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Antimicrobial testings, gas chromatographic analysis and olfactory evaluation of an essential oil of hop cones (*Humulus lupulus* L.) from Bavaria and some of its main compounds

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Summary

The essential oil of hop cones (*Humulus lupulus* L.) was analyzed by GC and GC-MS to assess the composition of volatiles and to evaluate olfactorially the principle aroma compounds. Myrcene (45.3%), α-humulene (31.2%) and β-caryophyllene (9.8%) were found to be the main constituents of this *H. lupulus* sample.

The essential hop oil, the 3 main compounds and three minor concentrated constituents, isobutyl isobutyrate, geraniol and β-pinene, were tested for their antimicrobial activities against some different strains of Gram-positive and Gram-negativebacteria as well as the yeast *Candida albicans* by using agar diffusion and agar dilution method, respectively. The same was done using the phenolic aroma compound eugenol and the synthetic antibiotics Ciproxin^R, Lidaprim^R and

tetracycline hydrochloride as reference-substance. It was found that the essential *H. lupulus* oil showed antimicrobial activities against the Gram-positive-bacteria *Staphylococcus aureus* and *Enterococcus faecalis*, the Gram-negative-bacteria *Escherichia coli* and *Salmonella* sp. as well as the yeast *Candida albicans*. This result was correlated to the antimicrobial testing of the mentioned main and minor constituents of the essential hop oil as well as of the 4 reference-compounds.
The obtained data were discussed to get more insight into the influence of pure aroma compounds on the antimicrobial activities of essential oils.

Keywords

Humulus lupulus L., cones, essential oil, composition, antimicrobial testing, olfactory evaluation, GC, GC-MS

Introduction

In continuation of our research work in the field of combined data interpretation of antimicrobial testing (agar diffusion and agar dilution methods) and gas chromatographic analysis (GC and GC-MS) as well as olfactory evaluation of aroma samples, including pure compounds, essential oils and extracts [1-10], a sample of essential oil of hop cones (*Humulus lupulus* L., Cannabaceae) from Bavaria was investigated.

The composition of volatiles and odor-active compounds of hop cones and the corresponding essential oils of various origins were reported in many papers [11-20] with prevailing sesquiterpenes (e.g. α-humulene and β-caryophyllene), monoterpenes (e.g. myrcene, linalool and geraniol) as well as some C_6-, C_{10}- and C_{11}-compounds as main aroma components.

Despite the importance of hop and its essential oil for the aroma and bitterness of beer production, few data of biological activities such as sedative effects caused by prenylflavonoids [21], as well as antimycobacterial [22] and antibacterial [23] effects were published, but no detailed information about the influence of some main and minor compounds of the essential oil on their antimicrobial activities are available until now.

Therefore, the objectives of this research work were to analyze the composition of the essential hop oil from Bavaria by using gas chromatographic methods (GC and GC-MS), to evaluate olfactorially the sample, and to compare this data with aroma impressions of the constituents. The essential *H. lupulus* oil and some main and minor compounds were also tested for antimicrobial activities by means of the usual methods agar diffusion and agar dilution with various strains of microorganisms [1-10].

As result of the combined data interpretation, the above mentioned influence of the main and minor components on the antimicrobial effects of the essential hop oil can be ascertained. On basis of these data, the systematic investigation of aroma compounds and odorous samples (essential oils and extracts) will be continued in a running, international project.

Results and discussion

The essential oil of *Humulus lupulus* L. cones from Bavaria was olfactorially evaluated by perfumers and aroma-chemists as follows: Herbal-green, spicy-aromatic, somewhat bitter and sweet with connotions of valerian.

To control the efficiency of the used microbiological testing methods, eugenol as a phenolic compound with well-known antimicrobial activity against many strains of microorganisms was tested. Eugenol shows excellent effects against all strains using both methods, agar diffusion and agar dilution (see **Table 1**).

In addition, the synthetic antibiotics Ciproxin^R, Lidaprim^R and tetracycline hydrochloride were used as reference substances for the antimicrobial tests. Tetracycline hydrochloride was effective against all Gram-positive and Gram-negative bacteria, surprisingly Ciproxin^R showed no antimicrobial activity against *Salmonella* sp. and Lidaprim^R against *Klebsiella pneumoniae* (**Table 1**).

The essential oil of *Humulus lupulus* L. cones from Bavaria showed high activity against both Gram-positive bacteria (*Staphylococcus aureus* and

Enterococcus faecalis), the Gram-negative bacteria *Escherichia coli* and *Salmonella* sp.) and the yeast *Candida albicans*, while no effects were observed against the Gram-negative-bacteria *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Klebsiella pneumoniae* (**Table 1**).

The monoterpenic target-compound myrcene was found to possess high antimicrobial activities only against *Escherichia coli* and *Proteus vulgaris*, but showed no effects against all other microorganisms of the test-series. Medium antimicrobial effects of the sesquiterpenic hydrocarbon α-humulene against the Gram-positive bacteria *Enterococcus faecalis* and Gram-negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella* sp. was found, whereas for the second tested sesquiterpenic hydrocarbon β-caryophyllene high antimicrobial activities against the Gram-negative-bacteria *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Salmonella* sp. as well as medium effects against the Gram-positive bacteria *Enterococcus faecalis* and the Gram-negative bacteria *Escherichia coli* could be observed. The monoterpenic hydrocarbon β-pinene showed high/medium antimicrobial effects against both Gram-positive bacteria *Staphylococcus aureus, Enterococcus faecalis* and a high activity against the Gram-negative-bacteria *Escherichia coli* and *Proteus vulgaris* as well as a medium one against *Candida albicans.* With the monoterpenic alcohol geraniol the only target-compound with medium up to high antimicrobial effects against all microorganisms was tested. Isobutyl isobutyrate was the second target-compound with medium up to high activities against all microorganisms except for the Gram-positive bacteria *Staphylococcus aureus,* where no effects were found (see **Table 1**).

A comparison of these data show that the antimicrobial effects of the essential hop oil against *Staphylococcus aureus* can be correlated only to the constituents geraniol and β-pinene and the effectivity against the yeast *Candida albicans* to geraniol, isobutyl isobutyrate and β-pinene. The antimicrobial activities of the essential *H. lupulus* oil against *Enterococcus faecalis*, *Escherichia coli* and *Salmonella* sp. results of medium up to high effects of some target-compounds.

	Staphylococcus aureus		*Enterococcus faecalis*		*Escherichia coli*		*Pseudomonas aeruginosa*	
	1.5×10^{13} cfu/cm^3		1×10^{13} cfu/cm^3		1×10^{11} cfu/cm^3		2×10^{13} cfu/cm^3	
Sample	IZ mm	MIC ppm	IZ mm	MIC ppm	IZ mm	MIC ppm	IZ mm	MIC ppm
Hop oil	9	600	8	600	7	600	-*	-
Myrcene	-	-	-	-	7	600	-	-
α-Humulene	-	-	10	6	9	60	9	60
β-Caryophyllene	-	-	11	60	10	6	8	600
Geraniol	15	60	12	60	15	60	11	60
Isobutyl isobutyrate	-	-	7	600	7	600	7	600
β-Pinene	10	600	10	60	8	600	-	-
Eugenol	15	6	10	600	12	6	10	600
Ciproxin^{R}	35	< 0.3	33	< 0.3	22	0.3	32	0.3
Lidaprim^{R}	27	< 60	27	< 60	11	60	-	-
Tetracycline Hydrochl.	15	< 3	22	60	11	600	15	600

*no inhibition observed

	Proteus vulgaris		*Klebsiella pneumoniae*		*Salmonella* sp.		*Candida albicans*	
	6×10^{13} cfu/cm^3		2×10^{13} cfu/cm^3		3×10^{12} cfu/cm^3		1.9×10^{13} cfu/cm^3	
Sample	IZ mm	MIC ppm	IZ Mm	MIC ppm	IZ mm	MIC ppm	IZ mm	MIC ppm
Hop oil	-	-	-	-	8	600	10	600
Myrcene	8	600	-	-	-	-	-	-
α-Humulene	-	-	-	-	11	60	-	-
β-Caryophyllene	8	600	-	-	8	600	-	-
Geraniol	12	60	10	600	10	60	25	60
Isobutyl isobutyrate	9	60	10	600	10	60	7	600
β-Pinene	8	600	-	-	-	-	10	60
Eugenol	12	60	8	600	27	0.6	27	0.6
Ciproxin^{R}	25	0.3	25	0.3	-	-	-	-
Lidaprim^{R}	23	60	-	-	8	60	-	-
Tetracycline Hydrochl.	16	600	20	60	10	600	-	-

*no inhibition observed

Tab. 1. Antimicrobial activities of the essential hop oil from Bavaria, some constituents and reference compounds

Using gas chromatography (GC-FID and GC-MS with 2 columns of different polarity) in combination with olfactoric evaluations, the purity of the target compounds as well as the composition of the essential hop oil was investigated.

In general, the purity of all tested components was found to be higher than 96% with a range from 96.8% for β-pinene up to 99.7% for α-humulene. The olfactoric evaluation with characteristic attributes for each sample proves the high purity and quality of these compounds (see **Table 2**).

Sample	%-Peak area*	Odor
Isobutyl isobutyrate	99.3[a]	Etheral, estery, fruity
Myrcene	98.9[b]	Fresh-terpeny, citric- and metallic-note
β-Pinene	96.8[a]	Terpenic, harsh-pine-like, weak herbal
Geraniol	99.5[a]	Floral, rose-like, sweet-fruity-notes
α-Humulene	99.7[b]	Soft-woody, reminding of fresh earth
β-Caryophyllene	97.3[a]	Dry- woody-spicy, somewhat oily

*relative %-peak-area using GC-FID with an [a]apolar OV-5-column or [b]polar carbowax-column

Tab. 2. GC analysis and olfactoric evaluation of target compounds

The gas chromatographic investigation of the essential oil of *Humulus lupulus* L. cones from Bavaria resulted in (see **Table 3**) the main compounds myrcene (45.3%), α-humulene (31.2%) and β-caryophellene (9.8%) as well as following compounds with concentrations higher than 1.0% (calculated as relative %-peak-area using GC-FID with an apolar column): geraniol (1.7%), δ-cadinene (1.7%), isobutyl isobutyrate (1.3%) and β-pinene (1.2%).

This result is also in accordance to published data of previous chemical analyses of different essential hop oils [11-20].

The olfactory data for each constituent of the essential hop oil by using the description given by perfumers and aroma-chemists as well as the correlation with published odor attributes [24-28] is also given (**Table 3**).

The odor-attributes of main and minor compounds of the essential *H. lupulus* oil are responsible for the characteristic odor-impression of this sample and additionally proof the high quality of this essential hop oil from Bavaria.

As a final remark of this combined investigation of antimicrobial activities, gaschromatographic (GC-FID and GC-MS) analysis and olfactory analysis of the essential oil of *Humulus lupulus* L. cones from Bavaria and some target compounds, we can state that the sample was described with a characteristic odor for essential hop oils and as main compounds myrcene, α-humulene and β-caryophyllene were identified. The essential hop oil showed high antimicrobial activities against Gram-positive-bacteria *St. aureus* and *Enterococcus faecalis*, Gram-negative-bacteria *Escherichia coli* and *Salmonella.* sp. as well as the yeast *Candida albicans*. These effects do not result from antimicrobial activities of a single constituent, but from the combination of main and minor compounds.

Compound	RI	Concen-tration	Odor in accordance to [24-28]
cis-3-Hexenol	859	tr[1]	fresh-green, leafy-like, grassy,
α-Pinene	939	0.1	terpenic, harsh-pine-like, weak herbal
Isoamyl propionate	953	0.1	fruity (pineapple-notes)
Sabinene	975	0.5	woody, terpenic, slightly citrus, weak spicy-herbal
Isobutyl isobutyrate	979	1.3	etheral, estery, fruity
β-Pinene	982	1.2	terpenic, harsh-pine-like, weak herbal
Myrcene	**991**	**45.3**	**fresh, terpenic, somewhat citrus and metallic**
α-Phellandrene	1003	tr	fresh, terpenic, citrus-like,
α-Terpinene	1017	0.2	terpenic, weak citrus-like
p-Cymene	1023	0.6	harsh terpene-like, limonen like
Methyl heptanoate	1025	0.4	green-fruity, green berry-like,
Limonene	1029	0.3	weak lemon and orange -like
β-Phellandrene	1031	0.2	fresh, somewhat minty, weak citrus
Amyl isobutyrate	1039	0.3	fruity, banana- and pineapple-notes
2-Methylbutyl isobutyrate	1042	0.5	sweet-fruity, tropical –notes and banana
trans-β-Ocimene	1049	0.6	fresh-terpenic, weak citrus-herbal
γ-Terpinene	1060	tr	citrus-terpenic, lime with a touch of herbs
Terpinolene	1089	tr	pleasant, sweet-piney, somewhat turpentine
Linalool	1097	0.2	fresh, floral and woody
Amyl 2-methylbutyrate	1124	0.2	exotic-fruity
Methyl octanoate	1127	0.1	green-fruity
Geraniol	1256	1.7	floral, rose-like, sweet- and fruity-notes
Methylnonyl ketone	1267	0.4	oily, citrus, orris connotations
Methyl geraniate	1323	0.3	Floral- and fruity-notes
α-Copaene	1377	0.2	weak woody, herbal
β-Cubebene	1389	0.3	weak woody, herbal, spicy
β-Caryophyllene	**1419**	**9.8**	**dry, woody-spicy, somewhat oily**
α-Humulene	**1455**	**31.2**	**soft-woody, reminding to fresh earth**
Geranyl propionate	1471	0.1	sweet-rose, weak fruity
β-Selinene	1492	0.6	weak spicy
α-Selinene	1497	0.2	weak spicy, balsamic, mild
γ-Cadinene	1513	0.4	dry-woody
δ-Cadinene	1524	1.7	dry-woody, mild
Geranyl isobutyrate	1517	0.1	sweet-fruity-rose
α-Cadinene	1540	0.3	woody, weak fruity
Spathulenol	1578	tr	woody, herbal
Caryophyllene oxide	1583	0.2	warm, soft-woody
Humulene oxide	1607	0.2	warm-woody, weak earthy

[1]trace compound less than 0.1%

Tab. 3. Composition of the essential hop oil (%-peak area of GC using an apolar fused silica OV-5-type column)

Experimental

Samples

The essential hop oil is a product of Kurt Kitzing Co., Germany as follows: Fresh cones of *Humulus lupulus* L. (Cannabaceae) from Bavaria (product-no 800387).

Target and reference compounds are products from 1.) Kurt Kitzing Co.: Isobutyl isobutyrate (700362), β-caryophyllene (800173), geraniol (800351), β-pinene (800140) and eugenol (800316); 2.) Sigma-Aldrich Austria Co. (Vienna): α-Humulene (6753-98-6), myrcene (123-35-3) and tetracycline hydrochloride (achromycine hydrochloride – 25g, T3383-25G); 3.) Bayer Austria Co. (Vienna): Ciproxin^{R} 500mg-tablet (1 tablet = 582mg ciprofloxacine hydrochloride/water); 4.) Nycomed Austria Co. (Vienna): Lidaprim^{R}-infusion-bottle (250mL contains 800mg sulfametrol and 160mg trimethoprim).

Antimicrobial testings

As test microorganisms, the Gram-positive-bacteria *Staphylococcus aureus* ATCC 25093 and *Enterococcus faecalis* (clinical isolated), the Gram-negative-bacteria *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 25517, *Klebsiella pneumoniae* (clinical isolated), *Proteus vulgaris* (clinical isolated) and *Salmonella* sp. (clinical isolated) as well as the yeast *Candida albicans* ATCC 10231 – all products from the National Bank of Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria – were used.

The antimicrobial activity was studied by two methods: Agar diffusion disc method using Whatman No. 1 filter paper discs (6mm) and quantities of 6μl of the sample each. After cultivation of the bacteria and the yeast at 37°C for 24^{h} the diameter of the inhibition zone (IZ) was measured [1-10] as well as agar serial tube dilution method which results as minimum inhibitory concentration (MIC) in accordance to [1-10] as follows: The essential oil and reference compounds were added to brine, containing 1.0% (v/v) Tween 80 at the appropriate volumes to produce final concentrations of the samples in the range of 1-1000 ppm; the Petri dishes were inoculated by pipetting $0.1cm^3$ of the desired culture and 6 µL of the samples as well as the reference compounds (the tablettes of Ciproxin^R were added as solution in saline at a quantity of 300 µg and 30 µg) on paper discs (6 mm) and then incubated at 37°C for 24h.

Olfactoric evaluation

All investigated samples were olfactorically evaluated by professional perfumers and/or olfactively trained chemists and the aroma described as mentioned in Table 1 and 2 and correlated with published odor impressions [24-28].

GC/FID

A GC-14A with FID and integrator C-R6A-Chromatopac (Shimadzu Co.) resp. a GC-3700 with FID (Varian Co.) and integrator C-R1B-Chromatopac (Shimadzu Co.) were used; carrier gas: hydrogen; injector-temp.: 250°C; detector-temp.: 300°C; temp.-progr.: 40°C/5 min. to 280°C/5 min. with a heating-rate of 6°C/min.; columns: 30m x 0.32mm bonded FSOT-RSL-200 (OV-5-type) fused silica (film thickness: 0.25 micron; Biorad Co.) and 60m x 0.32mm bonded Stabilwax (film thickness: 0.50micron; Restek Co.); quantification by %-peak-area-calculation and correlation of data cited [1-10].

GC/MS

A GC-17A with QP5000 (Shimadzu Co.) and data-system Compaq-ProLinea (Class5k-software, Shimadzu Co.), a GC-17A with QP5050 (Shimadzu Co.) with data-system PentiumII (Class5k-software, Shimadzu Co.), a GC-HP5890 with HP5970-MSD (Hewlett-Packard Co.) and Pentium-PC (Böhm Co., ChemStation-software) resp. a GCQ (Finnigan-Spectronex Co.) and Gateway-2000-PS75 (Siemens-Nixdorf Co.; GCQ-software) were used; carrier gas: helium; injector-temp.: 250°C; interface-heating: 300°C; ion-source-heating: 200°C; EI-mode, 70 eV; scan-range: 41-550 amu; other parameters see GC/FID-part. Mass spectra correlations with Wiley-, NBS-, NIST- and private library spectra on-line as well as correlation of data cited [1-10].

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