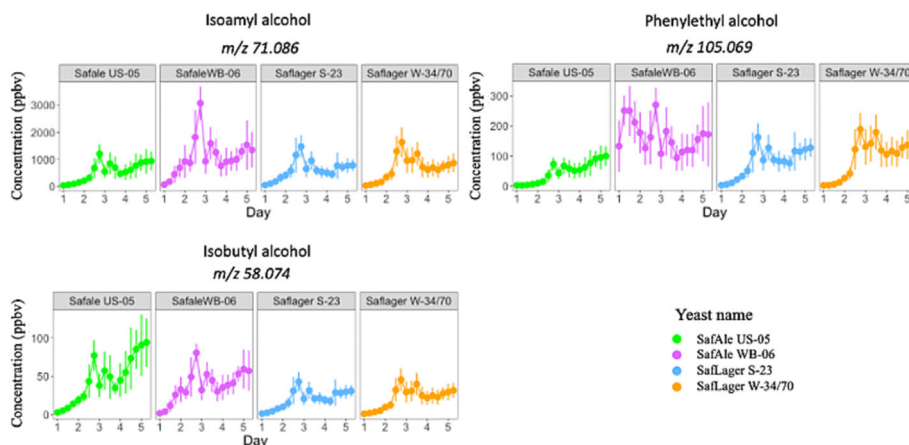
At the beginning of fermentation (first 2 days) and towards the end of the fermentation (half of Day 4 and all of Day 5), a significant

difference in the concentration of isobutyl alcohol between yeast strains was detected. The highest concentration at both these time points was present in the beer produced by *S. cerevisiae* strain SafAle US-05. In contrast, the lowest abundance was produced by *S. pastorianus* strain SafLager S-23 and SafLager W-34/70. Dack et al also identified that yeast strain affected the concentration of higher alcohols.¹⁶ Interestingly, lower levels of higher alcohols and esters were produced with *S. cerevisiae* strain A01 compared with S288c and L04. However, double the concentration of higher alcohols and 20 times the concentration of esters were detected with *S. cerevisiae* strain S288c when compared with *S. pastorianus* strain L04.¹⁶ The work displayed the need to gain a greater understanding of the different levels of higher alcohols and esters produced throughout fermentation by different yeast strains. Thus, implementing PTR-ToF-MS measurements could help reduce research and development (R&D) costs for breweries through a more thorough understanding of the importance of yeast selection.

3.4 | Comparison between PTR-ToF-MS and SPME/GC-MS to monitor the formation of compounds throughout beer fermentation

An advantage of PTR-ToF-MS is the ability to monitor the formation of compounds throughout fermentation, where the time required for measurements is considerably less than other methods such as GC-MS (3 min vs 60 min per sample). This enables more experimental replicates, thereby increasing reliability.⁷⁰ The GC-MS results of higher alcohols and esters produced by *S. cerevisiae* strains SafAle US-05 and SafAle WB-06 and *S. pastorianus* strains SafLager S-23 and SafLager W-34/70 are displayed in Figures 6 and 7. Because of the time required for measurement, each sample had only two independent measurements. A similar trend was observed when comparing the results from PTR-ToF-MS with GC-MS, which increased the confidence of compound identification. Between the two replicates, there was a greater variation than what was observed with PTR-ToF-MS. Therefore, PTR-ToF-MS enables the ability to rapidly measure multiple samples and obtain comparable results to established techniques

FIGURE 5 Mean concentration (ppbv) and standard deviation of higher alcohols: isoamyl alcohol (m/z 71.086), phenylethyl alcohol (m/z 147.127) and isobutyl alcohol (m/z 58.074) during fermentation by commercially available yeast: *Saccharomyces cerevisiae* strain SafAle US-05, *S. cerevisiae* var. *diastaticus* strain SafAle WB-06 and *S. pastorianus* strains SafLager S-23 and SafLager W-34/70. Data presented as mean \pm standard deviation of seven independent measurements measured with proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS).



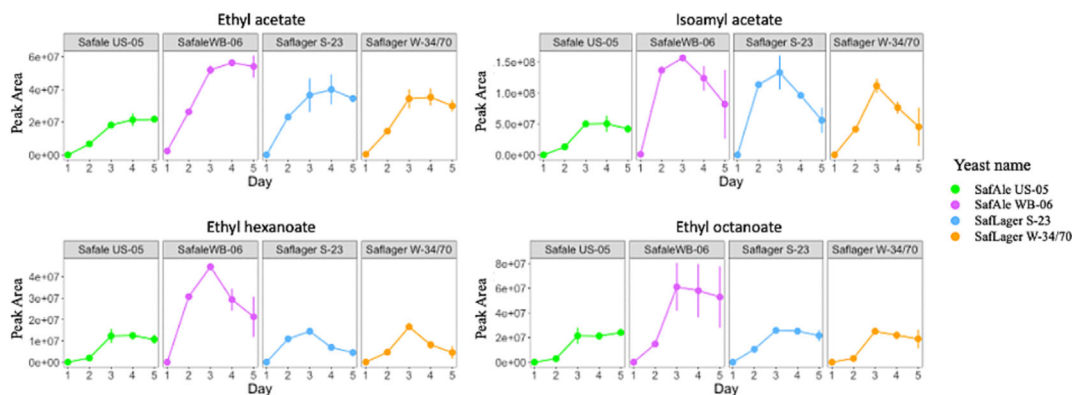


FIGURE 6 Mean peak area and standard deviation of esters: ethyl acetate, isoamyl acetate, ethyl hexanoate and ethyl octanoate during fermentation by commercially available yeast: *Saccharomyces cerevisiae* strain SafAle US-05, *S. cerevisiae* var *diastaticus* strain SafAle WB-06 and *S. pastorianus* strains SafLager S-23 and SafLager W-34/70 measured with solid phase microextraction–gas chromatography–mass spectrometry (SPME GC–MS).

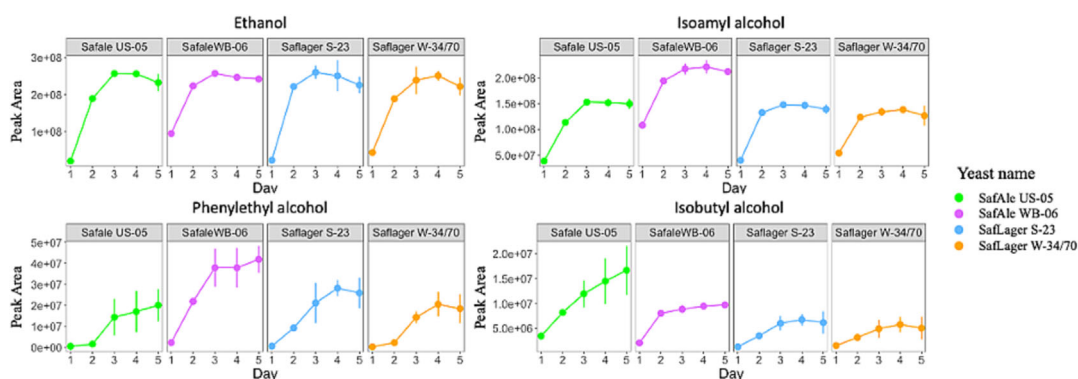


FIGURE 7 Mean peak area and standard deviation of ethanol and higher alcohols: isoamyl alcohol, phenylethyl alcohol and isobutyl alcohol during fermentation by commercially available yeast: *Saccharomyces cerevisiae* strain SafAle US-05, *S. cerevisiae* var *diastaticus* strain SafAle WB-06 and *S. pastorianus* strains SafLager S-23 and SafLager W-34/70 measured with solid phase microextraction–gas chromatography–mass spectrometry (SPME GC–MS).

like GC–MS. PTR–ToF–MS is reliable and highly sensitive, with a low detection limit (ppb_v) enabling small fluctuations during fermentation to be identified. These advantages illustrate why this method should be adopted as a gold standard to monitor VOC during fermentation for research. GC–MS is complementary to PTR–MS because it can support the identification of compounds and discrimination of isomers.

4 | CONCLUSION

The evolution of key higher alcohols and esters were measured during the beer fermentation by *S. cerevisiae* and *S. pastorianus* with PTR–ToF–MS and headspace SPME/GC–MS. The ability of yeast to form higher alcohols and esters during fermentation has previously been identified; however, this is the first study to monitor the changes in real-time using PTR–ToF–MS. The use of PTR–ToF–MS enabled yeast strain dependent differences to be identified. The implementation of

two separate analytical techniques allowed for the identification of compounds (GC–MS), online monitoring and quantitative determination (PTR–ToF–MS). The results from this study align with literature as lower concentrations of esters and higher alcohols were detected by *S. pastorianus* (SafLager S-23 and W-34-70) compared with *S. cerevisiae* var. *diastaticus* (SafAle WB-06). Strain dependent differences during fermentation were also observed as *S. cerevisiae* var *diastaticus* (SafAle WB-06) produced higher concentrations than *S. cerevisiae* (SafAle US-05). Future work should monitor other VOCs with PTR–ToF–MS and SPME/GC–MS to improve the current understanding of the biotransformations that may occur during beer fermentation.

AUTHOR CONTRIBUTIONS

Rebecca Roberts: Methodology; investigation; formal analysis; data curation; writing—original draft. **Iuliia Khomenko:** Methodology; formal analysis; data curation; writing—review and editing. **Graham T. Eyres:** Conceptualization; methodology; writing—review and

editing; supervision. **Phil Bremer:** Conceptualization; methodology; writing—review and editing; supervision. **Patrick Silcock:** Conceptualization; methodology; writing—review and editing; supervision. **Emanuela Betta:** Formal analysis; data curation. **Franco Biasioli:** Conceptualization; methodology; writing—review and editing; supervision. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

DATA AVAILABILITY STATEMENT

Data available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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