

## Identification Based on Quantitative Measurements and Aroma Recombination of the Character Impact Odorants in a Bavarian Pilsner-type Beer

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Application of aroma extract dilution analysis on the volatiles isolated from a Bavarian Pilsner-type beer revealed 40 odor-active constituents in the flavor dilution (FD) factor range of 16–2048, among which ethyl octanoate, (*E*)- $\beta$ -damascenone, 2- and 3-methylbutanoic acid, and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone showed the highest FD factor of 2048. After quantitation of the 26 odorants showing FD factors  $\geq 128$  by stable isotope dilution analysis and determination of their odor thresholds in water, odor activity values (OAVs) were calculated. The results indicated ethanol, (*E*)- $\beta$ -damascenone, (*R*)-linalool, acetaldehyde, and ethyl butanoate with the highest OAVs, followed by ethyl 2-methylpropanoate and ethyl 4-methylpentanoate, which was previously unknown in beer. Finally, the overall aroma of the beer could be mimicked for the first time by recombining 22 reference odorants in the same concentrations as they occurred in the beer using ethanol/water as the matrix.

**KEYWORDS:** Beer; aroma; aroma extract dilution analysis; stable isotope dilution analysis; odor activity value; aroma recombination

### INTRODUCTION

Up to now, numerous investigations aimed at identifying volatile compounds in different types of beer have been performed, and today more than 620 constituents have been reported (*1*). However, as recently proven for a significant number of foods (*2–4*), only a small number of the volatiles occurring in foods are able to activate the odorant receptors in the human nasal cavity and, thus, are responsible for aroma perception when food is eaten. Such aroma-active compounds can be selected from the bulk of “nonactive” volatiles by combining the human olfactory system with analytical techniques. Such methods are known as gas chromatography–olfactometry (GC-O), aroma extract dilution analysis (AEDA), or CHARM analysis (*2–6*). However, only a few applications of such methods on beer aroma volatiles can be found in the literature.

The first comprehensive approach to evaluate the sensorial contribution of single volatiles to beer flavor was performed by Meilgaard about 30 years ago (*7, 8*). He calculated the odor activity values (OAVs: ratio of concentration to odor threshold) of 239 beer constituents on the basis of quantitative data and odor thresholds determined in beer. The results indicated that, apart from ethanol and carbon dioxide, several esters (e.g., 3-methylbutyl acetate and ethyl hexanoate), higher alcohols (e.g., 3-methylbutanol), dialkyl sulfides (e.g., dimethyl sulfide), and

short-chain fatty acids (e.g., butanoic acid) were essential for the aroma of U.S. lager beers.

In a study on the aroma of a Bavarian pale lager beer, Schieberle (*9*) found that among the 22 odorants identified, (*E*)- $\beta$ -damascenone exhibited the highest odor activity. Furthermore, the author reported high OAVs for ethyl butanoate, 3-methylbutanol, ethyl hexanoate, and 2-phenylethanol, suggesting these compounds to be key contributors to the overall aroma. Compared to the pale lager beer, the OAV of the caramel-like smelling 4-hydroxy-2,5-dimethyl-3(2*H*)furanone in a dark beer and of the clove-like smelling 4-vinyl-2-methoxyphenol in a wheat beer were significantly higher (*9*). These odorants were, thus, suggested to contribute to the difference in the flavors of the different beer types. By applying AEDA on an extract prepared from commercial lager beers, Gijs et al. (*10*) confirmed 2-phenylethanol as an important beer odorant and suggested, in particular, 3-methyl-2-butene-1-thiol, dimethyl trisulfide, and *o*-aminoacetophenone as additional key beer aroma compounds.

In contrast to lager beers, Pilsner-type beers are usually produced by using higher amounts of hops in the brewing process. Such beers historically originate from Bohemia (Czech Republic) and are named after the city of Pilsen. Today, Pilsner beers amount to two-thirds of German beer consumption and are mainly consumed in the northern parts of Germany and Europe, respectively. Besides the characteristic bitter taste of such beers, a typical hoppy aroma note plays an important role as an additional sensory attribute. However, only in a few studies have attempts been made to identify substances being respon-

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sible for this hoppy note. Sanchez et al. (11) compared the odor-active volatiles in unhopped and hopped beers by using GC-O. The following compounds were reported to be more intensively perceived during GC-O in hopped beer than in unhopped beer: linalool, citronellol, 2-phenylethyl acetate, and 2-phenylethanol. In a study on beers prepared by using two different hops, Lermusieu et al. (12) reported that, in particular, 2-methyl-3-furanthiol, dimethyl trisulfide, linalool,  $\gamma$ -nonalactone,  $\beta$ -damascenone, ethyl cinnamate, humuladienone, and several unidentified compounds showed higher FD factors in a beer produced with Saazer hops as compared to an unhopped beer.

Although important odorants can be identified by applying GC-O and the OAV concept, only aroma recombination experiments based on the natural concentrations of the aroma-active compounds in the food under consideration are able to address the fact that interactions between the aroma attributes of all odor-active constituents finally generate the typical food aroma. Such recombination experiments have recently been successfully applied by our group to a variety of foods, such as strawberries (13), potato chips (14), or orange and grapefruit juices (15).

The purpose of the present investigation was therefore, first, to identify the potent odorants of a Bavarian Pilsner-type beer by application of the AEDA, second to verify the importance of identified odorants by calculating OAVs, and, third, to simulate the overall odor of the beer by blending the key odorants in their "natural" concentrations, that is, as these occurred in the beer.

## MATERIALS AND METHODS

Several batches of a Pilsner-type beer were obtained from a Bavarian brewery immediately after production. Within the frame of this study, three different batches of the same brand have been analyzed.

**Chemicals.** The reference compounds of the odorants listed in the tables were obtained commercially: acetaldehyde, *o*-aminoacetophenone, 1,1-diethoxyethane, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone, dimethyl sulfide, ethyl hexanoate, 5-ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone, (*S*)-ethyl 2-methylbutanoate, ethyl 2-methylpropanoate, ethyl octanoate, ethyl pentanoate, ethyl-3-phenylpropanoate, hexanoic acid, (*R/S*)-linalool, 3-(methylthio)propanal, 3-(methylthio)propanol, 3-methylbutanal, 2- and 3-methylbutanoic acid, 3-methylbutanol, 2-methylpropanol,  $\gamma$ -nonalactone, phenylacetic acid, phenylacetaldehyde, and phenylethyl acetate (Aldrich, Sigma-Aldrich Chemie, Taufkirchen, Germany); acetic acid, butanoic acid, ethanol, 2-methoxyphenol, (*R/S*)-2-methylbutanol, and vanillin (Merck, Darmstadt, Germany); ethyl butanoate, (*R*)-linalool, and 2-phenylethanol (Fluka, Neu-Ulm, Germany); 2- or 5-ethyl-4-hydroxy-5- or 2-methyl-3(2*H*)-furanone (Givaudan, Dübendorf, Switzerland); 1-octen-3-one and 4-vinyl-2-methoxyphenol (Lancaster, Mühlheim/Main, Germany). (*E*)- $\beta$ -Damascenone was a gift from Symrise, Holzminden, Germany.

3-Methyl-2-butene-1-thiol was synthesized according to the procedure described by Holscher et al. (16).

**Isotopically Labeled Compounds.** The following compounds were prepared as described previously: [ $^2\text{H}_2$ ]-3-methylbutanal (17), [ $^{13}\text{C}_2$ ]-diethoxyethane (18), [ $^2\text{H}_2$ ]-3-methylbutanol (19), [ $^2\text{H}_3$ ]ethyl butanoate (18), [ $^2\text{H}_3$ ]-3-methyl-2-butene-1-thiol (20), [ $^2\text{H}_3$ ]ethyl-2-methylbutanoate (21), [ $^2\text{H}_3$ ]-3-(methylthio)propanol (22), [ $^2\text{H}_3$ ]ethyl hexanoate (18), [ $^2\text{H}_2$ ]-linalool (23), [ $^2\text{H}_2$ ]-2-phenylethanol (19), [ $^2\text{H}_3$ ]ethyl octanoate (19), [ $^2\text{H}_3$ ]-4-vinyl-2-methoxyphenol (24), [ $^2\text{H}_6$ ]-(*E*)- $\beta$ -damascenone (25), [ $^2\text{H}_2$ ]butanoic acid (26), [ $^2\text{H}_2$ ]-3-methylbutanoic acid (24), [ $^2\text{H}_3$ ]-2-methoxyphenol (27), [ $^{13}\text{C}_2$ ]-4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (28), [ $^2\text{H}_3$ ]-4-hydroxy-2- or 5-ethyl-5-, or 2-methyl-3(2*H*)-furanone (29), and [ $^{13}\text{C}_2$ ]-3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (30).

[ $^{13}\text{C}_2$ ]Acetaldehyde was purchased from Promochem (Wesel, Germany), and [ $^{13}\text{C}_2$ ]phenylacetic acid and [ $^2\text{H}_6$ ]dimethyl sulfide were purchased from Aldrich (Sigma-Aldrich Chemie, Taufkirchen, Germany).

**Isolation of the Volatiles.** Beer (500 mL) was filtered through a paper filter to avoid foaming during extraction and was repeatedly extracted with diethyl ether (total volume = 2.4 L). The extract was dried over  $\text{Na}_2\text{SO}_4$  and finally concentrated to 100 mL by distilling off the solvent at 38 °C using a Vigreux column (60 cm  $\times$  1 cm i.d.). To remove the nonvolatile material, the concentrate was distilled using the solvent-assisted flavor evaporation (SAFE) method (31). By treatment of the distillate with aqueous sodium bicarbonate (0.5 mol/L, 200 mL), a fraction of the acidic volatiles (AV) and of the neutral/basic volatiles (NBV) was obtained (32). After drying over  $\text{Na}_2\text{SO}_4$ , both fractions were concentrated by microdistillation (32) to 250  $\mu\text{L}$ .

**High-Resolution GC–Olfactometry (HRGC-O) and High-Resolution GC–Mass Spectrometry (HRGC-MS).** HRGC was performed by means of a type 8000 gas chromatograph (Fisons, Mainz, Germany) and by using the following capillaries: SE-54 (30 m  $\times$  0.32 mm fused silica capillary DB-5, 0.25  $\mu\text{m}$ ) (J&W Scientific, Fisons, Germany); free fatty acid phase FFAP (30 m  $\times$  0.32 mm fused silica capillary; 0.25  $\mu\text{m}$ ) (Chrompack, Mühlheim, Germany); and OV-1701 (30 m  $\times$  0.32 mm fused silica capillary DB-1701, 0.25  $\mu\text{m}$ ) (Chrompack). The samples were applied by using the cold on column technique at 35 °C. After 2 min, the temperature of the oven was raised at 40 °C/min to 50 °C for the SE-54 or to 60 °C for the FFAP and OV-1701 capillaries, respectively, held for 2 min isothermally, then raised at 6 °C/min to 180 °C, and finally raised at 20 °C/min to 230 °C and held for 10 min. The flow rate of the carrier gas helium was 2.5 mL/min. At the end of the capillary, the effluent was split 1:1 (by volume) into an FID and a sniffing port using two deactivated but uncoated fused silica capillaries (50 cm  $\times$  0.32 mm). The FID and the sniffing port were held at 220 and 240 °C, respectively. Linear retention indices (RI) of the compounds were calculated as previously described (32). Mass spectrometry (MS) was performed by means of a MAT 95 S (Finnigan, Bremen, Germany) in tandem with the capillaries described above. Mass spectra were generated in the electron impact mode at 70 eV and in the chemical ionization mode at 110 eV with isobutane as the reagent gas.

**Aroma Extract Dilution Analysis (AEDA).** The flavor dilution (FD) factors of the odorants were determined by AEDA (4) of the following dilution series: the extracts containing the neutral/basic and the acidic volatiles (AV and NBV; each 250  $\mu\text{L}$ ) from 500 mL beer were diluted stepwise with diethyl ether (1+1 by volume). GC-O was performed with aliquots (0.5  $\mu\text{L}$ ) using the capillaries SE-54 (for NBV) and FFAP (for AV). The original extract was sniffed by at least four experienced panelists in order to avoid overlooking that odor-active areas. The AEDA was then performed on three different batches of the same beer by two panelists, and the FD factors obtained were averaged.

**Aroma Dilution Analysis (ADA).** Beer (50 mL) was pipetted into a vessel (volume = 300 mL), sealed with a septum, and equilibrated for 20 min at room temperature. Headspace samples of decreasing volumes (from 20 to 0.1 mL) were withdrawn from different vessels containing the same amount of beer using a gastight syringe (Hamilton, Australia) and then analyzed by headspace HRGC-O as described previously (33).

**Quantitation by Stable Isotope Dilution Assays (SIDA) Coupled with Two-Dimensional High-Resolution Gas Chromatography (TD-HRGC-SIDA).** Beer (1–1000 mL, depending on the concentration of the respective odorant determined in a preliminary experiment) was pipetted into a vessel (100–1000 mL) and spiked with an ethanolic solution of the internal standards using the amounts given in Table 1. The amount of the respective internal standard was chosen in a similar concentration range as compared to the analyte. The samples were equilibrated for 15 min with stirring and then repeatedly extracted with diethyl ether and distilled using the SAFE apparatus (31). The distillate was separated into the AV and NBV, and both fractions were concentrated to 250  $\mu\text{L}$  as described above.

The two-dimensional HRGC-MS was performed by means of an HRGC Mega 2 series (Fisons, Mainz, Germany) coupled with an HRGC 5160 (Carlo Erba, Hofheim, Germany). The separation of the extract in the first dimension was achieved on an SE-54 column (NBV) or on an FFAP (AV), respectively. The elution range containing the respective odorant and the internal standard, respectively, was transferred to the

**Table 1.** Amount of Beer and Selected Ions ( $m/z$ ) of Analytes and Isotope-Labeled Standards (IST) Used in the Stable Isotope Dilution Assays

compound	amount of beer (mL)	analyte ( $m/z$ )	IST	
			isotope label	$m/z$
3-methyl-2-butene-1-thiol	3000	69	$^2\text{H}_8$	77
ethyl 4-methylpentanoate	1000	131	$^{-a}$	134
( <i>E</i> )- $\beta$ -damascenone	1000	191	$^2\text{H}_{5-7}$	196–198
2-methoxyphenol	1000	125	$^2\text{H}_3$	128
3-hydroxy-4,5-dimethyl-2(5 <i>H</i> )-furanone	1000	129	$^{13}\text{C}_2$	131
diethoxyethane	50	73	$^2\text{H}_2$	75
ethyl butanoate	50	117	$^2\text{H}_3$	120
ethyl hexanoate	50	145	$^2\text{H}_3$	148
linalool	50	137	$^2\text{H}_2$	139
ethyl octanoate	50	173	$^2\text{H}_3$	176
4-vinyl-2-methoxyphenol	50	151	$^2\text{H}_3$	154
4-hydroxy-2,5-dimethyl-3(2 <i>H</i> )-furanone	50	129	$^{13}\text{C}_2$	131
2- or 5-ethyl-4-hydroxy-5- or 2-methyl-3(2 <i>H</i> )-furanone	50	143	$^2\text{H}_3$	145
phenylacetic acid	50	137	$^{13}\text{C}_2$	139
3-methylbutanal	20	69	$^2\text{H}_{2-3}$	70–71
dimethyl sulfide	10	63	$^2\text{H}_6$	69
3-(methylthio)propanol	10	89	$^2\text{H}_3$	92
butanoic acid	10	89	$^2\text{H}_{2-4}$	91–93
2- and 3-methylbutanoic acid <sup>b</sup>	10	103	$^2\text{H}_2$	105
acetaldehyde	1	45	$^{13}\text{C}_2$	47
3-methylbutanol	1	71	$^2\text{H}_2$	73
2-phenylethanol	1	105	$^2\text{H}_2$	107

<sup>a</sup> [ $^2\text{H}_3$ ]Ethyl 3-methylbutanoate was used as internal standard. <sup>b</sup> Isomers could not be separated during GC.

second column by means of the MCSS system (moving capillary stream switching; Thermoquest, Germany) via a cold-trap, which was cooled to  $-100\text{ }^\circ\text{C}$ . With heating to  $200\text{ }^\circ\text{C}$  the analyte and the internal standard were then transferred onto the second column with different polarities (for NBV, FFAP; and for AV, OV-1701). Quantitation was done by means of an ITD 800 ion trap detector (Finnigan MAT, Bremen, Germany), which was coupled to the HRGC in an open-split mode. Spectra were generated in the chemical ionization mode with methanol as the reagent gas (ionization energy =  $70\text{ eV}$ ).

**Determination of Enantiomeric Ratios.** The enantiomeric ratios in linalool and ethyl 2-methylbutanoate were determined in the NBV by two-dimensional GC using a chiral column (BGB 176,  $30\text{ m} \times 0.25\text{ mm}$ , film thickness =  $0.25\text{ }\mu\text{m}$ ) (BGB Analytik, Rothenfluh, Switzerland) in the second dimension. The temperature program used for the chiral separation started at  $35\text{ }^\circ\text{C}$ . After 1 min, the temperature of the oven was raised at  $40\text{ }^\circ\text{C}/\text{min}$  to  $50\text{ }^\circ\text{C}$ , held for 1 min isothermally, then raised at  $6\text{ }^\circ\text{C}/\text{min}$  to  $180\text{ }^\circ\text{C}$ , and finally raised at  $20\text{ }^\circ\text{C}/\text{min}$  to  $230\text{ }^\circ\text{C}$  and held for 10 min. The flow rate of the carrier gas, helium, was  $2.5\text{ mL}/\text{min}$ . For calculation of enantiomeric distribution, the intensities of selected ions were recorded.

**Quantitation of Highly Volatile Components.** The quantitation of the highly volatile odorants acetaldehyde, dimethyl sulfide, and 3-methylbutanal was performed by SIDA using static headspace coupled with HRGC-MS. For this purpose, aliquots of beer (1 mL for quantitation of acetaldehyde, 10 mL for dimethyl sulfide, and 20 mL for 3-methylbutanal) were pipetted into an Erlenmeyer flask (250 mL) and spiked with the respective isotopically labeled standard (Table 1). The sample was equilibrated for 20 min at  $45\text{ }^\circ\text{C}$ , and then 5 mL of the headspace was withdrawn with a gastight syringe (Hamilton, Australia); the volatiles were cryofocused in a trap before these were injected onto a capillary column SE-54 installed in a type CP-9001 GC (Chrompack, Frankfurt, Germany). The GC column was coupled to a type Inco XL quadrupole mass spectrometer (Finnigan Mat, Bremen), and mass spectra were generated in the chemical ionization mode ( $115\text{ eV}$ ) using methane as reactant gas.

**Quantitation of Ethanol.** Ethanol was quantified using an enzymatic test kit from R-Biopharm (Darmstadt, Germany). Beer (10 mL) was degassed over a filter and diluted with water 1+999 before the enzymatic test was applied.

**Determination of Odor Thresholds.** All reference odorants were first checked for the absence of contaminating odorants by HRGC-O and purified by distillation, if necessary. Ethanol ( $10\text{ }\mu\text{L}$ ), containing a defined amount of the respective odorant, was pipetted into 25 mL of tap water. After 2 min of stirring in glass vessels (height =  $65\text{ mm}$ ; i.d. =  $40\text{ mm}$ ; total volume =  $45\text{ mL}$ ), the sample was judged by a panel of 10 persons by means of triangular tests using 25 mL of tap water spiked with  $10\text{ }\mu\text{L}$  of ethanol as the control (13). The samples were presented in order of increasing concentrations of the odorant (1+1 dilutions), and the odor thresholds were calculated according to the method of section 35 LMBG, methods 00.90-9 and 00.90-7 (34).

**Pilsner Aroma Model.** Tap water was carbonated with a Soda-Maker, and the pH was adjusted to 4.3 (the same as the pH of the beer investigated), using hydrochloric acid ( $0.5\text{ mol/L}$ ). The following mixture of 22 odorants, dissolved in ethanol, was added to 1 L of water: 3-methylbutanol ( $50000\text{ }\mu\text{g}$ ), 2-phenylethanol ( $15000\text{ }\mu\text{g}$ ), 2-methylbutanol ( $14400\text{ }\mu\text{g}$ ), acetaldehyde ( $5100\text{ }\mu\text{g}$ ), butanoic acid ( $1740\text{ }\mu\text{g}$ ), 3-(methylthio)propanol ( $998\text{ }\mu\text{g}$ ), 3-methylbutanoic acid ( $860\text{ }\mu\text{g}$ ), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone ( $310\text{ }\mu\text{g}$ ), ethyl hexanoate ( $200\text{ }\mu\text{g}$ ), ethyl butanoate ( $200\text{ }\mu\text{g}$ ), ethyl octanoate ( $160\text{ }\mu\text{g}$ ), 4-vinyl-2-methoxyphenol ( $140\text{ }\mu\text{g}$ ), dimethyl sulfide ( $60\text{ }\mu\text{g}$ ), diethoxyethane ( $50\text{ }\mu\text{g}$ ), (*R*)-linalool ( $45\text{ }\mu\text{g}$ ), 2- or 5-ethyl-4-hydroxy-5- or 2-methyl-3(2*H*)-furanone ( $20\text{ }\mu\text{g}$ ), 3-methylbutanal ( $5\text{ }\mu\text{g}$ ), ethyl 2-methylpropanoate ( $3.2\text{ }\mu\text{g}$ ), (*E*)- $\beta$ -damascenone ( $2.3\text{ }\mu\text{g}$ ), 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone ( $1\text{ }\mu\text{g}$ ), ethyl 4-methylpentanoate ( $0.28\text{ }\mu\text{g}$ ), and 3-methyl-2-butene-1-thiol ( $0.01\text{ }\mu\text{g}$ ). The ethanol content (23rd aroma compound) of the aroma model was finally adjusted to  $40\text{ g/L}$ .

**Descriptive Profile Test.** The beer sample and the Pilsner aroma model were orthonasally evaluated by 10 trained panelists, who were asked to rank the intensity of the following odor attributes: malty, honey-like, fruity, citrus-like, hoppy, and sweaty. The respective odor intensities were rated as 0 (not perceivable), 1 (weak), 2 (significant), and 3 (strong) using a scale of 0, 0.5, 1.0, 1.5, etc.

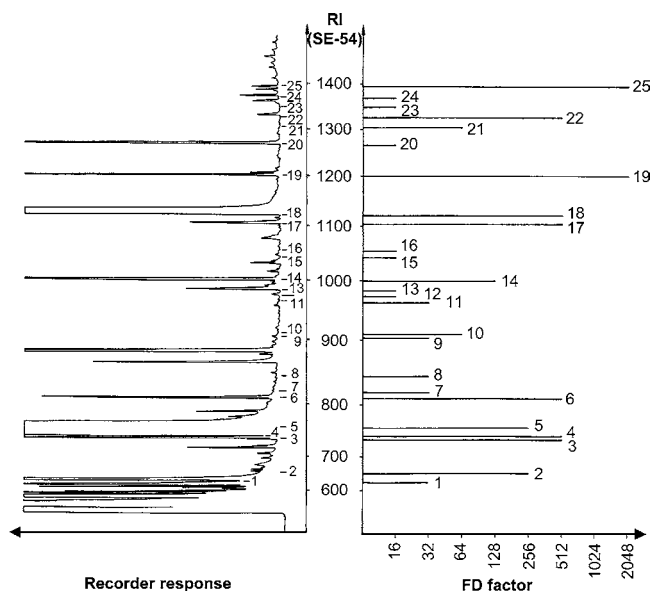
Panelists underwent training for odor qualities and odor intensities using aqueous solutions containing the respective odorant responsible for a certain odor attribute, for example, phenylacetaldehyde for honey-like, in different concentrations.

## RESULTS AND DISCUSSION

**Identification of Odor-Active Constituents.** Isolation of the Pilsner beer volatiles by extraction of a fresh beer with diethyl ether followed by SAFE resulted in a distillate eliciting a beer-like smell, in which odor attributes such as malty, fruity, and flowery (honey-like) predominated when aliquots of the extract on a strip of filter paper were evaluated. Before application of the AEDA, acidic compounds were separated from the distillate by treatment eliciting sodium bicarbonate. Preliminary experiments showed that this separation step was necessary to avoid interference with acidic compounds, such as acetic acid, during GC-O.

The major volatile constituents eliciting an odor were compounds **4** and **18** (Figure 1, left side) with malty and flowery odor notes, respectively. However, some major peaks did not show any odor activity. Sniffing of serial dilutions (AEDA) resulted in 25 odor-active compounds, which were sensorially detected in the FD factor range of 16–1024 (Figure 1, right side). The highest FD factors were determined for compounds **19** (fruity) and **25** (cooked-apple like).

In the fraction containing the acidic volatile compounds, an additional set of 16 odor-active compounds was detected by applying the AEDA (Figure 2). Among them, compounds **28** (sweaty) and **33** (caramel-like) exhibited the highest odor activities (based on their FD factor).



**Figure 1.** High-resolution gas chromatogram (left) and flavor dilution chromatogram obtained by applying the AEDA on an extract containing the neutral and basic volatiles (NBV) of Pilsner beer.

To characterize the compounds responsible for the odors perceived during GC-O, mass spectrometric data and retention indices on two columns of different polarities were determined for the respective odor-active areas. On the basis of the data obtained, reference compounds were proposed and then analyzed by GC-MS and checked for identical retention indices. If these results agreed with the data obtained for the sensorially active analyte in the beer extract, the odor qualities of the analyte and the reference compound were compared by GC-O. Finally, also the odor potencies of the analyte and reference compound were checked for identity. For instance, if the analyte elicited a strong aroma without displaying an FID signal, also the reference compound must show the same property. This procedure is necessary to exclude that, for example, a coeluting constituent in the extract will be identified on the basis of MS and RI data, even though the odor is caused by a coeluting trace constituent.

On the basis of this procedure, (*E*)- $\beta$ -damascenone and ethyl octanoate were identified with the highest FD factor among the NBV constituents (Table 2). Odorants with somewhat lower

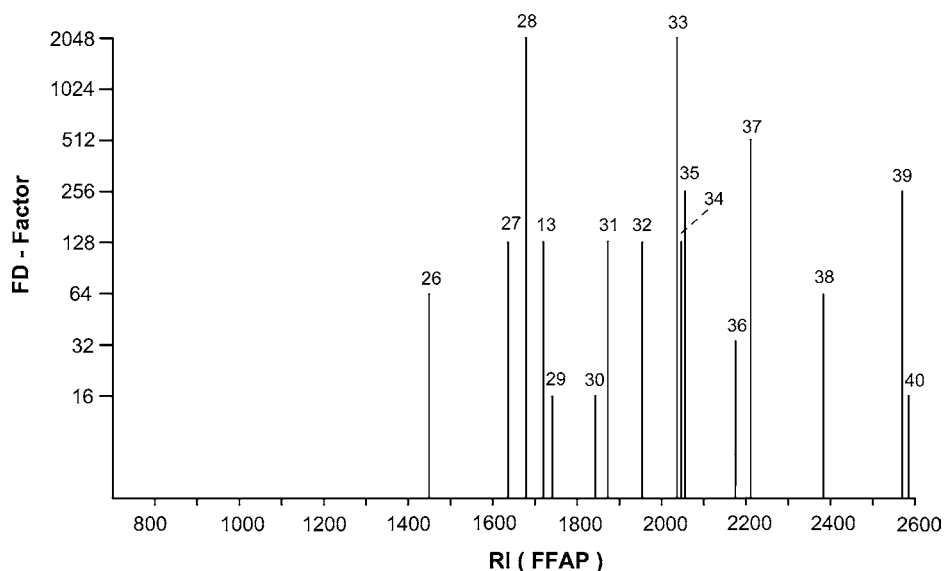
FD factors were characterized as 1,1-diethoxyethane (**3**, fruity), 2- and 3-methylbutanol (**4**, malty), ethyl butanoate (**6**, fruity), (*R*)-linalool (**17**, flowery, citrus-like), 2-phenylethanol (**18**, flowery, honey-like), and 4-vinyl-2-methoxyphenol (**22**, spicy, clove-like). Except for ethyl 4-methylpentanoate (**11**), which was identified for the first time in beer, the other compounds had earlier been identified as volatile beer constituents, but previously their odor contribution was unclear. The results of all identification experiments are summarized in Table 2.

The most odor-active compounds in the acidic fraction were identified as 2- and 3-methylbutanoic acid (**28a/b**) and 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (**33**) (Table 3). Somewhat lower FD factors were shown by 3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolon, **37**), 5-ethyl-4-hydroxy-2-methyl-3(*2H*)-furanone (**35**), and phenylacetic acid (**39**).

Very volatile odorants, such as acetaldehyde, will coelute during GC-O with the solvent used for beer extraction. Therefore, the aroma contribution of such compounds can only be estimated by directly analyzing decreasing volumes of the headspace above beer. Application of this technique, assigned as aroma dilution analysis (ADA), revealed 19 odorants in a headspace volume of 40 mL (Table 4). By GC-O of decreasing headspace volumes, taken from separate vessels but containing the same amount of beer, ethyl octanoate and ethyl butanoate followed by dimethyl sulfide, acetaldehyde, ethanol, 3-methylbutanal, and ethyl 2-methylpropanoate were characterized with the highest relative FD factors in the beer headspace. Compared to the results obtained in the AEDA (Table 2), acetaldehyde, ethanol, and dimethyl sulfide were detected by ADA as additional odorants.

**Quantitation of Odor-Active Compounds.** All techniques based on GC-O and thus, also, dilution to odor threshold technique, such as AEDA or CHARM, do not allow a final conclusion on the contribution of a single compound to the overall aroma. One main reason for this is the fact that the entire amount of a compound present in a distillate is volatilized during GC-O, whereas from a food, only the amount of an odorant present in the headspace above the food is available for the odorant receptors in our nose and, thus, is "odor-active".

Therefore, to get closer to the foods themselves, OAVs are a helpful means, because these data correlate quantitative data to odor thresholds in a matrix and, thus, address the influence of the beer matrix on the volatility of a given odorant. For this



**Figure 2.** Flavor dilution chromatogram of the acidic volatiles (AV) isolated from a Pilsner beer.

**Table 2.** Most Odor-Active (FD Factor  $\geq 16$ ), Neutral–Basic Volatile Constituents in Pilsner Beer

no. <sup>a</sup>	odorant <sup>b</sup>	odor quality <sup>c</sup>	RI <sup>d</sup> on		FD factor <sup>e</sup>	earlier identified as volatile constituent in beer (ref)
			SE-54	FFAP		
1	2-methylpropanol	malty	640	1096	32	35
2	3-methylbutanal	malty	652	925	256	36
3	1,1-diethoxyethane	fruity	729	900	512	35
4	2- and 3-methylbutanol	malty	732	1204	512	35
5	ethyl 2-methylpropanoate	fruity	760	944	256	37
6	ethyl butanoate	fruity	805	1021	512	35
7	3-methyl-2-butene-1-thiol	sulfury	818	1100	32	38
8	(S)-ethyl 2-methylbutanoate	fruity	845	1045	32	–
9	ethyl pentanoate	fruity	904	1133	32	7
10	3-(methylthio)propanol (methionol)	potato-like	906	1458	64	40
11	ethyl 4-methylpentanoate	fruity	968	1189	32	–
12	1-octen-3-one <sup>f</sup>	mushroom-like	979	1304	16	7
13	3-(methylthio)propanol (methionol)	potato-like	987	1716	16	40
14	ethyl hexanoate	fruity	1000	1235	128	35
15	phenylacetaldehyde	sweet, honey-like	1043	1643	16	41
16	unknown	flowery	1057	–	16	–
17	(R)-linalool	flowery, citrus-like	1104	1548	512	42
18	2-phenylethanol	flowery, honey-like	1115	1911	512	35
19	ethyl octanoate	fruity	1200	1431	2048	35
20	phenylethyl acetate	flowery	1260	1816	16	35
21	2-aminoacetophenone	flowery	1306	2200	64	43
22	4-vinyl-2-methoxyphenol	smoky, clove-like	1317	2206	512	9
23	ethyl 3-phenylpropanoate <sup>f</sup>	sweet	1350	1876	16	44
24	$\gamma$ -nonalactone	coconut-like	1362	2015	16	45
25	(E)- $\beta$ -damascenone	honey-like	1390	1821	2048	46

<sup>a</sup> Numbering refers to **Figure 1**. <sup>b</sup> The compound was identified by comparing it with the reference substance on the basis of the following criteria: retention index (RI) on the capillaries detailed in the table, mass spectra obtained by MS(EI) and MS(CI), and odor quality as well as odor quality and odor intensity perceived at the sniffing port. <sup>c</sup> Odor quality perceived at the sniffing port. <sup>d</sup> RI = linear retention index. <sup>e</sup> FD = flavor dilution factor. <sup>f</sup> The MS signals were too weak for an unequivocal interpretation. The compound was identified on the basis of the remaining criteria given in footnote *b*.

**Table 3.** Most Odor-Active (FD Factor  $\geq 16$ ) Acidic Volatiles in Pilsner Beer

no. <sup>a</sup>	odorant <sup>b</sup>	odor quality <sup>c</sup>	RI <sup>d</sup> on		FD factor <sup>e</sup>	earlier identified in beer (ref)
			FFAP	OV-1701		
26	acetic acid	sour, pungent	1453	790	64	35
27	butanoic acid	buttery, rancid	1629	991	128	35
28	2- and 3-methylbutanoic acid	sweaty, rancid	1671	1034	2048	47
13	3-(methylthio)propanol (methionol)	potato-like	1716	1150	128	40
29	unknown	flowery	1737	–	16	–
30	hexanoic acid	sweaty	1844	1176	16	35
31	2-methoxyphenol	smoky	1870	1232	128	37
32	unknown	sweaty	1957	–	128	–
33	4-hydroxy-2,5-dimethyl-3(2H)-furanone	caramel-like	2035	1248	2048	45
34	unknown	sweaty	2047	–	128	–
35	4-hydroxy-2- or 5-ethyl-5- or 2-methyl-3(2H)-furanone	caramel-like	2059	1317	256	48
36	unknown	spicy	2152	–	32	–
37	3-hydroxy-4,5-dimethyl-2(5H)-furanone	spicy	2206	1352	512	45
38	unknown	flowery	2380	–	64	–
39	phenylacetic acid	flowery	2561	1525	256	37
40	vanillin	vanilla-like	2580	1642	16	45

<sup>a</sup> Numbering refers to **Figure 2**. <sup>b–e</sup> Refer to **Table 2**.

reason, all 26 odorants, which had shown FD factors of 128 or above, were quantified. Except for ethanol, SIDAs previously developed by our group were used in the quantitative study.

As to be expected, ethanol was present at 40800 mg/L as the compound with the highest concentration (**Table 5**), followed by 3-methylbutanol (49.6 mg/L), 2-phenylethanol (15.1 mg/L), and 2-methylbutanol (14.4 mg/L). On the other hand, for example, 3-methyl-2-butene-1-thiol (0.01  $\mu$ g/L) and ethyl 4-methylpentanoate (0.28  $\mu$ g/L) were present in the lowest concentrations.

To correlate the quantitative data with the aroma contribution, odor thresholds of all odorants were then determined in water.

Although odor thresholds in water are, for sure, different from odor thresholds in beer, there is no appropriate matrix available, in particular, simulating the interaction of odorants with the nonvolatile constituents of beer.

In this study, the odor thresholds for (*R*)-linalool (**Table 6**), ethyl 4-methylpentanoate, diethoxyethane, and 3-methylthiopropyl were determined for the first time. All other thresholds were taken from results of our previous studies (49). The enantiomeric distribution of chiral odorants, such as linalool, has to be taken into consideration when the influence of this compound on the overall beer aroma is determined. Therefore, the enantiomeric distribution of linalool in the beer extract was

**Table 4.** Highly Volatile Odor-Active Constituents Detected by an Aroma Dilution Analysis in the Headspace above Pilsner Beer

odorant <sup>a</sup>	odor quality <sup>b</sup>	RI <sup>c</sup> on SE-54	vol <sup>c</sup> (mL)	rel FD factor <sup>d</sup>
acetaldehyde	sweet, pungent	<600	0.5	40
ethanol	ethanolic	<600	0.5	40
dimethyl sulfide	canned maize	<600	0.2	100
ethyl acetate	solvent-like	<600	20	1
2-methylpropanol	malty	600	5	4
3-methylbutanal	malty	640	0.5	40
1,1-diethoxyethane	fruity	736	5	4
2- and 3-methylbutanol	malty	740	2.5	8
ethyl 2-methylpropanoate	fruity	763	0.5	40
ethyl butanoate	fruity	806	0.1	200
3-methyl-2-butene-1-thiol	sulfury	833	10	2
(S)-ethyl 2-methylbutanoate	fruity	855	1	20
3- and 2-methylbutanoic acid	sweaty, rancid	879	10	2
ethyl pentanoate	fruity	907	10	2
ethyl 4-methylpentanoate	fruity	967	5	4
ethyl hexanoate	fruity	1000	2.5	8
(R)-linalool	flowery, citrus-like	1100	10	2
ethyl octanoate	fruity	1200	0.1	200
(E)- $\beta$ -damascenone	honey-like	1400	2.5	8

<sup>a</sup> Refer to **Table 2**. <sup>b</sup> Refer to **Table 2**. <sup>c</sup> Smallest headspace volume in which the odorant was detectable. <sup>d</sup> Calculated by dividing the largest headspace volume analyzed (20 mL) by the smallest volume, in which the odorant was detectable by GC-O.

**Table 5.** Concentrations of 26 Potent Odorants in Pilsner Beer

odorant	concn ( $\mu$ g/L)	SD <sub>M</sub> <sup>b</sup> ( $\mu$ g/L)
ethanol	40800000	$\pm$ 489600
3-methylbutanol	49600	$\pm$ 744
2-phenylethanol	15100	$\pm$ 257
2-methylbutanol	14400	$\pm$ 226
acetaldehyde	5100	$\pm$ 536
butanoic acid	1800	$\pm$ 104
3-(methylthio)propanol	991	$\pm$ 44
3-methylbutanoic acid <sup>c</sup>	855	$\pm$ 62
2-methylbutanoic acid <sup>c</sup>	438	$\pm$ 35
4-hydroxy-2,5-dimethyl-3(2H)-furanone	312	$\pm$ 32
phenylacetic acid	257	$\pm$ 15
ethyl hexanoate	205	$\pm$ 4
ethyl butanoate	198	$\pm$ 4
ethyl octanoate	160	$\pm$ 3
4-vinyl-2-methoxyphenol	137	$\pm$ 10
dimethyl sulfide	59	$\pm$ 5
diethoxy ethane	50	$\pm$ 4
(R)-linalool	45	$\pm$ 3
4-hydroxy-2- or 5-ethyl-5- and 2-methyl-3(2H)-furanone	19	$\pm$ 2
3-methylbutanal	4.0	$\pm$ 0.4
ethyl 2-methylpropanoate	3.2	$\pm$ 0.2
(E)- $\beta$ -damascenone	2.3	$\pm$ 0.3
2-methoxyphenol	2.0	$\pm$ 0.2
3-hydroxy-4,5-dimethyl-2(5H)-furanone	1.0	$\pm$ 0.2
ethyl 4-methylpentanoate	0.28	$\pm$ 0.03
3-methyl-2-butene-1-thiol	0.01	$\pm$ 0.001

<sup>a</sup> Mean value obtained by analyzing three different batches of the same brand. <sup>b</sup> Standard deviation of the mean value. <sup>c</sup> Isomers were separately quantified using *m/z* 74 (2-methylbutanoic acid) and *m/z* 60 (3-methylbutanoic acid) for differentiation.

determined by application of two-dimensional HRGC-MS using a chiral column in the second dimension. In linalool, the (R)-enantiomer predominated (82%). The enantiomeric distribution was also determined for ethyl 2-methylbutanoate (**8**, fruity), which occurred in beer almost as pure (S)-enantiomer (98%).

By dividing the concentrations by the odor thresholds, the OAVs summarized in **Table 6** were finally calculated. On the

**Table 6.** Orthonasal Odor Thresholds and Odor Activity Values (OAV) of 26 Beer Odorants

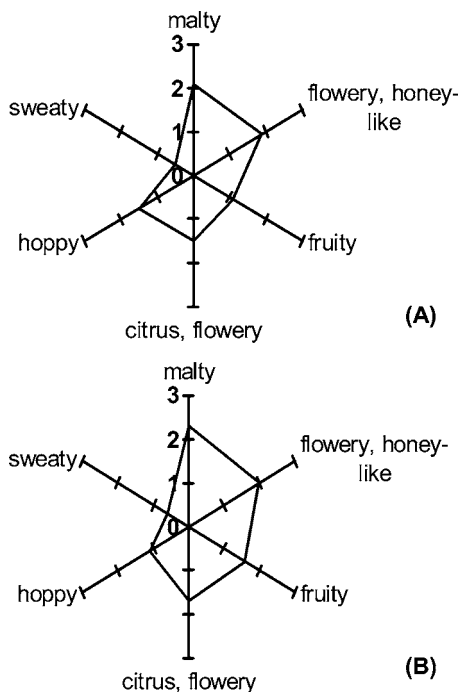
odorant	odor threshold in water <sup>a</sup> ( $\mu$ g/L)	OAV <sup>b</sup>
ethanol	24900	1639
(E)- $\beta$ -damascenone	0.004	575
(R)-linalool	0.14 <sup>c</sup>	321
acetaldehyde	25	204
ethyl butanoate	1.0	198
ethyl 2-methylpropanoate	0.02	160
ethyl 4-methylpentanoate	0.003 <sup>c</sup>	93
dimethyl sulfide	1.0	59
3-methylbutanol	1000	50
2-methylbutanol	320	45
ethyl hexanoate	5	41
4-hydroxy-2- or 5-ethyl-5- or 2-methyl-3(2H)-furanone	1.15	17
2-phenylethanol	1000	15
4-hydroxy-2,5-dimethyl-3(2H)-furanone	25	13
diethoxyethane	4.9 <sup>c</sup>	10
3-methylbutanal	0.4	10
3-methyl-2-butene-1-thiol	0.0012	8
3-(methylthio)propanol (methionol)	250 <sup>c</sup>	4
3-hydroxy-4,5-dimethyl-2(5H)-furanone	0.3	3
butanoic acid	1000	2
ethyl octanoate	70	2
3-methylbutanoic acid	740	1
4-vinyl-2-methoxyphenol	100	1
2-methylbutanoic acid	540	<1
2-methoxyphenol	2.5	<1
phenylacetic acid	1000	<1

<sup>a</sup> Odor threshold taken from ref 49. <sup>b</sup> OAVs were calculated by dividing the concentrations by the respective odor thresholds in water. <sup>c</sup> Odor thresholds in water were determined in this study.

basis of the results obtained, ethanol, (E)- $\beta$ -damascenone, (R)-linalool, acetaldehyde, ethyl butanoate, and ethyl 2-methylpropanoate showed the highest OAVs (>100). In addition, ethyl 4-methylpentanoate, 3-methylbutanal, dimethyl sulfide, 2- and 3-methylbutanol, and ethyl hexanoate can also be proposed as important contributors to the overall aroma. Three compounds, namely, 2-methylbutanoic acid, 2-methoxyphenol, and phenylacetic acid, should not contribute much to the aroma, because their OAVs were below 1 (**Table 6**).

**Aroma Simulation.** To establish that the odorants showing high OAVs are actually the key odorants of Pilsner beer, an aroma model was prepared by the following procedure: All odorants with OAVs >1 (altogether, 23 aroma compounds including ethanol) were dissolved in tap water in concentrations equal to those determined in the Pilsner beer. Before the addition of odorants, the water was enriched with carbon dioxide and adjusted to a pH value of 4.3. A sensory panel consisting of 10 panelists performed descriptive profiles tests, and odor attributes of the original beer and the model mix were compared. Additionally, all panelists judged the similarity between the overall odor of both samples on a given scale from 0 = no similarity to 3 = very good similarity. The results of the sensory evaluation are displayed in **Figure 3**. A comparison of the aroma profile of the beer and the aroma recombine showed a clear similarity, which was judged with 2.5 points of 3.0 (100%).

The results of the aroma simulation experiment show that it is possible to create the typical aroma of the investigated beer by combining 23 odorants in the exact concentrations as occur in beer using water as the matrix. This fact demonstrates that all important odorants of the investigated Pilsner beer have been detected by applying AEDA and ADA and that the quantitation



**Figure 3.** Aroma profile analysis of the fresh Pilsner-type beer (A) and the aroma recombine (B).

experiments were performed with highest accuracy. Furthermore, the data suggest that the nonvolatile fraction of beer does not seem to be significantly involved in the modification of orthonasal beer aroma characteristics, for example, by influencing the aroma release. Otherwise, the simulation would not have been successful in such a simple matrix as carbonated water.

Previous studies on pale lager (9) beer had proposed that (*E*)- $\beta$ -damascenone, 3- and 2-methylbutanoic acid, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, 3-methylbutanol, ethyl butanoate, 2-phenylethanol, and 4-vinyl-2-methoxyphenol play an important role in beer flavor. In this study, the importance of these compounds was confirmed also for the overall flavor of a Bavarian Pilsner-type beer.

In the literature, many compounds have been proposed to be responsible for the hoppy flavor of Pilsner beer, for example, linalool, linalool oxides, 3-methyl-2-butene-1-thiol, citronellol, geraniol, geranyl acetate,  $\alpha$ -terpineol,  $\alpha$ -eudesmol, T-cadinol, humulene epoxide I, humulenol II, and humuladienone (50, 51). However, with the exception of linalool, none of these compounds were detected by GC-O in the Bavarian Pilsner beer during this investigation. With an OAV of 321 (*R*)-linalool appeared as one of the most important odorants in Pilsner beer, but was absent in a GC-O study on pale lager beer (9). In previous studies, it has been shown that (*R*)-linalool is one of the key odorants in hops (52) and is transferred into the final beer during kettle boiling (53). For this reason, it might be speculated that (*R*)-linalool plays an important role, in particular, in the aroma of Pilsner-type beers.

Besides linalool, another hop-specific compound was found as a contributor to Pilsner flavor: For the first time, ethyl 4-methylpentanoate could be detected in Pilsner beers. This compound has been identified previously in hops (52) but has not yet been discussed in the literature in context with the hoppy note of Pilsner type beers. This odorant may possibly be transferred from hops into the beer during boiling of wort. However, to get more information about the influence of hops on beer flavor, it would be necessary to systematically compare

odorants of hops as well as of unhopped and hopped beers by applying the approach described in this study.

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