ANALYSIS OF POLYFUNCTIONAL THIOLS IN BEER

Developing a novel method of extraction and analysis using GC-FID

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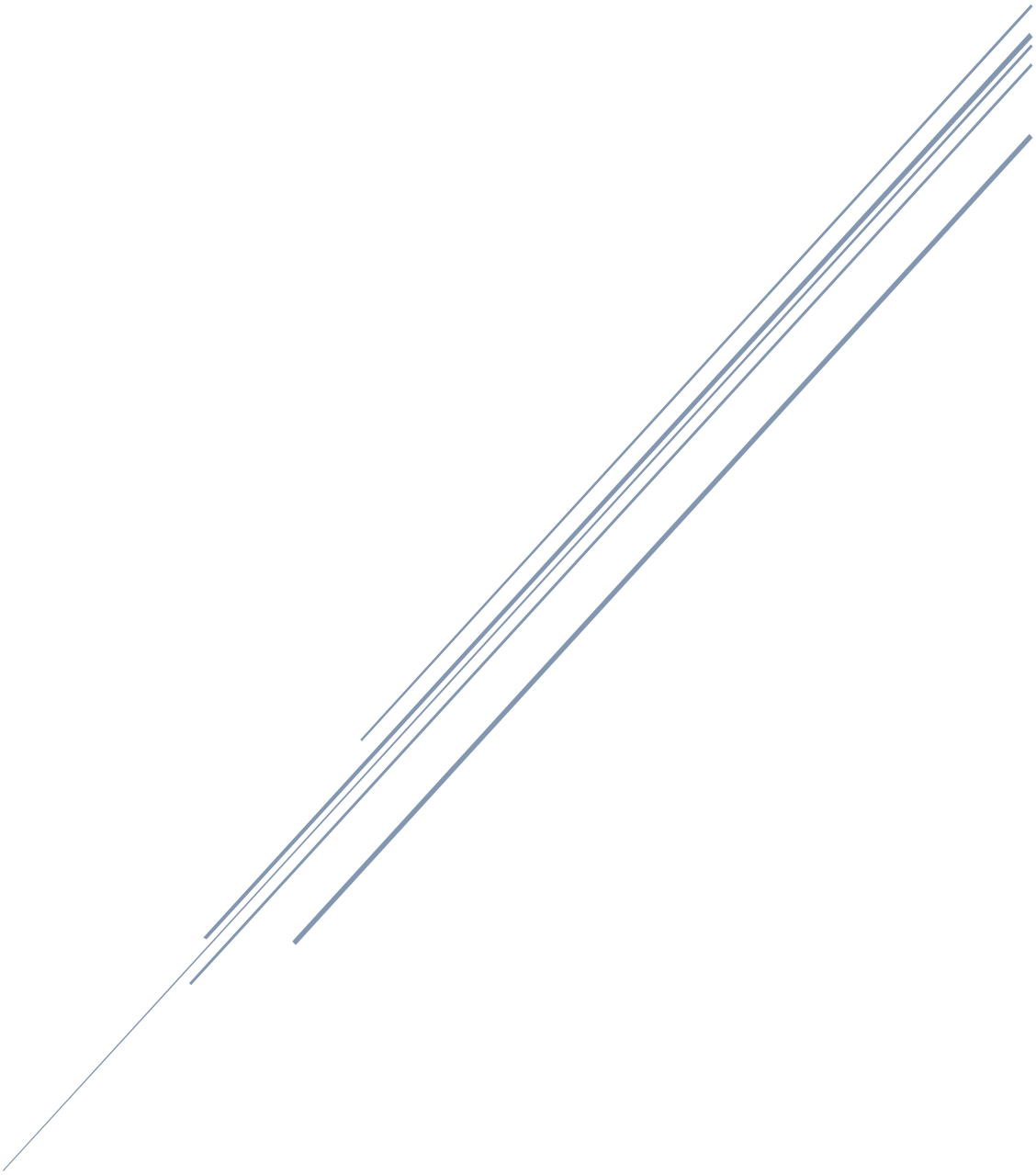

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Abstract

In this project, attempts were made to identify and quantify two polyfunctional thiols – 4-mercapto-4-methyl-pentanone and 3-mercaptohexyl acetate – and three monoterpene alcohols – linalool, geraniol, and β-citronellol – in beer samples. In order to avoid the use of hazardous compounds such as dichloromethane and hydroxymercuriobenzoate, common in traditional methods, two novel techniques were tested: a pure liquid-liquid extraction of the beer samples with cyclohexane, and a liquid-liquid extraction with cyclohexane followed by solid phase extraction on silver ion columns. Analyses were then performed with GC-FID. Evaluation of the methods showed both to be insufficiently sensitive to measure the low concentrations of thiols in the beer samples. The complexity of the beer matrix also proved cumbersome, as the analyte peaks of the more abundant monoterpene alcohols in the chromatograms were often blotted out by non-analyte peaks. To improve this method towards thiol analysis, it is recommended to further enrich the extracts, such as by allowing the volatile solvent to evaporate and thereby increase the concentration of analyte in the remaining solvent, or to use a different detector such as a mass spectrometer or a sulfur chemiluminescence detector. To improve the methods towards monoterpene alcohols, it is recommended to use a column that more specifically retains the compounds of interest or use a slower temperature programme to improve resolution between peaks.

Detta projekt har syftat till att identifiera och kvantifiera två polyfunktionella tioler – 4-merkapto-4-metyl-pentanon och 3-merkaptohexylacetat – och tre monoterpenalkoholer – linalool, geraniol och β-citronellol – i öl. För att undvika de hälsofarliga ämnena diklormetan och hydroxymerkuriobensoat, som är vanligt förekommande i traditionella metoder, testades två nya metoder: en ren vätske-vätskeextraktion av ölproverna med cyklohexan, och en vätske-vätskeextraktion med cyklohexan följt av en fastfasextraktion på silverjonkolonner. Analyser gjordes därefter med GC-FID. Utvärdering av metoderna visade att ingen av dem var tillräckligt känslig för att kunna detektera de låga tiolhalterna i ölproverna. Ölmatrisens komplexitet visade sig också vara ett problem, då analyttopparna för de mer rikligt förekommande monoterpenalkoholerna i kromatogramen ofta överskuggades av toppar för icke-analyter från ölen. För att förbättra metoden gentemot tiolanalys rekommenderas det att extrakten anrikas ytterligare, till exempel genom att indunsta proverna och därigenom öka koncentrationen av analyt, eller genom att använda en annan detektor såsom masspektrometer eller svavelkemiluminescensdetektor. För att förbättra metoden gentemot monoterpenalkoholer rekommenderas det att använda en kolonn som retenterar de intressanta ämnena bättre, eller använda ett långsammare temperaturprogram för att därigenom öka upplösningen i kromatogramet.

Analysing flavour-potent thiols in beer – what makes a beer smell like passion fruit?

Understanding the flavour chemistry of beer is one of the most effective ways of improving it. In this study, attempts were made to analyse the content of polyfunctional thiols in beers treated with products intended to increase the content of thiols. The analyses were performed using two novel methods, developed to avoid the hazardous chemicals used in traditional analysis.

Over the last decade, the popularity of hazy beers with fruity aroma and low bitterness has spiked massively. As a consequence of this much effort has been put into understanding the key to producing these delicate beer styles, and by applying methods and previous findings from the wine industry to beer and hops, scientists have been able to isolate so-called polyfunctional thiols as an explanatory factor. Thanks to their incredibly low detection threshold, some of these substances – although only present in nanogram per litre levels – give beer or wine a pleasant smell of tropical fruit, box tree or black currant. In order to evaluate whether a process, a product, or a hop variety contributes to the overall thiol content in a beer, a reliable method of analysis is required. This is challenging as the concentration of thiols is so low, and traditional methods have had to rely on using hazardous compounds to achieve good results. In this study, beers treated with products meant to increase their thiol content were analysed using two novel methods involving less hazardous chemicals. The thiols were extracted using either cyclohexane – a relatively common organic solvent popular for its low toxicity – or using solid phase extraction on silver ions – chosen because silver ions have a similar affinity to thiols as do the more traditionally used mercury compounds but are much less toxic.

After extraction the extracts were analysed in a gas chromatograph, which separates volatile compounds based on their volatility and their affinity to the column on which the separation is performed. As the compounds leave the gas chromatograph at different times, they are detected in a flame ionization detector, and an amount of each substance can be determined.

Unfortunately for this study, the results proved to be inconclusive: while some hop compounds, such as the common monoterpene alcohol linalool, could be detected in many of the studied samples, no thiols were reliably detected. It is likely that this is because of the low concentration in the beer samples, and thus the method would need to be revised to include some concentrating step in order to be effective. This could be done by including an evaporation step, where a large amount of the solvent is evaporated and the concentration of analyte is thus increased, however this would risk losing or altering some of the analytes, which would require further evaluation.

Preface

The following is a report on the master's thesis project performed by me, Arvid Lindmark, for a Master of Science in Engineering in Biotechnology degree. The project was conducted at the Faculty of Engineering at Lund University between January and May of 2022.

Many people have helped me greatly in realizing this project, and I would like to especially thank Olexandr Fedkiv and Hans Bolinsson of the Department of Food Technology, Engineering and Nutrition, Peter Spégel, Margareta Sandahl and Mynta Norberg of the Centre for Analysis and Synthesis, and Herje Schagerlöf and Mats Galbe of the Department of Chemical Engineering – for lending me their time, their lab space, and their expertise. I would also like to thank my supervisor, Lars Nilsson, who has helped me navigate the shifting fortunes of research, and my examiner, Karolina Östbring, for helping me improve my work.

Furthermore, I am grateful to all those whose names I cannot mention for reasons of confidentiality – you know who you are. Lastly, to all those friends and family members who've helped me in a myriad of different ways, without who's love and support I would not be where I am today – I am ever in your debt.

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Introduction

Beers displaying fruity, tropical aroma and flavour have gained massively in popularity over the last decade. The exact cause of these flavour is, as with most foods, a combination of a vast number of compounds which work in unison to produce the sought-after effect. However, one group of compounds can be singled out as being a major part of the fruity aroma in beer: volatile, polyfunctional thiols. While low molecular weight thiols such as hydrogen sulfide and methanethiol may impact the flavour and aroma of a product negatively, larger molecules such as 3-mercaptohexanol (3MHA), 3-mercaptohexyl acetate (3MHA), and 4-mercapto-4-methylpentanone (4MMP) will impart often desired aromas of for example grapefruit, black currant, and passion fruit (Bonnaffoux, et al., 2021). Whilst these compounds are present in their free form in hops – which are used in the brewing process – recent studies have shown that there is an abundance of bound 3MH and 4MMP in both hops and malt. These bound precursors are not flavour-active and can make up as much as 99% of the total thiol content (Roland, et al., 2016), which means that a more efficient release of thiols from their precursor form can lead to decreased material costs for the brewer, fewer transports for the environment, and more flavourful beer for consumer.

In this paper, it is examined whether it is possible to detect and quantify thiols using a novel method of extraction and analysis, and whether two different products can influence the levels of total free 3MHA and 4MMP in beer. If these products can aid in releasing the compounds of interest from their precursor form, they will indeed be a powerful addition to the brewers' arsenal in crafting the best product possible.

Objective

The objective of this study is three-fold: the primary objective is to investigate whether two nutrient blends, herein referred to as Products X and Y due to confidentiality, can enhance the release of 3MHA and 4MMP in beer by stimulating the enzymatic activity of the yeast. However, as analysis of thiols is a challenging task due to their low concentration in beer and relative instability, the first objective of the study will be to develop and evaluate a novel method for extraction and analysis of thiols from the beer matrix. The second objective is thus to determine, using the developed method, whether products X and Y can impact the total concentration of free thiols in beer. The third objective, to give a more detailed picture of the composition of the studied beer, is to determine whether products X and Y influence the content of linalool, geraniol and β-citronellol in beer. This is done partly because these monoterpene alcohols may have a sensory impact that works in synergy with that of the studied thiols, and thus a more complete understanding of the chemical composition may help inform further sensory analysis, but also to study whether the products used can impact the yeast's biotransformation of geraniol to β-citronellol.

A short note on nomenclature

According to IUPAC, the herein referenced thiols should be referred to as 3-sulfanylhexanol, 3-sulfanylhexyl acetate and 4-sulfanyl-4-methylpentanone, as the use of the "mercapto-" suffix is no longer advised (Hellwich, et al., 2020). However, as much of the available literature still refer to these compounds using their former name (and corresponding abbreviations), this paper will do the same and refer to the compounds of interest as 3MH, 3MHA, and 4MMP respectively.

Background

The brewing process

The brewing of beer consists of three principal steps: mashing, boiling, and fermenting. During the mashing step, crushed malt (germinated cereal grains, almost exclusively barley but other germinated grains such as wheat or rye can also be used) is mixed with warm water to activate the endogenous enzymes of the malt and initiate the enzymatic breakdown of predominantly starch and proteins. In this step, other starch-rich adjuncts such as maize or flaked wheat can also be added. These non-enzymatic adjuncts benefit from the enzymatic power of the malt and thus have their starches and proteins degraded as well.

Once the mash is completed, the liquid, which is now called wort, is separated from the grains. The wort is now rich in dissolved sugars, proteins, and other compounds from the malt which will later serve as nutrients for the yeast during fermentation. Once all liquid has been separated from the grains, the grains are sparged with more hot water to achieve maximum extraction of sugars, and the combined liquid is then boiled. Hops, the flower of the hop plant *Humulus lupulus*, are added to the wort during the boil for flavour, aroma, and bitterness. The exact timing of the hop addition(s) varies depending on the sought-after effect: adding the hops early during the boil will give a high bitterness, as the very bitter α-acids in the hops are isomerised and dissolved as a function of time and temperature. Adding the hops later in the boil however will lead to a more aromatic final product, as the more volatile components of the hops are evaporated during long boiling times.

After boiling, the wort is cooled and transferred to a fermentation vessel. Here, yeast is added, and fermentation occurs, usually for about 5-14 days until all available sugar is gone and the fermentation is deemed "completed". During this period, hops can also be added directly to the fermenter in a process known as dry hopping. Analogously to the hop additions during the boil, this will not result in much bitterness, but the beer will retain the volatile components of the hops much better.

Once fermentation and dry hopping is done, the beer is carbonated, packaged, and shipped to consumers.

Occurrence of thiols and their sensory impact

Thiols are present in many foods and have gained interest due to their generally very low odour threshold. The aromas of these thiols are very diverse, ranging from aromas of decay and putrefaction from simpler thiols such as hydrogen sulfide or methanethiol to the more pleasant aromas of box tree or passion fruit displayed by the compounds relevant in this study (Rodriguez-Bencomo, et al., 2009). These compounds have been studied in a number of food matrixes such as coffee, fruit juices or Chinese *baijiu* (Bonnaffoux, et al., 2021) (Song, et al., 2021). However, most of the relevant findings for application in beer has been done in the field of oenology, as these thiols are an important part of the aroma of many wines. It is in this field that much of the studies regarding the precursors of the free thiols, which will be discussed more in depth in the next segment, have been done (Bonnaffoux, et al., 2017). The issue when

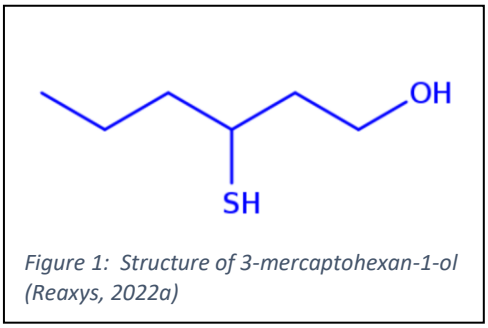

Figure 1: Structure of 3-mercaptohexan-1-ol (Reaxys, 2022a)

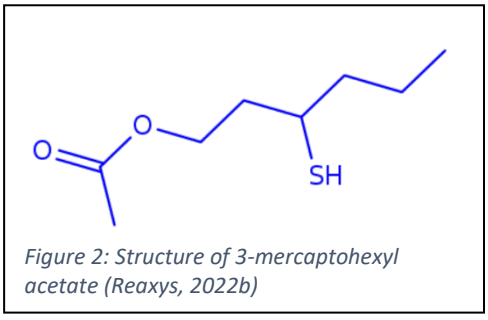

Figure 2: Structure of 3-mercaptohexyl acetate (Reaxys, 2022b)

analysing thiols is their relatively low abundance. In a study performed by Takazumi et al. (2017) analysing thiols in beers it was found that the concentration of 3MH was on average 112.5 ng/L, 4MMP on average 9.25 ng/L (although there were only detectable amounts in 60% of the samples), and 3MHA was not detectable in any of the samples. In another study (Gros, et al., 2012), 3MH was found to occur in the studied beers at a concentration of 135.75 ng/L on average, whereas 4MMP was not quantifiable in any of the samples (and 3MHA was not measured).

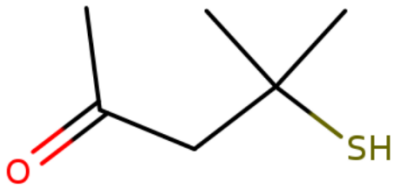

Figure 3: Structure of 4-mercapto-4-methylpentan-2-one (Reaxys, 2022c)

The concept of "bound thiols"

It has long been observed that highly aromatic wines can be made from less aromatic grapes, which has led scientists to theorise that some step in the production of wine causes the release or creation of aroma-active compounds. Apart from yeast producing esters and carboxylic acids, it has been noticed that many grape varieties contain the thiols 3MH and 4MMP in a conjugated form, where the sulphur atom of the thiol is conjugated with the sulphurous amino acid cysteine. This has been observed in a number of constellations, such as pure cysteine *S*-conjugate, glutathione *S*-conjugate and S-3-(hexan-1-ol)-γ-glutamyl-cysteine (Bonnaffoux, et al., 2017). During fermentation, these thiols are cleaved from the precursor form by β-lyase activity displayed by yeast. While this is true for fermentations using only *Saccharomyces cerevisiae*, some studies have shown that the effect can be enhanced by using other yeasts – such as *Pichia kluyveri* or *Saccharomyces bayanus* – together with *S cerevisiae,* or by using a hybrid *S cerevisiae x S bayanus* strain (Anfang, et al., 2009) (Masneuf-Pomarède, et al., 2006).

While this is of interest to wine producers, the connection to beer brewing might seem farfetched. This connection is made clearer when we learn that recent studies have shown that hops show many of the same precursors as wine, and in even greater quantities (Roland, et al., 2016). Interestingly, the study by Roland et al. also shows that an astonishing quantity of the total thiols present in the studied hop samples are present in their conjugated form. For 3MH, the percentage of free vs bound species (expressed as 3MH equivalents) ranged from 0 to 0.7%, while for total thiols studied (3MH and 4MMP) the average was 0.04%.

Monoterpene alcohols

Among the many studied odour active compounds in hops are the monoterpene alcohols. These include five substances: linalool, geraniol, β-citronellol, nerol, and α-terpineol. Out of these, linalool, geraniol, and β-citronellol are often regarded as having the largest sensory impact on beer as they generally occur in higher concentrations relative to their flavour threshold (Takoi, et al., 2010a). These compounds are very similar in structure, and some can be created from others by biotransformative action of the yeast during fermentation, as shown in figure 4 below.

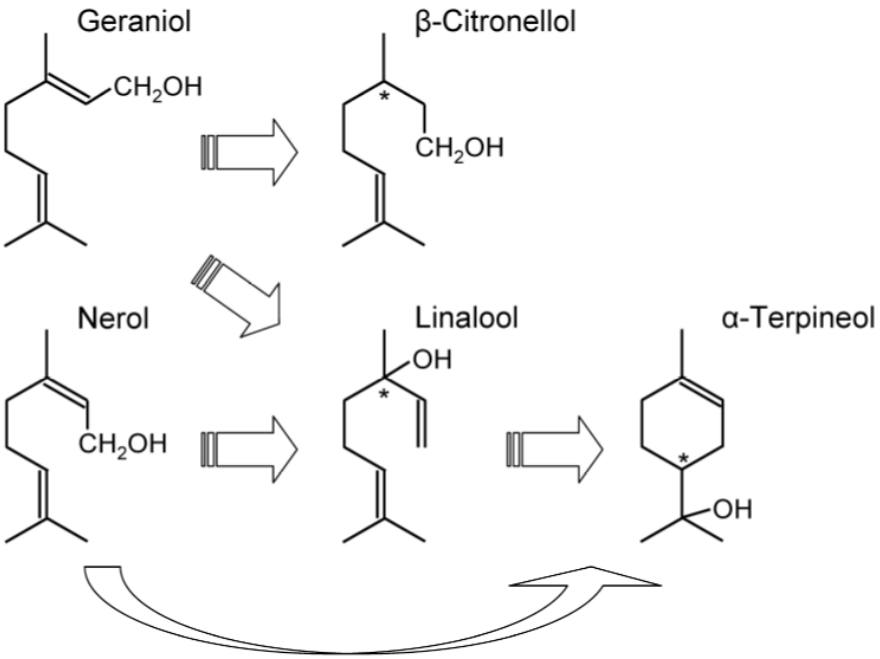

Figure 4: Schematic representation of the metabolism cascades of monoterpene alcohols by S. cerevisiae *and* S. pastorianus *(Takoi, et al., 2010b), asterisks indicate chiral centra.*

Furthermore linalool, geraniol, and β-citronellol have been shown to have a synergistic sensory effect, with as little as 5 µg/litre each of geraniol and β-citronellol having a significant impact on the overall sensory characteristic (Takoi, et al., 2010a). As Takoi et al. have also shown β-citronellol to be virtually absent from wort prior to fermentation, it can be assumed that the biotransformation of geraniol to β-citronellol has a substantial impact on the sensory characteristics of the finished beer.

The concentrations of linalool, geraniol, and β-citronellol vary considerably depending on what amount and type of hops are used for brewing, but in the study by Takoi et al. (2010a) the values range from approximately 2 µg/L to 100 µg/L, with a mean of 23 µg/L. Of the three monoterpene alcohols of interest, linalool is the most abundant with a mean concentration of 55 µg/L, followed by geraniol and β-citronellol, both at 7 µg/L. In another study by Takoi et al. (2010b), beers were hopped with the Citra cultivar at various concentrations (0.4 - 0.8 g/L at 5 minutes left of the boil), and the concentration of linalool was determined to be on average 58 µg/L, geraniol 15 µg/L, and β-citronellol 18 µg/L. Lastly, in a third study (Takoi, et al., 2014), beers were hopped at different times with three different hop cultivars, and the average concentrations were determined. For linalool, the average concentration was 65 µg/L, for geraniol 84 µg/L, and for β-citronellol 32 µg/L. However, it should be remembered that these studies, particularly the 2014 one, are aimed at maximising the content of geraniol to increase the content of β-citronellol through biotransformation, thus the values may be higher than normally expected.

Extraction and analysis

Previous methods to extract thiols have relied on quite hazardous chemicals, primarily hydroximercuribenzoic acid and/or dichloromethane (Vermeulen, et al., 2006) (Takazumi, et al., 2017). While these have shown to be effective in the extraction, they come with serious risks such as carcinogenicity, acute toxicity and high toxicity to aquatic life with long lasting effects (Sigma-Aldrich, 2021a) (Sigma-Aldrich, 2021b). As the studied compounds are quite hydrophobic (Reaxys, 2022b) (Reaxys, 2022a) (Reaxys, 2022c) this study will instead attempt to extract the thiols using the organic solvent cyclohexane which is significantly less hazardous to work with. Two extraction methods are to be attempted: one using only liquid-liquid extraction with

cyclohexane on the beer matrix, and one using solid phase extraction on a silver ion column, adapted from the method described by Takazumi et al. (2017).

Methods

Materials

Samples of 3MHA, linalool, geraniol, β-citronellol, 2-mercapto-3-butanol and heptanol, Discovery® Ag Ion SPE tubes, and thioglycerol were purchased from Merck. A sample of 4MMP was obtained from Fischer Scientific. Cyclohexane and acetonitrile were obtained from VWR Chemicals, and ethanol from Solveco.

Unfortunately, samples of 3MH could not be acquired due to shipping issues and the limited timeframe of the project, and the analysis has therefore focused completely on the thiols 4MMP and 3MHA.

The beer samples

The beer used for the analysis was brewed using 79.0% pale ale malt and 10.5% each of oat malt and wheat malt, hopped with Saaz hops at 60 and 0 minutes left of the boil, and fermented with an ale yeast to a final alcoholic strength of approximately 5.94% (standard deviation 0.07%, likely caused by differences in boil off rate and yeast performance). The exact addition times of Products X and Y are not disclosed, but the beers are divided into Control (having received no product), 1 (having received a single dose of product at time 1), and 1+2 (having received one dose of product at time 1, and another dose at time 2). The beers were packaged carbonated in PET bottles and shipped to Lund University, where they were stored cold while waiting for analysis.

Gas chromatography

Analysis was performed on an Agilent Technologies 7890B gas chromatograph equipped with an Agilent 19095F-123 fused silica capillary column (30 m×530 µm×1 µm) and a flame ionization detector under a nitrogen flow. Samples were injected in splitless mode at an injection volume of 1.5 µL. The oven temperature was held at 65˚C for 3 minutes, increased at a rate of 5˚C/min to 240˚C and then held for 15 minutes before terminating the cycle.

Calibration and identification

Peak identifications were made by diluting the standard samples and the two internal standards to correspond to a concentration of approximately 15.5×10^6 ng/L for the thiols and 340×10^6 ng/L for the monoterpene alcohols and heptanol. These were then run individually through the gas chromatograph at the pre-defined temperature programme and the retention time of each substance was noted.

To quantify the amount of analyte in the samples as well as establishing the limits of quantification and limit of detection of the method (defined as corresponding to a signal-to-noise ratio of 10 and 3 respectively), a 10-point calibration curve was prepared and run through the gas chromatograph, with concentrations as depicted in table 1.

Table 1: showing the concentrations used in the calibration curves

Calibration level	Concentration (ng/L)						
	3MH	4MMP	2M3B	Linalool	Geraniol	Citronellol	Heptanol
1	1.000×10^6	1.000×10^6	1.000×10^6	1.000×10^6	1.000×10^6	1.000×10^6	1.000×10^6
2	0.500×10^6	0.500×10^6	0.500×10^6	0.500×10^6	0.500×10^6	0.500×10^6	0.500×10^6
3	0.250×10^6	0.250×10^6	0.250×10^6	0.270×10^6	0.270×10^6	0.270×10^6	0.270×10^6
4	0.125×10^6	0.125×10^6	0.125×10^6	0.250×10^6	0.250×10^6	0.250×10^6	0.250×10^6
5	62.50×10^3	62.50×10^3	62.50×10^3	0.125×10^6	0.125×10^6	0.125×10^6	0.125×10^6
6	8100	8100	8100	90.00×10^3	90.00×10^3	90.00×10^3	90.00×10^3

7	2700	2700	2700	62.50×10^3	62.50×10^3	62.50×10^3	62.50×10^3
8	900.0	900.0	900.0	30.00×10^3	30.00×10^3	30.00×10^3	30.00×10^3
9	300.0	300.0	300.0	10.00×10^3	10.00×10^3	10.00×10^3	10.00×10^3
10	100.0	100.0	100.0	3333	3333	3333	3333

Extraction evaluation

To evaluate the extraction efficiency a model beer solution was also made by mixing water and ethanol to achieve a final concentration of 6 v%, and then spiking the solution with the standard solutions to achieve a final concentration of 100 ppt of each thiol and 30 000 ng/L of the monoterpene alcohols. This beer was then extracted using the two methods described below, and the two samples were analysed in the gas chromatograph.

A sample of water spiked with heptanol to a final concentration of 30 000 ng/L was also extracted using the liquid extraction method described below.

Sample preparation

The liquid extraction samples were prepared by mixing 35 mL of beer sample, 10 mL of cyclohexane and 10 g of NaCl in a centrifuge tube. These tubes were shaken for 15 minutes and thereafter centrifuged for 15 minutes at 1800 *g*. The organic phases were removed with a pipette and analysed in the gas chromatograph.

Solid phase extraction (SPE) samples were prepared by mixing 20 mL of beer with 20 mL of cyclohexan and 6 g of NaCl in a centrifuge tube. These tubes were shaken for 15 minutes and thereafter centrifuged for 15 minutes at 1800 *g*. The organic phase (~20 mL) was obtained and then applied to Discovery® Ag Ion SPE tubes – conditioned with 6 mL of cyclohexane – run over a Supelco Visiprep™ 12 at -20 kPA. The SPE tubes were then rinsed, first with 10 mL of cyclohexane and then with 20 mL of acetonitrile. Then the tubes were eluted with 6 mL of 1v% thioglycerol dissolved in cyclohexane. The eluate was then mixed with 30 mL of water and 6 g of salt, shaken for 15 minutes, and then centrifuged at 1800 *g* for 15 minutes. The organic phase was then obtained and analysed in the gas chromatograph.

A third sample preparation was also performed, where 35 mL of beer was first spiked with heptanol to reach a concentration of 30 000 ng/L, and then mixed with 5 mL of cyclohexane and 10 g of NaCl, shaken for 15 minutes and centrifuged at 1800 *g* for 15 minutes. The organic phase was then obtained with a pipette and analysed.

Statistics

All liquid-liquid extractions, except for the extraction evaluation sample, were performed in triplicates. Mean values and standard variations were calculated using Microsoft Excel. Single factor Anova analyses were also performed in Microsoft Excel to check for significant differences in the measured concentrations between samples.

To calculate the standard deviation after a number of operations, two formulas for propagation of uncertainty are used – one for additions and one for division and multiplication. For addition, the formula for propagation of uncertainty is:

$$STD(y) = \sqrt{STD(x_1)^2 + STD(x_2)^2 + \cdots + STD(x_n)^2}$$

where *STD(y)* is the standard deviation of the total sum and $STD(x_1)$ to $STD(x_n)$ is the standard deviation of each operation. To calculate the standard deviations when two quantities with associated standard deviations are multiplied or divided, the following formula is used:

$$STD(y) = \sqrt{y^2 * \left(\frac{STD(x_1)^2}{{x_1}^2} + \frac{STD(x_2)^2}{{x_2}^2}\right)}$$

In this formula, y is the result of the division or multiplication, x_1 and x_2 are the ingoing values, and STD indicates the standard deviation of each quantity accordingly.
To calculate the standard deviation presented in table 3, the standard deviation in each operation – i.e. each measurement for diluting, mixing etc. of the final sample – was calculated in sequence using the two formulas above. When further calculating the standard deviation of the extraction factor, which is done by dividing the measured concentration (with no standard deviation) by the actual sample concentration (with a known standard deviation), the standard deviation is given by assuming the relative standard deviation to be the same throughout the operation, i.e.:

$$STD(c * x_1) = STD(x_1) * c$$

Results

Calibration and identification

All compounds except 2-mercapto-3-butanol (2M3B) were identified using the standard solutions described in the method. All identified compounds had a retention time between 13.132 minutes (4MMP) and 23.630 minutes (geraniol). It was also discovered that 2M3B emerged from the column simultaneously with the solvent plug, and it was therefore excluded from further analysis. After evaluating the results from the calibration solutions, it was decided to only include calibration levels 1 to 5 in the actual calibration, as the background noise and low concentrations in calibration levels 6 to 10 made the results quite unreliable. The calibration curves used, along with their curve fitting equations, can be found in the appendix. The curves achieved good linearity with R^2-values ranging from 0.966 to 0.999. The limit of detection (LOD) and limit of quantitation (LOQ) of all substances (defined as a signal-to-noise ratio of 3 and 10 respectively) are presented in table 2, calculated from a linear regression of the S/N ratios plotted against concentration.

Table 2: Estimated limits of detections and quantification of the different analytes

Substance	LOD [ng/L]	LOQ [ng/L]	R^2
3MHA	91 000	230 000	0.981
4MMP	390 000	1 400 000	0.987
Linalool	33 000	110 000	0.999
Geraniol	27 000	200 000	0.999
Citronellol	23 000	93 000	1.000
Heptanol	45 000	110 000	0.995

Extraction evaluation

Liquid-liquid extraction

In the spiked sample extracted only with the liquid-liquid extraction technique, only three substances could be identified: 3MHA, linalool and geraniol. Their concentrations, original concentrations in the sample and calculated extraction coefficients are presented in table 3 below.

Table 3: Measured concentrations and calculated extraction coefficients of the liquid-liquid extraction evaluation. Figures in italics indicate a measurement with a signal-to-noise ratio between 3 and 10.

Substance	Measured concentration in extract [ng/L]	Concentration in original sample [ng/L]	Extraction coefficient
3MHA	*160 000*	100 ± 12	1600 ± 190
Linalool	270 000	30 000 ± 104	9.0 ± 0.03
Geraniol	420 000	30 000 ± 106	14 ± 0.05

Solid phase extraction

In the spiked sample extracted using the solid phase extraction technique described in the method, only 3MHA could be identified. However, the signal-to-noise ratio of this peak was 2.67, i.e. below the LOD, meaning that it cannot with certainty be said to be an actual analyte peak and not simply baseline noise. Therefore, it is discarded from the results, and thus no results were obtained from the solid phase extraction evaluation.

Internal standard blank

The water sample spiked with heptanol and extracted using the liquid extraction technique did not yield a peak corresponding to the previously determined retention time of heptanol. As a heptanol peak was very clearly observed in the spiked beer samples (as described below in *Internal Standard Samples* below), it can be assumed that this lack of results is due to some mishandling during preparation. Unfortunately, due to the time constraints, a second sample could not be analysed, and therefore the average concentration of heptanol in the internal standard samples was used to determine the extraction coefficient of heptanol from the beer matrix to the extract, as shown in table 4.

Table 4: Mean value and standard deviation of heptanol in the internal standard samples and calculated extraction coefficient.

Substance	Measured concentration in extract (average of 18 measurements) [ng/L]	Concentration in sample [ng/L]	Extraction coefficient
Heptanol	170 000 ± 59 000	30 000 ± 100	6 ± 2.0

Sample analysis

Liquid-liquid extraction samples

The results of the liquid extraction samples are presented in table 5 below.

Table 5: Results from the liquid-liquid extraction samples, presented as mean values with standard deviations. Numbers in italics indicate one measurement in the triplicate having a signal-to-noise ratio below 10, numbers in red indicate one measurement in the triplicate was not detected. If more than one measurement in the triplicate was not detected or detected but having a signal-to-noise ratio below 10, it reads as "Not detected" in the table.

Sample	3MHA [ng/L]	4MMP [ng/L]	Linalool [ng/L]	Geraniol [ng/L]	Citronellol [ng/L]	Heptanol [ng/L]
X 1	Not detected	Not detected	*180 000 ± 51 000*	Not detected	Not detected	Not detected
X 1+2	Not detected	Not detected	240 000 ± 35 000	Not detected	Not detected	Not detected
X Control	Not detected	Not detected	240 000 ± 17 000	Not detected	Not detected	Not detected
Y 1	Not detected	Not detected	*190 000 ± 87 000*	Not detected	Not detected	Not detected
Y 1+2	Not detected	Not detected	200 000 ± 25 000	Not detected	Not detected	Not detected
Y Control	Not detected	Not detected	190 000 ± 18 000	Not detected	Not detected	Not detected

For linalool, the only substance detected both in the majority of the liquid extraction samples and in the extraction sample, the original concentrations of the sample, based on the extraction coefficient calculated above, are presented in table 6 below.

Table 6: Calculated concentration of linalool in the beer samples, presented with mean value and standard deviation.

Compound	X 1 [ng/L]	X 1+2 [ng/L]	X Control [ng/L]	Y 1 [ng/L]	Y 1+2 [ng/L]	Y Control [ng/L]
Linalool	*30 000 ± 9000*	40000 ± 6000	40 000 ± 3000	*30 000 ± 15 000*	33 000 ± 4000	32 000 ± 3000

Solid phase extraction samples

The results of the solid phase extraction samples are presented in table 7 below.

Table 7: Results of the solid phase extraction samples. Where no peak could be observed, this is stated as "Not detected". When a peak is observed but the signal-to-noise ratio is below 3, this is stated as "Below LOD".

Sample	3MHA [ng/L]	4MMP [ng/L]	Linalool [ng/L]	Geraniol [ng/L]	Citronellol [ng/L]	Heptanol [ng/L]
X 1	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
X 1+2	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
X Control	Below LOD	Not detected	Not detected	Not detected	Not detected	Not detected
Y 1	Below LOD	Not detected	Not detected	Not detected	Not detected	Not detected
Y 1+2	Below LOD	Below LOD	Not detected	Not detected	Not detected	Not detected
Y Control	Below LOD	Not detected	Not detected	Not detected	Not detected	Not detected

Internal standard samples

The results of the internal standard liquid extracted samples, performed in triplicates, are presented in table 8 below.

Table 8: Results from the internal standard samples, presented as mean values with standard deviations. Numbers in italics indicate one measurement in the triplicate having a signal-to-noise ratio below 10, numbers in red indicate one measurement in the triplicate was not detected. If more than one measurement in the triplicate was not detected or detected but having a signal-to-noise ratio below 10, it reads as "Not detected" in the table.

Sample	3MHA [ng/L]	4MMP [ng/L]	Linalool [ng/L]	Geraniol [ng/L]	Citronellol [ng/L]	Heptanol [ng/L]
X 1	Not detected	Not detected	*220 000 ± 46 000*	Not detected	Not detected	290 000 ± 51 000
X 1+2	Not detected	Not detected	*150 000 ± 50 000*	Not detected	Not detected	170 000 ± 33 000
X Control	Not detected	Not detected	*140 000 ± 42 000*	Not detected	*14 900 ± 180*	200 000 ± 27 000
Y 1	Not detected	Not detected	190 000 ± 52 000	Not detected	*30 000 ± 12 000*	160 000 ± 54 000
Y 1+2	Not detected	Not detected	*200 000 ± 45 000*	Not detected	*30 000 ± 13 000*	170 000 ± 28 000

Y Control	Not detected	Not detected	200 000 ± 54 000	Not detected	*30 000 ± 12 000*	180 000 ± 33 000

Based on these results and the previously determined extraction coefficient, the original sample concentration of the analytes – corrected for sample-to-sample variation assuming that changes in extraction of heptanol applies equally to all analytes – can be calculated as shown in table 9 below. Unfortunately, as only linalool gave results for both the liquid extraction evaluation and the internal standard samples, the calculations could only be performed on linalool.

Table 9: Calculated concentrations of analytes in beer samples, presented as mean values with standard deviation.

Sample	Linalool extract concentration [ng/L]	Sample concentration corrected for extraction coefficient [ng/L]	Correction factor based on extraction of heptanol	Sample concentration corrected for heptanol extraction [ng/L]
X 1	*220 000 ± 46 000*	*24 000 ± 5000*	*1.6 ± 0.6*	*39 000 ± 17 000*
X 1+2	*150 000 ± 50 000*	*17 000 ± 6000*	*0.9 ± 0.4*	*16 000 ± 8000*
X Control	*140 000 ± 42 000*	16 000 ± 5000	1.1 ± 0.4	18 000 ± 8000
Y 1	190 000 ± 52 000	21 000 ± 6000	0.9 ± 0.4	19 000 ± 10 000
Y 1+2	*200 000 ± 45 000*	*22 000 ± 5000*	*0.9 ± 0.4*	*21 000 ± 10 000*
Y Control	200 000 ± 54 000	22 000 ± 6000	1 ± 0.4	22 000 ± 11 000

Statistics

Due to analytes not being detectable in all replicates, only linalool and heptanol could be compared conclusively with the Anova analysis. When including measurements with a signal-to-noise ratio between 3 and 10, β-citronellol could also be compared between samples. None of these Anova analyses, the results of which can be seen in table 10 below, indicated any significant difference in concentration of the analytes between data sets, and it can thus not be stated that any of the products X or Y, regardless of addition method, has any significant impact on the concentration of linalool or β-citronellol in the finished beer.

Table 10: Results of the performed Anova analyses.

Sample	Number of samples	F	F_{crit}
Linalool	5	0.511452	3.47805
	11	0.594812	2.296696
Heptanol	6	2.998256	3.105875
B-citronellol	5	0.043844	3.47805

During sample preparation, evaluation of the micropipettes used were performed regularly by pipetting and weighing a fixed amount of distilled water. From this, the standard deviation of each pipette used, for the specific volume pipetted or as close as possible to it, was calculated, and is presented in table 11 below.

Table 11: Showing the standard deviations of 2 different pipettes for a total of 4 different volumes.

Pipette	1000 µl pipette		200 µl pipette	
Volume	1000 µl	500 µl	200 µl	20 µl

Standard deviation (µl)	1.075	1.302	0.789	0.887

Discussion

As evidenced by the results above, the methods used in this project are far from ideal for detection and quantification of both thiols and monoterpene alcohols in a beer matrix. This could be due to a number of factors, which will be discussed further below.

Results evaluation

In the Results section above, all values apply to identified peaks with a signal-to-noise ratio of at least 3. The absence of results in many of the measurements does not however imply that the compound is not present, or not present in a significant amount, but might simply mean that another compound eclipses the peak of the analyte of interest. This is likely the case with geraniol for example, which gave very clear peaks and a high extraction coefficient in the extraction evaluation but did not show up in any of the sample measurements. Instead, an unidentified but very large peak emerges at almost the same time as the expected retention time of the geraniol, thus likely blotting out any possible analyte response. This can be seen when comparing figure 5, which is a chromatogram of one of the calibration points, and figure 6, which is a rather typical chromatogram for the studied samples. For further chromatograms and injection results, please refer to the appendix.

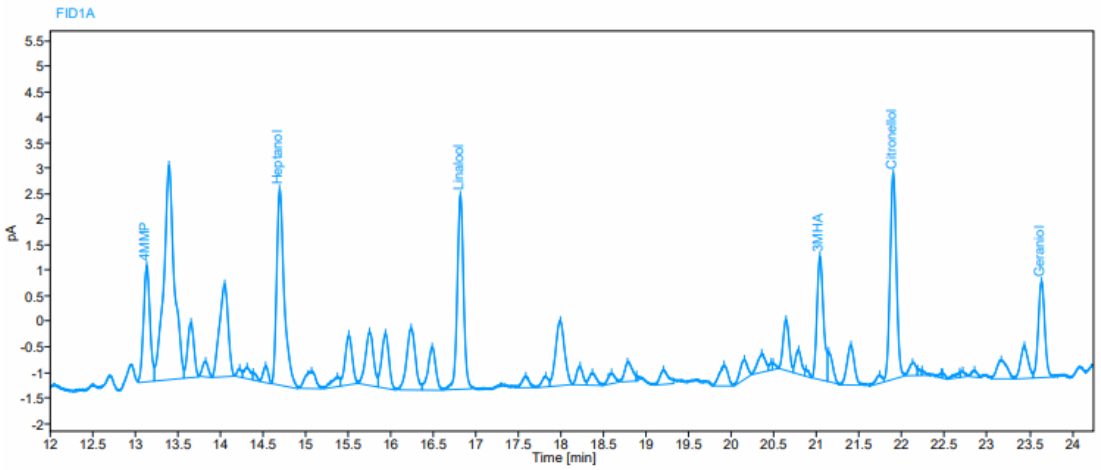

Figure 5: The chromatogram of the highest calibration concentration, with all analyte peaks labelled.

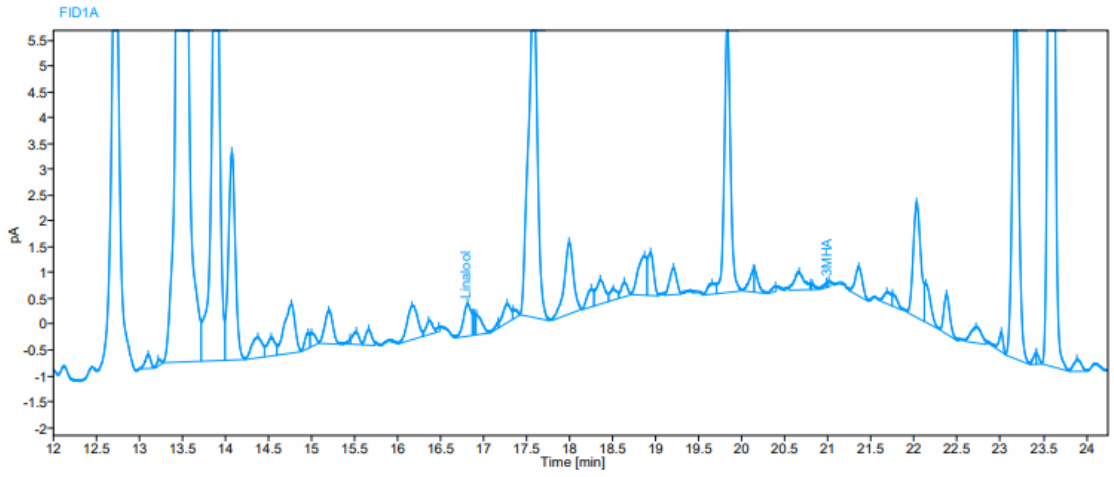

Figure 6: The chromatogram of one of the X 1+2 liquid-liquid extraction samples. Note the abundance of peaks that are not present in the calibration chromatogram.

A different mechanism can be seen in the measurements of 4MMP, which does produce some peaks, but none with a signal-to-noise ratio greater than 3. This is likely caused by the unusually large noise at the retention time of 4MMP, as seen in both the calibration measurements and any sample measurement where a peak at the correct retention time is observed. The baseline noise at the retention time of 4MMP is almost 10 times higher than the noise for any other peak, which also explains the very high LOD and LOQ values in table 2. This is likely caused by some impurity in the solvent that emerges at roughly the same time as the 4MMP, which is especially crucial for 4MMP which according to literature is present only in very small amounts in the beer.
Whilst it is possible that an interfering compound eclipses the signal from an analyte as described above, the opposite can also be true – an impurity in the sample may emerge at the same time as the analyte, and thus create a larger peak than could have been created by the analyte alone. There is no certain way of avoiding this without changing the method, but in this study care has been taken not to include any peaks which have a slightly different retention time or clearly different signal response than expected from the analyte in question.
To conclude, the results of this experiment are uncertain, and it is unclear whether any conclusion can be drawn more than to say that further trials are necessary if the method is to be fully evaluated.

Method evaluation

Although the results of the measurements were less than satisfactory, the method did prove effective in some respects. The calibration curves exhibit excellent linearity (within the given calibration range), linalool is identified in almost all samples (except for the SPE samples, which are meant to specifically measure thiols), and the extraction coefficients of the samples that were detected in the liquid extraction analysis were quite high, meaning that the analytes are not only extracted but also quite significantly enriched in the extract.
However, none of the applied methods of analysis succeeded in delivering reliable results for all of the studied compounds, meaning that they are clearly unsatisfactory.

As for the use of internal standards, heptanol proved to work well as an internal standard, especially for the monoterpene alcohols, as it showed roughly the same extraction behaviour and signal strength. 2-mercapto-3-butanol however proved unsatisfactory, as it was not possible to detect within the given conditions. This is likely because the volatility of the substance is so high that it emerges from the column along with the solvent plug, thus making it unidentifiable. While a column with a higher specificity towards thiols might solve this problem, it would probably be better to choose an internal standard with a boiling point more similar to that of the analytes.

Possible sources of error

There are a few sources of error that are quickly noticed, primarily the high background noise and non-analyte peaks as seen in the blank, calibration, extraction evaluation, and identification measurements.
The noise and non-analyte peaks in the blank are likely explained by a combination of contamination of the cyclohexane from previous experiments, contamination from improperly washed glass ware used when preparing the samples, or any slowly released contaminants in the liner of the column.
In the case of the calibration these peaks are even more pronounced, which cannot be explained by the factors described above except for unclean laboratory equipment, as these samples require more preparation than the blank sample and there is thus more chance of contamination. Instead, these peaks could be explained by impure standard, although all standards are labelled as being

of 95% purity and above, and possibly contamination when handling the samples – though this is also unlikely, as much care was taken to avoid this. A further possibility is thermal breakdown of the standards caused by the high temperature of the injector, meaning that the observed peaks could be products of the analytes rather than external contaminants.
For the liquid-liquid extraction evaluation sample, the only major differences from the calibration samples are contact with water, salt, and a centrifugation tube, and even more preparation. Out of these, it would seem most likely that some contaminant made its way from the salt to the sample, as quite a large amount (~18 w%) of salt was used and the salt was in contact with the extract for 15 minutes before centrifugation – thus, any non-polar contaminants in the salt should be transferred to the organic cyclohexane phase. To illustrate the high background noise in the liquid-liquid extraction evaluation sample, please refer to figure 7 below, showing the chromatogram of the liquid-liquid extraction evaluation sample.

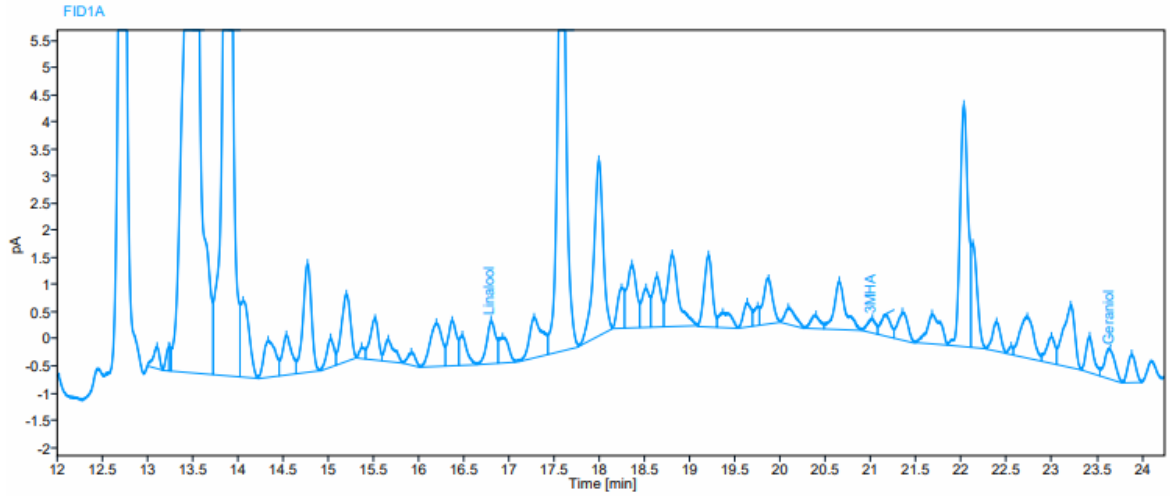

Figure 7: Chromatogram of the liquid-liquid extraction evaluation sample, with the three identified analyte peaks labelled

Lastly, in the solid phase extraction evaluation sample, all of the above applies plus any contamination from acetonitrile, thioglycerol, or the SPE tubes themselves. Here it should also be remembered that "contamination" in this regard does not only apply to unwanted substances in the respective solvents, but also traces of the solvent that are not properly rinsed out of the extract before analysis.

Possible improvements

Analytes

Apart from considering and, if possible, eliminating some sources of error, there are other improvements that can be made to the methods used. For the thiol analysis, the most obvious fault in this method is the exclusion of 3MH from the analyte set. This is especially crucial as it is, according to the studied literature, the most abundant of the polyfunctional thiols of interest, and should thus be easiest to detect and require a less fine-tuned analytical process. Including it would not require any real change in the method, as the method was developed with the intention of being used to measure 3MH, but would only require that the standard sample can be delivered in time for analysis. As mentioned above, the use of a different internal thiol standard is also advised.

Gas chromatography

To improve the resolution and decrease the effect of non-analyte peaks interfering with the peaks of interest, an even slower temperature programme or a longer column could be applied. Although this would require more time for the analysis, and possibly make the peaks of interest less distinct,

it might still make the analysis more reliable. Furthermore, changing the detector from a flame ionization detector to a mass spectrometer, a flame photometric detector or adding a sulfur chemiluminescence detector to the pre-existing flame ionization detector might improve the detection limit, particularly of sulfur compounds. The exact detection limit is quite difficult to determine based on literature alone though: Harris (2016) states that the flame ionization detector has a detection limit of approximately 2 pg/s, which – if we assume that all analyte emerges in one second – would translate to an extract concentration of approximately 1300 ng/litre at an injection volume of 1.5 µl. This can be compared to the lowest detection limit that was calculated in this study: 23 000 ng/l. While Harris' definition of "detection limit" likely refers to the absolute limit of detection, meaning the concentration where no signal at all is detected, rather than the signal-to-noise ratio definition used in this paper, it clearly signals how much values can differ from literature to actual application. Bearing that in mind, Harris suggests that a mass spectrometer might detect as little as 25 fg of analyte in a sample, whereas a sulfur chemiluminescence detector has a response to sulfur that is 10^7 times higher than the response to carbon, and a flame photometric detector has a response to sulfur that is 10^5 times higher than the response to carbon. For the flame photometric and sulfur chemiluminescence detector, the main advantage over the simple flame ionization detector would be less interference from carbon atoms in the sample, which would be very beneficial in reducing the noise in the measurements – however, Harris also states that the detection limit of sulfur using the sulfur chemiluminescence detector might be as low as 100 fg/s, i.e. a ten-fold increase compared with just the flame ionization detector. Lastly, changing the column to one that has a greater specificity for the compounds of interest, such as the Supelco SPB-1 SULFUR or Agilent DB-Sulfur SCD, might also improve the resolution and thereby the detection.

Sample preparation

When the method used in this project was adapted from that used by Takazumi et al. (2017), there were two main changes made: the solvent was changed from dichloromethane to cyclohexane, and the drying and concentrating under a nitrogen flow was omitted. The choice to use cyclohexane instead of dichloromethane was a conscious one, as described above, to investigate the effectiveness of a less hazardous solvent. While the extraction coefficient of most analytes when using cyclohexane seems satisfactory, a possible route of improvement would be to optimise the choice of solvent to further increase the extraction. As for the omission of the concentration step, this was done in an effort to save time, as the concentration and subsequent evaluation of analyte loss during concentration would have been quite time consuming. Additionally, more steps in the sample preparation would also mean more time for changes in the sample to occur, such as the thiols reacting with oxygen, thus making the analysis less reliable. However, it is very possible that by concentrating the analytes, especially in combination with the above-mentioned effort to improve resolution of peaks, more compounds could have been identified. Furthermore, increasing the signal of a peak without increasing the noise would also mean a higher signal-to-noise ratio, and thereby better opportunity to conclusively identify and quantify compounds.

Conclusion

To conclude and answer the questions posed in the introduction of this paper, the methods used in this project were less than ideal for measuring thiols and monoterpene alcohols in beer. Linalool, the only monoterpene alcohol that was reliably detectable in a number of samples, did not occur in significantly different concentrations between samples, indicating that products X and Y did not significantly impact the amount of linalool in the finished beer. Further research

should focus on improving the extraction and enrichment to better analyse thiols and improve resolution to better analyse monoterpene alcohols.

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Appendix

The appendices to this report can be found separately, and include the results of the calibration curves, identification of compounds and all samples. Samples are referenced as their product and addition method (i.e. "X 1" for product X, addition 1), followed by the extraction method (no reference for pure liquid-liquid extraction, IST for internal standard and SPE for solid phase extraction) and replicate marker (no reference for replicate 1, "Dup" for replicate 2, and "Trip" for replicate 3). The extraction evaluation samples are referenced as "Analys" followed by "liq ext" for liquid-liquid extraction or "SPE" for solid phase extraction. Please note that the calibration levels for the calibration curves differ between thiols and monoterpene alcohols. The correspondence of calibration samples to the calibration levels used in this report is:

Calibration level	Monoterpene alcohol	Thiols
1	Kal A	Kal A
2	Kal B	Kal B
3	Kal C	Kal C
4	Kal D	Kal D
5	Kal 1	Kal E
6	Kal E	Kal 1
7	Kal 2	Kal 2
8	Kal 3	Kal 3
9	Kal 4	Kal 4
10	Kal 5	Kal 5