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Enantiospecific determination of the odour threshold concentrations of (*R*)- and (*S*)-linalool in water and beer

Hop-derived linalool can substantially impact beer aroma. However, during the processing and storage of beer, the (*R*)-linalool predominating in hops can undergo racemisation to the less potent (*S*)-enantiomer. The aroma impact of the (*S*)-linalool was difficult to assess, because reliable threshold data were unavailable. To fill this gap, enantiopure (*S*)-linalool was isolated from the racemate by HPLC using a chiral column. This (*S*)-linalool was used in parallel to enantiopure (*R*)-linalool to determine enantiospecific odour threshold concentrations of linalool in water and unhopped beer with a trained sensory panel. Results revealed orthonasal odour detection threshold concentrations of 0.82 and 8.3 µg/kg in water and 6.5 and 53 µg/kg in beer, for (*R*)- and (*S*)-linalool, respectively. The higher odour potency of the (*R*)-linalool was thus confirmed, however, its odour threshold concentrations were found to be only 8 to 10 times lower than the odour threshold concentrations of the (*S*)-linalool and not 80 times as previously approximated from GC-O data. The data will help to better understand the aroma impact of (*R*)- and (*S*)-linalool in beer.

Descriptors: hop aroma, beer, linalool, 3,7-dimethylocta-1,6-dien-3-ol, enantiomer, odour threshold concentration

1 Introduction

Hops provide microbial stability and bitterness to beer, but can also considerably impact beer aroma. A major hop-derived beer aroma compound is citrusy, floral smelling linalool (3,7-dimethylocta-1,6-dien-3-ol). It was already identified in 1903 in hops and its characteristic odour led hop chemist *Alfred C. Chapman* to hypothesise that it contributes significantly to the characteristic aroma of hops [2]. *Peacock* et al. [15] were the first to recognize the importance of linalool for the hop aroma note in beer, which was later confirmed by *Fritsch* et al. [5, 6].

Peacock had not considered that linalool is chiral; Fritsch, however, showed that the linalool in a commercial late-hopped Pilsner beer was predominantly the (*R*)-isomer. While the odour qualities of (*R*)- and (*S*)-linalool are very similar, their odour potencies were considered to differ substantially. A previous study had reported that the odor threshold concentration (OTC) of (*R*)-linalool in air was 80 times lower that the OTC of (*S*)-linalool [8]. When Fritsch spiked an unhopped beer with the amount of (*R*)-linalool previously determined in the Pilsner beer, the hoppy aroma note increased from zero to the intensity perceived in the hopped beer, and the sensory profiles of the hopped and the spiked beer were virtually identical, thus indicating that solely (*R*)-linalool accounted for the hoppy aroma in this beer.

Although new hop varieties and alternative hopping techniques such as dry-hopping, both widely applied in craft beer making, have increased the number of hop-derived compounds transferred into beer in aroma-active amounts (e.g. [12, 13, 15, 19]), linalool still ranks among the most important contributors to the hoppy aroma in beer.

During brewing as well as during beer ageing, the (*R*)-linalool predominating in hops with > 90 % can undergo racemisation, sometimes leading to (*R*)/(*S*) ratios in beer close to 50/50, particularly when the pH is low [5, 10, 11, 21]. While it is unquestionable that racemisation leads to aroma loss, the contribution of the (*S*)-linalool formed is still not fully understood. Different from (*R*)-linalool, which is readily available commercially, pure (*S*)-linalool is difficult to obtain. Accordingly, OTC data in beer have been determined experimentally for (*R*)-linalool and racemic linalool, but not for (*S*)-linalool. For example, Fritsch [5] reported an OTC of 2.2 µg/L for the (*R*)-linalool and *Takoi* et al. [20] recently published an OTC of 5 µg/L for the racemate. The aroma impact of the (*S*)-linalool was typically only estimated from the ratio of the OTCs of (*R*)- and (*S*)-linalool in air which had been approximated by GC-O [16] with a chiral column in a study on pepper odorants [8]. The OTCs in air were 0.036 and 2.9 ng/L, respectively, and provided the basis for the previously mentioned factor of 80, which was discussed e.g. in [9, 10, 17, 20]. Given, however, that the OTC determinations in

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air were only performed by a single assessor, the validity of the factor is somewhat questionable.

Therefore the parallel determination of the OTCs of (*R*)- and (*S*)-linalool using a well-established sensory approach and a panel of assessors would fill an important knowledge gap. Results would particularly lead to a better understanding of the role of the (*S*)-linalool in beer aroma. A crucial requirement is, that both isomers are available in sufficient enantiomeric purity. Even a minor impurity of the more potent enantiomer could significantly falsify the OTC determination of the less potent enantiomer. Thus, our study aimed to separate a linalool racemate into the enantiomers, verify the purity of the individual enantiomers, and finally determine enantiospecific OTCs of (*R*)- and (*S*)-linalool in beer and water.

2 Materials and methods

2.1 Chemicals

The following substances were purchased from the commercial sources given in parentheses: linalool, racemic (97 %); (*R*)-linalool, analytical standard (99.9 %); methyl octanoate (Merck, Darmstadt, Germany); ethanol (Honeywell, Offenbach, Germany); *n*-hexane (Thermo Fisher Scientific, Dreieich, Germany); dichloromethane; diethyl ether; sodium sulphate; methanol, LiChrosolv® (VWR, Darmstadt, Germany). Dichloromethane and diethyl ether were freshly distilled through a column (120 cm × 5 cm) packed with Raschig rings before use. ($^2H_2$)Linalool was synthesised as detailed in the literature [17].

2.2 Unhopped beer: preparation and testing for absence of linalool

Liquid malt extract (Bavarian Pilsner; Weyermann, Bamberg, Germany; 7.1 kg) was diluted with demineralised water (37.9 L) at 85 °C to 12 °P. The wort was boiled for 5 min, and the hot trub was removed in a whirlpool. The major part of the wort (25 L) was cooled (12 °C) and transferred to a disinfected fermentation tank. A minor amount of the wort (10 L) was autoclaved and then used for the hop-free propagation of the yeast, strain TUM 34/70. For this purpose, yeast cells taken from an agar slant were first shaken with sterile wort (60 mL) in a 100 mL flask at 80 rpm for 72 h. After the yeast had settled, it was added to 60 mL wort and the mixture was shaken at 80 rpm for 72 h. This procedure was repeated 3 times until no linalool (which could have come from the slant agar) was detectable. The entire mixture was then added to 2.5 L of sterile wort, and the yeast was further propagated for 72 h. 2.1 L of the final yeast suspension (190 × 10^6 cells/mL) was added to the wort in the fermentation tank. Fermentation and maturation proceeded at 12 °C until the diacetyl content as determined according to MEBAK [14] was < 0.1 mg/L, which was the case after 12 days. At 70 % fermentation degree, the bung valve was closed to trap the carbon dioxide in the beer. The beer was stored for 3 weeks at 0 °C, filtered with Seitz KS 80 depth filter sheets (Pall, Dreieich, Germany), bottled, and stored at 4 °C.

The linalool concentration in the unhopped beer was analysed by GC-MS. For this purpose, a portion of the beer was degassed by filtration through a folded filter and a portion (2 g) was diluted with water (4 mL). Diethyl ether (16 mL) spiked with the internal standard ($^2H_2$)linalool (0.575 µg) in dichloromethane (50 µL) was added, and the mixture was stirred overnight to ensure equilibration. The organic phase was dried over anhydrous sodium sulphate, and non-volatiles were removed by solvent-assisted flavour evaporation (SAFE) [4] at 40 °C. The volatile fraction was concentrated to 1 mL by using a Vigreux column (50 cm × 1 cm) followed by a *Bemelmans* microdistillation device [1]. The concentrate was subjected to GC-MS analysis using the ZB-1701 column (cf. section 2.4). The linalool concentration was supposed to be calculated as mean after triplicate analyses from the area count of the analyte peak (m/z 137), the area count of the internal standard peak (m/z 139), the amount of beer sample, and the amount of standard added, by using a calibration line equation obtained after analysis of analyte/standard mixtures in five different concentration ratios (5:1, 3:1, 1:1, 1:3, and 1:5) by linear regression. However, no peak could be detected for the target analyte. Thus the maximum possible linalool concentration was calculated by integrating the background noise at the elution time of the analyte.

2.3 High-performance liquid chromatography (HPLC)

The AZURA high-performance liquid chromatography (HPLC) system (Knauer, Berlin, Germany) consisted of an autosampler AZURA 6.1 L, a binary high-pressure pump system P6.1 L HPG, a UV detector MWD 2.1 L, and a fraction collector LABOCOL Vario 4000. The PurityChrome software (Knauer) was employed for data analysis. The system was used with an analytical column, Lux 5 µm i-Amylose-1, 250 × 4.6 mm in combination with a precolumn SecurityGuard SemiPrep Cartridge, Lux i-Amylose-1, 4 × 3 mm, or a semipreparative column, Lux 5 µm i-Amylose-1, 250 × 10 mm in combination with a precolumn SecurityGuard SemiPrep Cartridge, Lux i-Amylose-1, 10 × 10 mm (Phenomenex, Aschaffenburg, Germany). The optimised analytical method included isocratic elution with *n*-hexane/ethanol (98 + 2, v + v), a starting concentration of the racemic linalool of 5 mg/mL, and an injection volume of 25 µL, which translated to a starting concentration of 20 mg/mL in combination with an injection volume of 80 µL when the semipreparative column was used. The run time was 30 min. Eluate fractions corresponding to (*R*)- and (*S*)-linalool were collected from a total of 150 runs. The solvents were removed from the (*R*)- and (*S*)-linalool fractions by rotary evaporation, and the residues were dissolved in 50 mL absolute ethanol, respectively, to obtain ethanolic stock solutions. Ethanolic stock solutions were also prepared from the commercial racemic linalool and the commercial (*R*)-linalool. Dilutions of these stock solutions in dichloromethane were used for GC-MS and GC-O analysis.

2.4 Gas chromatography-mass spectrometry (GC-MS)

A 7890B GC was equipped with a GC 80 autosampler and a multimode injector (Agilent Technologies, Waldbronn, Germany). The column was either a BGB-176, 28 m × 0.25 mm i.d., 0.25 µm film thickness (BGB Analytik, Rheinfelden, Germany) with a stationary phase consisting of 30 % 2,3-dimethyl-6-tert-butyldimethylsilyl-β-cyclodextrin in BGB-15 (15 % phenyl-, 85 % methylpolysiloxane) or a ZB-1701, 30 m × 0.25 mm i.d., 0.25 µm film thickness (Phenomenex). The carrier gas was helium at a constant flow of 1.0 mL/min. The injector was used in splitless mode with a start temperature of 40 °C, and an end temperature of 250 °C. The injection volume was 1 µL. The initial oven temperature of 40 °C was held for 2 min, followed by a gradient of 4 °C/min until 140 °C and a gradient of 8 °C/min until the final temperature of 230 °C. The GC was connected to a Saturn 220 ion trap mass spectrometer (Agilent Technologies). Mass spectra were generated in chemical ionisation (CI) mode with methanol as reagent gas. The MS Workstation 7.0.2 software (Agilent Technologies) was used for data analysis.

2.5 Gas chromatography-olfactometry (GC-O)

A Trace GC Ultra gas chromatograph (Thermo Fisher Scientific) was equipped with a cold on-column injector, a flame ionization detector (FID), and a tailor-made sniffing port [18]. The column was the BGB-176 detailed in section 2.4. The carrier gas was helium at 90 kPa constant pressure. The injection volume was 1 µL. The initial oven temperature of 40 °C was held for 2 min, followed by a gradient of 2 °C/min until the final temperature of 220 °C. The column effluent was divided 1:1 using a deactivated Y-shaped glass splitter and two deactivated fused silica capillaries (50 cm × 0.25 mm i.d.) connecting the splitter to the FID and the sniffing port. The sniffing port was mounted on a heated (250 °C) detector base of the GC.

2.6 Gas chromatography-flame ionisation detector (GC-FID)

A Trace GC Ultra gas chromatograph (Thermo Fisher Scientific) was equipped with a Triplus autosampler, a cold on-column injector, and an FID. The column was a DB-FFAP, 30 m × 0.32 mm i.d., 0.25 µm film thickness (Agilent Technologies). The carrier gas was helium at 64 kPa constant pressure. The injection volume was 2 µL. The initial oven temperature of 40 °C was held for 2 min, followed by a gradient of 8 °C/min until the final temperature of 230 °C. The ChromQuest 5.0 software (Thermo Fisher Scientific) was used for data analysis.

2.7 OTC determinations

OTCs were determined orthonasally by a series of 3-AFC tests according to the American Society for Testing and Materials (ASTM) standard practice for determination of odour and taste thresholds by a forced-choice ascending concentration series method of limits [7]. The matrix was tap water or the unhopped beer (cf. section 2.2). The highest concentrated test sample was prepared from the ethanolic stock solution and matrix. The exact concentration of (*R*)-linalool in the (*R*)-linalool stock solution was determined from the original weight and the enantiomeric purity of 98.7 %. The exact concentration of (*S*)-linalool in the (*S*)-linalool stock solution was determined by GC-FID (cf. section 2.6) using methyl octanoate as internal standard and a response factor of 0.923 determined from the GC-FID analysis of an (*R*)-linalool/methyl octanoate mixture. The ethanol content in the highest concentrated water sample was below its OTC. Diluted test samples were prepared by stepwise dilution of the highest concentrated test sample with matrix, the dilution factor in each step was three [3]. This approach finally covered an overall concentration range of 2 ng/kg to 5 mg/kg. Reference samples consisted of the pure matrix. All samples were presented to the assessors in cylindrical polytetrafluoroethylene vessels (5.7 cm height × 3.5 cm i.d.) with lids. The sample temperature was 20 °C. The sensory evaluation room was divided into individual booths. The room temperature was 22 ± 2 °C. The four OTCs were determined in two sessions on separate days, the first being dedicated to the OTCs in water, the second to the OTCs in the unhopped beer. The panel con-

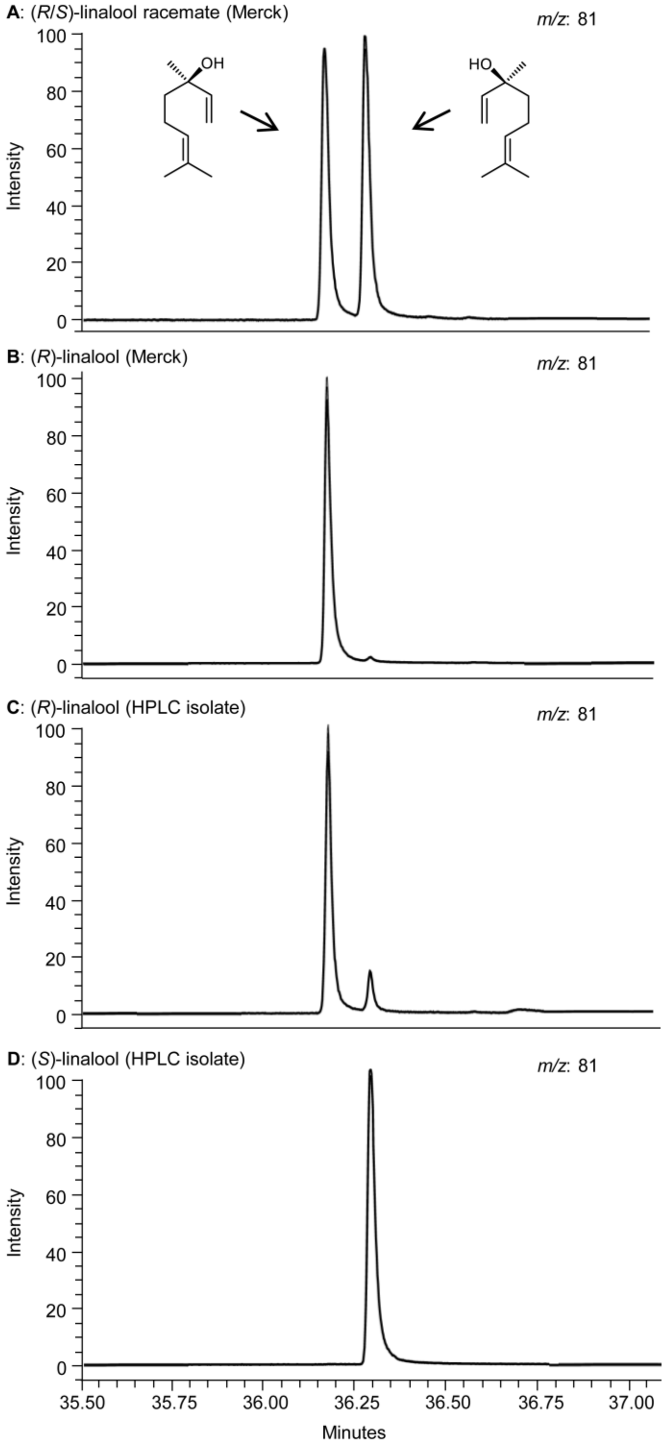

Fig. 1 **GC-MS chromatograms showing the enantiomeric distribution of linalool in the commercial racemate (A), the commercial (*R*)-linalool (B), and the (*R*)- and (*S*)-linalool isolates obtained by HPLC in this study (C and D)**

sisted of 17 trained assessors and included males and females aged 25 – 59 years. The training was done in weekly sessions and included flavor language development, olfactory profiling of test mixtures and authentic food samples, 3-AFC tests, and OTC determinations in different matrices.

3. Results and discussion

3.1 Preparation of (*R*)- and (*S*)-linalool

At first, an HPLC method for the separation of (*R*)- and (*S*)-linalool was developed. Different chiral columns were tested and the amount of injected racemic linalool, the eluent composition, and the flow rate were varied to optimise the separation (data not shown). The final method was scaled up by using a semipreparative column with the same amylose-based chiral stationary phase. This allowed the preparative isolation of an (*R*)-linalool fraction and an (*S*)-linalool fraction in sufficient amounts within a reasonable time frame.

The (*R*)- and (*S*)-linalool fractions were checked for their enantiomeric purity by GC-MS analysis using a β-cyclodextrin-based chiral GC column. Results are depicted in figure 1. Figure 1A shows the commercial racemic mixture with the two enantiomers virtually baseline-separated. The elution order was confirmed by analysis of the commercial (*R*)-linalool (Fig. 1B). The enantiomeric purity of the isolated (*S*)-linalool fraction was > 99.9 % (Fig. 1D), however, the enantiomeric purity of the (*R*)-linalool fraction was only 87.6 % (Fig. 1C), thus lower than that of the commercial (*R*)-linalool, which amounted to 98.7 % (Fig. 1B).

With the high enantiomeric purity of the isolated (*S*)-linalool, a major goal of the study was achieved. As discussed in the introduction section, even a minor amount of the more potent (*R*)-linalool could have the potential to substantially falsify the OTC determination of the (*S*)-linalool. Vice versa, a minor amount of the less potent (*S*)-linalool is less critical for the OTC determination of the more potent (*R*)-linalool. Therefore, the 98.7 % purity of the commercial (*R*)-linalool might be acceptable for the following OTC determinations. However, to ultimately confirm this and additionally exclude the presence of other odour-active impurities in the commercial (*R*)-linalool and the isolated (*S*)-linalool, both were subjected to GC-O using the chiral GC column in combination with an aroma extract dilution analysis based on 1:10 dilutions. For both samples, the FD factors of all impurities including the respective enantiomeric compounds were at least 100 times lower than the FD factor of the target compound, thus providing final evidence of sufficient olfactory purity for the enantiospecific threshold determinations [3, 16].

3.2 Determination of enantiospecific odour threshold concentrations of (*R*)- and (*S*)-linalool

A fixed sensory panel consisting of 17 experienced assessors was employed to determine the orthonasal odour threshold concentrations of (*R*)- and (*S*)-linalool in beer and water according to the American Society for Testing and Materials (ASTM) standard practice [7]. In all four cases, not only the detection thresholds but also the recognition thresholds, i.e. the concentration at which the characteristic odour was perceived, were determined [3].

Table 1 Orthonasal odour threshold concentrations (µg/kg) of (*R*)- and (*S*)-linalool in water and beer

Compound	Water		Beer	
	Recognition threshold	Detection threshold	Recognition threshold	Detection threshold
(*R*)-linalool	1.9	0.82	13	6.5
(*S*)-linalool	18	8.3	110	53

The results are shown in table 1. Data confirmed the higher odour potency of the (*R*)-linalool, however, its odour threshold concentrations were found to be only 8 to 10 times lower than the odour threshold concentrations of the (*S*)-linalool and not 80 times as previously assumed [8]. The threshold concentrations in beer were clearly higher than in water. This was to be expected given that many additional odorants were present in the beer and potentially able to mask the odour of linalool present at low concentrations. By contrast, a contribution of innate linalool in the unhopped beer to the difference between the water and the beer threshold could be clearly excluded. No evidence of any linalool in the unhopped beer was achieved. Background noise integration resulted in a maximum concentration of 0.054 µg/kg, which would still be negligible considering that the detection threshold concentration of the more potent (*R*)-linalool in water amounted to 0.82, thus 15 times more. The factor by which the beer thresholds were higher than the water thresholds varied between 6 and 8. For both, water and beer, the recognition threshold concentrations exceeded the detection threshold concentrations approximately by a factor of 2.

4 Conclusion

For the first time, reliable odour threshold concentrations of the individual linalool enantiomers (*R*)- and (*S*)-linalool were determined in water and beer. The data confirmed the higher odour potency of the (*R*)-isomer, however, disproved the factor of 80 by which the odour potency of the (*S*)-isomer was reported to be lower. Our data instead suggest a factor of 8 to 10. This finding will shed new light on the individual aroma impact of (*R*)- and (*S*)-linalool in beer and help to better understand aroma changes associated with the racemisation of hop-derived linalool during brewing and beer ageing.

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