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A Comparison of Quantitative Composition and Bioactivity of Oils Derived from Seven North American Varieties of Hops (*Humulus lupulus* L.)

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Abstract: Seven commercial hop (*Humulus lupulus* L.) oils originating from a selection of North American hop varieties (Amarillo, Azacca, Cascade, Centennial, Chinook, Saaz, and Ahhhroma) and six homemade hop oils hydrodistilled from the same commercial hop pellets (except Ahhhroma) were compared. Seven terpenes regarded as hop oil markers (i.e., α-pinene, β-pinene, β-myrcene, β-ocimene, limonene, β-caryophyllene, and α-humulene) and methyl heptanoate were identified and quantified by GC–MS and GC-FID. The antioxidant potential of the commercial hop oil samples was evaluated using electron paramagnetic resonance (EPR) spectroscopy, while their components' antibacterial (against *Aliivibrio fischeri*) and enzyme (α-glucosidase and lipase) inhibition activities were screened using high-performance thin-layer chromatography (HPTLC)-based assays. A distinct feature of five of the commercial hop oils (except Saaz and Ahhhroma) was relatively high contents of β-myrcene (between 4.21 and 6.40 μg mg^{-1} hop oil). Azacca, Cascade, and Centennial hydrodistilled oils had perceptibly higher contents of β-caryophyllene than the rest, and most of them (except Chinook) contained relatively high amounts of α-humulene. Differences between the terpene profiles of the commercial and homemade hydrodistilled hop oils suggested that the commercial hop oils were derived from hop cones in a process different from hydrodistillation. The oils showed relatively low antioxidant potential, comparable to that of popular beers and white wines. The highest antioxidant potential was observed in Ahhhroma oil, while it was very low in Centennial oil, and no antioxidant potential was observed in Cascade and Saaz oils. The developed streamlined workflow, including parallel HPTLC-directed bioassays and HPTLC—TLC–MS Interface—SPME–GC–MS, enabled the identification of β-myrcene, dimyrcenes, β-farnesene, and 2-methylbutyl isobutyrate as anti-obesity compounds and β-farnesene, β-myrcene, and 2-methylbutyl isobutyrate as weak antibacterial hop oil components.

Keywords: antibacterial effect; anti-obesity effect; antioxidants; electron paramagnetic resonance spectroscopy; gas chromatography; high-performance thin-layer chromatography–effect-directed analysis; hop oil; *Humulus lupulus* L.

Citation: Móricz, Á.M.; Bartoszek, M.; Polak, J.; Marczewska, P.; Knaś, M.; Böszörményi, A.; Fodor, J.; Kowalska, T.; Sajewicz, M. A Comparison of Quantitative Composition and Bioactivity of Oils Derived from Seven North American Varieties of Hops (*Humulus lupulus* L.). *Separations* **2023**, *10*, 402. https://doi.org/10.3390/separations10070402

Academic Editor: Paraskevas D. Tzanavaras

Received: 1 June 2023
Revised: 3 July 2023
Accepted: 10 July 2023
Published: 12 July 2023

1. Introduction

Hop oil is among the most complex plant oils and accounts for up to 4% of the dried hop weight [1]. It can be obtained by hydrodistillation (essential oil) or extraction from hop (*Humulus lupulus* L.) cones and then is analysed for its volatile and non-volatile components [2]. Most of the volatile components of hop oil belong to the three chemical groups (hydrocarbons, oxygenated, and sulphur-containing compounds), and the non-volatile ones include carboxylic acids, hop resins, carbohydrates, amino acids, and polyphenols [3]. Gas chromatography (GC), implemented with increasingly more precise detection systems, has been the main analytical tool used to study the volatile components of hop oil since the early days of this technique, and nowadays their number is estimated to be higher than 1000 [4]. An extensive and informative review on the composition of hop oil and the chemical and biological properties of individual oil components is given in reference [5]. This review mentions the curative properties of hop oil (sedative, anti-cancer, analgesic, and anti-inflammatory) [6,7] and also harmful ones (allergic reactions, neurotoxicity, and abortion when used at higher doses) [8]. Hops and hop-derived products have also been tested for their antioxidant [9,10] and antibacterial [11,12] properties.

The volatile and non-volatile components of hop oil are largely responsible for the taste, flavour, and aroma of beers [13], although this effect is not straightforward and depends upon numerous reactions among different volatile and non-volatile components. These complex and still largely unknown processes can be classified as synergistic, antagonistic, additive, and masking [14]. Structural transformation of compounds belonging to the non-volatile fraction occurs at the wort boiling step, but the most important transformation is the isomerisation of hop α-acids to iso-α-acids, which are responsible for the bitter taste of beers [15]. The contents of volatile and non-volatile hop oil components depend on several factors, such as the genetic characteristics of a given hop variety [16,17], the plant rootstock age, geographical location of the hop plantation, climatic and soil conditions, and harvest time [18,19].

The traditional beer brewing processes are kettle, late, and dry hopping, and these processes differ regarding the stage at which hops are introduced to the beer brewing system. A drawback of kettle hopping is that up to 85% of the components of the most volatile fraction evaporate from the system due to thermal exposure [20,21]. This drawback can be mitigated by applying late hopping, which results in strongly aromatic yet moderately bitter beers [21,22]. This compromise can be circumvented by dry hopping (i.e., adding hops to the beer fermentation vessel, or cold lagering beer), which helps to achieve a kind of balance among beer flavour, aroma, and bitterness [23]. Hops available on the global market can be divided into two groups: bittering hops (characterized by high contents of bitter acids) [14] and finishing hops (rich in volatile aroma components) [11,24–26]. Summing up, the steering of traditional beer production needs outstanding expertise and is far from an easy task [16,27].

Seasoning of post-fermentation beers with commercially available hop oils hydrodistilled from different hop varieties is regarded as an emerging alternative to dry hopping and manufacturers of commercial hop oils carefully see to it that hop cones are harvested during the best phase of the hop vegetation season [28]. This approach is a relatively new option of considerable economic and environmental importance, as it saves energy and limits brewery waste. Reference [29] reports on the GC-flame ionization detection (FID) analysis of hop oils hydrodistilled in-home from ten different hop cultivars of Belgian, Dutch, German, and North American types, and a comparison is made of their chemical composition. A similar comparison of hop oil composition for seven different hop cultivars of European and North American types based on GC/mass spectrometry (MS) results is given in reference [27]. Neither reference [29] nor [30] used phytochemical standards of the hop oil components for quantification purposes and the contents of individual components were comparatively assessed against the FID-derived total sum of peak areas [29] or the MS-derived total ion current [30]. To our best knowledge, so far, quantification of the components of different commercially available hop oils has not been performed based

on chemical or phytochemical standards, except for sporadic cases (such as the use of isobutyric, 2-methylisobutyric, and isovaleric esters, as reported in reference [24]).

High-performance thin-layer chromatography in combination with effect-directed analysis (HPTLC–EDA) is an efficient tool to obtain the bio-profiles of samples with various matrices [31], including essential oils [32]. The results obtained can point out the separated compounds responsible for antimicrobial and/or enzyme inhibitory activities, thus allowing their further characterization and identification. So far, HPTLC has been used for the analysis of hops or beers in a few cases only. For example, an HPTLC method was developed to quantify xanthohumol in hops [33], and the antibacterial components of aqueous and ethanol hop extracts have been traced by TLC-directed bioautography using *Bacillus subtilis* and *Escherichia coli* [34]. Demonstration of phenolic, antioxidant, antibacterial, and acetylcholinesterase inhibitory profiles of various beers was carried out utilising HPTLC and HPTLC–EDA [35,36]. Compounds with an anti-obesity effect can be screened using HPTLC–α-glucosidase [37,38] and HPTLC–lipase [39] assays. The inhibition of α-glucosidase delays the digestion of polysaccharides, thus reducing the release of glucose [40]. Pancreatic lipase inhibition results in reduced fat absorption [41]. Therefore, such inhibitors improve energy metabolism in obese individuals.

The aim of this study was to compare the chemical and biological profiles of various hop oils. Using GC/MS and GC-FID, we compared the contents of seven terpenes and methyl heptanoate, considered chemical markers, of seven commercial North American hop oils and six hop oils hydrodistilled from commercial hop pellets. To our best knowledge, these are the first quantification results with selected compounds contained in hop oils derived from seven North American hop varieties. For all commercial hop oils considered in this study, their antioxidant potential was evaluated with the use of electron paramagnetic resonance (EPR) spectroscopy, and their enzyme (α-glucosidase and lipase) inhibitory and antibacterial profiles were revealed using HPTLC–EDA. The components in the active HPTLC zones were further characterized by HPTLC—TLC–MS Interface–SPME—GC–MS.

2. Materials and Methods

2.1. Materials

Commercial hop oils of the following American hop varieties (*Humulus lupulus* L.) were examined: Amarillo, Azacca, Cascade, Centennial, Chinook, Saaz, and Ahhhroma. Hop oils were hydrodistilled in our laboratory from hop pellets of the same varieties (except for Ahhhroma, which was not available). Commercial hop oils and hop pellets were provided by Hopzoil™ (Glacier Hops Ranch, Whitefish, MT, USA). Analytical purity grade standards (α-pinene, β-pinene, β-myrcene, methyl heptanoate, β-ocimene, d-limonene, β-caryophyllene, α-humulene, β-farnesene, and geraniol) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Working samples of commercial and hydrodistilled hop oils and terpene standards were prepared for GC analysis as 5 mg mL^{-1} solutions in *n*-hexane.

Standard solutions of Trolox (TR) (Acros Organics, Geel, Belgium), gallic acid (GA), and ascorbic acid (AA) (P.P.H. POCh, Gliwice, Poland) were prepared for the needs of EPR spectroscopic analysis as a quantitative measure of the antioxidant activity of the commercial hop oils.

Glass and aluminium-backed HPTLC silica gel 60 F_{254} layers (all 20 cm × 10 cm) and lipase (from porcine pancreas) were purchased from Merck (Darmstadt, Germany). Solvents of analytical grade were obtained from Molar Chemicals (Halásztelek, Hungary). Bovine serum albumin, *p*-anisaldehyde, 1,1-diphenyl-2-picrylhydrazyl (DPPH•), and Fast Blue B Salt were obtained from Sigma-Aldrich (Budapest, Hungary). α-Glucosidase (yeast maltase) was obtained from NOACK (Budapest, Hungary). 2-Naphthyl-α-D-glucopyranoside, 4-methylumbelliferyl-α-D-glucopyranoside, and 1-naphthyl acetate were purchased from Biosynth (Bratislava, Slovakia). *Aliivibrio fischeri* (DSM 7151), a naturally luminescent marine bacterium strain, was supplied by Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures (Berlin, Germany).

2.2. Hydrodistillation of Hop Pellets

Twenty-five grams of hop pellets, 300 mL double distilled water, and 0.5 mL *o*-xylene were placed in a 500-mL round-bottomed flask in a Deryng apparatus. Hydrodistillation was carried out for 4 h once the boiling process began. Then, the *o*-xylene solutions of hop oil (of light yellow or yellow colour) were dried over anhydrous sodium sulphate, transferred to tightly closed amber glass vials, and finally stored at −20 °C in a freezer. This procedure complied with the regulations of the Polish Pharmacopoeia regarding hydrodistillation and quantification of essential oils from plant material [42].

2.3. Gas Chromatography Working Conditions

For qualitative analysis, a GC–MS system (Thermo Electron Corporation, Austin, TX, USA) was used that consisted of a capillary gas chromatograph (Thermo-Quest CE Instruments Trace GC 2000 series) and an MS detector (Finnigan Trace). For analysis, a Quadrex 5MS fused silica capillary column (column length (l) = 30 m, inner diameter (i.d.) = 0.32 mm, stationary phase film thickness (d_f) = 0.5 µm; Quadrex Corporation, Woodbridge, CT, USA) and on-column injection (2 µL) were used. The flow rate of the helium (He) carrier gas was 5 mL min^{-1}. The temperature gradient was as follows: 2 min at 50 °C; temperature rise to 200 °C at a rate of 5 °C min^{-1}; 2 min at 200 °C; temperature rise to 220 °C at a rate of 15 °C min^{-1}; and 10 min at 220 °C. The temperature of the injector was 220 °C. Identification of the separated analytes was performed using the NIST mass spectral library (National Institute of Standards and Technology, Gaithersburg, MD, USA).

A capillary gas chromatograph (Fisons Instruments GC 8000 Series) with a flame ionisation detector (FID) (Fisons Instruments, Milan, Italy), on-column injection (5 µL), and a Chromapack CP-Sil 8CB capillary column (column length (l) = 50 m, inner diameter (i.d.) = 0.53 mm, stationary phase film thickness (d_f) = 1 µm; Agilent, Santa Clara, CA, USA) were used for quantitative analysis. Helium (He) was used as the carrier gas at the flow rate of 4 mL min^{-1}. The injector temperature was set at 250 °C. The temperature gradient was as follows: 5 min at 50 °C; temperature rise to 150 °C at a rate of 15 °C min^{-1}; temperature rise to 220 °C at a rate of 15 °C min^{-1}; 5 min at 200 °C; temperature rise to 250 °C at a rate of 20 °C min^{-1}; and 20 min at 250 °C. Calibration curves for the eight compounds were constructed using the commercial standards by injection of 1, 2, 3, 4, and 5 µL aliquots of 5 mg mL^{-1} *n*-hexane solutions into the GC-FID system in triplicate (n = 3). The respective limits of detection (LOD) and limits of quantification (LOQ) were determined based on the standard deviation of the peak height taken as the noise measure (SD) and the slope (a) of the corresponding calibration curve (y = ax + b). The used equations were as follows: LOD = 3.3 × SD/a and LOQ = 10 × SD/a.

For identification of the bioactive compounds, prior to SPME–GC–MS analysis, the HPTLC zones of interest were eluted with ethanol into vials sealed with a silicon/PTFE septum (20 mL headspace) with the use of the TLC–MS Interface (CAMAG) [43]. A CTC Combi PAL (CTC Analytics AG, Zwingen, Switzerland) automatic multipurpose sampler with a 65 µm StableFlex carboxen/polydimethylsiloxane/divinylbenzene (CAR/PDMS/DVB) SPME fibre (Supelco, Bellefonte, PA, USA) was used for static headspace-solid phase microextraction (sHS-SPME). A 5 min incubation at 100 °C was followed by a 10 min extraction at 100 °C exposing the fibre to the headspace. Then, the fibre was transferred immediately to the GC–MS injector port and desorbed for 1 min at 250 °C. A Fibre Bakeout Station was applied to clean and condition the SPME fibre in a pure nitrogen atmosphere at 250 °C for 15 min. Analyses were performed using an Agilent 6890N/5973N GC-MSD (Santa Clara, CA, USA) system equipped with a capillary column (Agilent SLB-5MS, 30 m × 250 µm × 0.25 µm). The temperature gradient was as follows: 3 min at 60 °C; temperature rise to 250 °C at a rate of 8 °C min^{-1}; and 1 min at 250 °C. As the carrier gas, high purity helium (6.0) was used at 1.0 mL min^{-1} (37 cm s^{-1}) in constant flow mode. The injector was operated in splitless mode at 250 °C. To obtain the mass spectra, electron ionisation at 70 eV and a quadrupole analyser in full scan mode (41–500 amu at 3.2 scan s^{-1}) were

utilised. MSD ChemStation D.02.00.275 software (Agilent) was used to perform the data evaluation. Compounds were identified by comparing the recorded spectra and retention times with those of the standards, and the NIST 2.0 library was also consulted.

2.4. EPR Spectroscopic Assessment of Antioxidant Capacities of Hop Oils

The antioxidant capacity of the commercial hop oils was determined using the method previously described in reference [44]. A Bruker EMX EPR spectrometer (Bruker-Biospin, Germany), operating at room temperature (21 ± 0.5 °C) at the X-band frequency, provided the electron paramagnetic resonance (EPR) spectra. The typical working parameters of the instrument were: microwave power, 20.12 mW; time constant, 40.96; gain, 1×10^4 G; central field, 3480 G; and modulation amplitude, 2.0 G. The regression equations for the linear relationship between percent inhibition of the EPR signal intensity and the mole number of a given standard were assessed as y = 1547.8x + 4.7 for TR, y = 3767.6x + 2 for GA, and y = 1627.8x + 0.8 for AA, where y is inhibition [%] and x is sample volume [mL]. Based on these equations, we calculated the antioxidant capacity per 100 mL of the studied samples in µmol TR, µmol GA, and µmol AA, respectively.

A typical reaction mixture contained 1 mL of 200 µmol L^{-1} DPPH• solution in ethanol and 0.025 to 0.100 mL of sample. For each sample, the regression equation of the linear relationship was determined between the percent inhibition (% I) of the EPR signal intensity and the volume of sample (*V*). This equation was used to calculate the % I value corresponding to 100 mL of the studied sample. Then, from the standard curves obtained for TR, GA, and AA, we calculated the antioxidant activity per 100 mL sample expressed in µmol TR, µmol GA, and µmol AA, respectively. All measurements were performed in triplicate (n = 3).

2.5. High-Performance Thin-Layer Chromatography—Effect-Directed Analysis (HPTLC–EDA) of Hop Oils and Selected Essential Oil Standards

Hop oils (50 µL mL^{-1} in cyclohexane—acetone, 1:1) and standards (5 µL mL^{-1} in ethanol) were manually applied using a 10 microliter syringe (Hamilton, Bonaduz, Switzerland) in 0.5–5.0 µL $band^{-1}$ aliquots as 5 mm bands with a 9 mm track distance onto the HPTLC layers, at8 mm from the bottom. HPTLC separations were carried out with *n*-hexane or chloroform in an unsaturated 20 cm × 10 cm Twin Trough Chamber (CAMAG, Muttenz, Switzerland) up to the migration distance of 65 or 75 mm, respectively. After development, the plates were dried in a cold stream of air and cut into segments using a smartCUT Plate Cutter (CAMAG) or a blade. Plate segments were derivatised by dipping them into anisaldehyde reagent (50 µL *p*-anisaldehyde, 10 mL methanol, 1 mL acetic acid, and 500 µL concentrated sulfuric acid (96%)), heated at 110 °C for 5 min, and then documented (digital camera, Cybershot DSC-HX60, Sony, Neu-Isenburg, Germany) under white light illumination in transmittance mode (Vis; 96891 Salobrena 2 LED lamp, Eglo Lux, Dunakeszi, Hungary). For SPME–GC–MS analysis, 70 µL aliquots of Azacca and Cascade oils and 30 µL of Azacca oil were applied as 7 cm bands and developed with chloroform or n-hexane, respectively. Zones of interest were eluted with ethanol using the TLC–MS Interface (CAMAG).

Free radical scavenging activity was detected by dipping the chromatogram into a DPPH• solution (0.02% in methanol). Antioxidants were indicated by bright zones against a purple background [45].

The HPTLC–α-glucosidase assays were performed based on the previously described methods [37,38], but with modifications. Substrates (2-naphthyl-α-D-glucopyranoside or 4-methylumbelliferyl-α-D-glucopyranoside) were dissolved in DMSO (10 mg in 0.4 mL) and diluted with 9.6 mL phosphate buffer (0.1 M, pH 7.5). The developed and dried chromatograms were first sprayed (airbrush, Revell, Bünde, Germany) with one of the substrate solutions and then with α-glucosidase solution (5 units mL^{-1} in 0.1 M phosphate buffer, pH 7.5). After spraying with each solution, the layers were allowed to become unglazed. Then, the bioautograms were incubated at 37 °C and 100% humidity. After 20 min incu-

bation, the bioautograms prepared with 2-naphthyl-α-D-glucopyranoside substrate were sprayed with Fast Blue Salt B solution (chromogenic reagent, 1 mg/mL in water) and allowed to dry. Bright inhibition zones against a violet background were documented under white light illumination (in reflectance mode). In the case of 4-methylumbelliferyl-α-D-glucopyranoside substrate, the wet bioautograms were documented after 60 min incubation under a UV lamp (CAMAG) at 365 nm. Dark zones indicated inhibition against the blue fluorescent background (active enzyme was revealed from the blue fluorescent 4-methylumbelliferol substrate).

Employing the previously published HPTLC–lipase assay [39] with a slight modification, the plate was immersed in the substrate solution (α-naphthyl acetate, 1 mg mL^{-1} in ethanol), dried with a cold air stream, then sprayed with the enzyme solution (0.8 mg mL^{-1} lipase and 1 mg mL^{-1} bovine serum albumin in 0.1 M phosphate buffer, pH 7.5), incubated in a vapor chamber at 37 °C for 15 min, sprayed with Fast Blue Salt B solution (1 mg mL^{-1} in water), dried, and documented under white light illumination. Inhibition zones were revealed as bright zones against a violet background.

The antibacterial *A. fischeri* [37] assay was carried out based on the previously described method. Briefly, the developed and dried plates were dipped into the cell suspension for 8 s, the unglazed bioautograms were immediately placed in a dark box under a cooled camera (iBright™ FL1500 Imaging System, Thermo Fisher Scientific, Budapest, Hungary), and they were documented using an exposure time of 1 min. Active zones were indicated by dark (or bright) zones against the luminescent background.

3. Results and Discussion

3.1. GC–MS and GC-FID Analyses of Commercial Hop Oils and Hop Oils Hydrodistilled from Commercial Hop Pellets

GC–MS was used to confirm the identity of seven targeted terpenes and methyl heptanoate contained in seven commercial hop oils and six hop oils hydrodistilled from commercial hop pellets (i) by comparison of their respective retention time (t_R) values with those of commercial terpene standards, and (ii) by comparison of their mass spectra with those of standards and in the virtual NIST library.

Quantification of the eight targeted compounds in the hop oils was carried out by GC-FID. Figure 1 shows the chromatograms derived from GC-FID analysis for two hop oils of the same hop variety (Azacca), one commercially obtained and the other hydrodistilled from the hop pellets, as a kind of fingerprint for visual inspection. The parameters of the calibration curves obtained for the eight compounds are summarized in Table 1.

Table 1. Retention times (t_R), calibration curves, and LOD and LOQ values obtained by GC-FID for standard samples of the investigated terpenes and methyl heptanoate (n = 3).

Compound	Retention Time, t_R [min]	Calibration Curve $y = ax + b$	Correlation Coefficient, r	LOD [ng mL^{-1}]	LOQ [ng mL^{-1}]
α-Pinene	16.1	$y = 257.4\,x - 227.6$	0.989	0.51	1.59
β-Pinene	17.6	$y = 256.0\,x - 252.7$	0.986	1.80	5.47
β-Myrcene	17.9	$y = 233.1\,x - 204.6$	0.978	0.01	0.04
Methyl heptanoate	18.7	$y = 179.4\,x - 156.0$	0.955	2.26	6.86
β-Ocimene	19.4	$y = 227.4\,x - 140.4$	0.963	0.58	1.76
Limonene	19.8	$y = 176.6\,x - 164.4$	0.977	1.87	5.66
β-Caryophyllene	30.5	$y = 267.2\,x - 113.1$	0.971	2.72	8.23
α-Humulene	31.1	$y = 248.2\,x - 115.7$	0.975	20.47	62.13

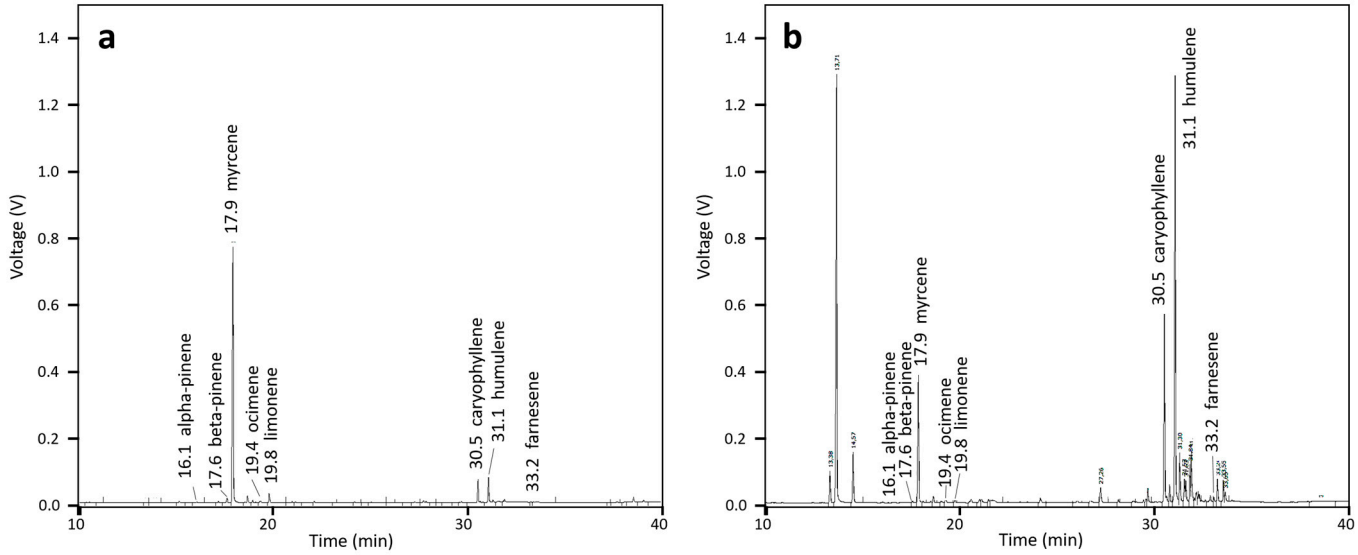

Figure 1. Chromatograms recorded with use of the GC-FID system for 5 µL Azacca hop oil; (**a**) commercial sample and (**b**) hydrodistilled sample.

The contents of the seven terpenes and methyl heptanoate present in the commercial hop oils and the hydrodistilled hop oils are given in Table 2. From the comparison of quantitative results, it was evident that the differences in the terpene profiles of a set of terpene oils from one technological process (either commercial oils or those hydrodistilled in our lab) were quite similar because all of the investigated hop varieties were from the same botanical species. A distinct feature of the commercial hop oils was a relatively high content of β-myrcene (between 4.21 and 6.40 µg mL^{-1} hop oil) in five oils (except Saaz and Ahhhroma). With the hydrodistilled hop oils, the quantitative differences were not very significant either, although Cascade demonstrated an above average content of β-myrcene (6.40 µg mg^{-1} dry hop pellet) compared with the remaining varieties. Azacca, Cascade, and Centennial demonstrated perceptibly higher contents of β-caryophyllene than the rest, and most hydrodistilled oils (except Chinook) demonstrated relatively high contents of α-humulene. Some differences between the terpene profiles of individual hop oils presented in Table 2 suggested that the commercial hop oils were derived from hop cones using a process different from hydrodistillation, e.g., solid–liquid extraction with organic solvents or sub- or supercritical fluid extraction with carbon dioxide [46].

3.2. EPR Spectroscopic Assessment of Antioxidant Capacity of Hop Oils

The antioxidant capacity of the seven commercial hop oils was evaluated using electron paramagnetic resonance (EPR) spectroscopy and compared against three different free radical scavengers, i.e., Trolox (TR), gallic acid (GA), and ascorbic acid (AA), with the results expressed in equivalent antioxidant capacity (EAC) units, i.e., in TREAC, GAEAC, and AAEAC, respectively (Table 3). Graphical illustration of the assumed EPR spectroscopic method, in the form of the series of EPR spectra of DPPH• recorded for the commercial Ahhhroma hop oil, clearly showed a decrease in the EPR signal intensity with increased sample volume (Figure 2).

The numerical values of TREAC and AAEAC obtained for the individual hop oil samples were similar, and the numerical values of GAEAC were lower than the remaining ones by ca. 55–60%. This might have resulted from the different molecular structures of TR, GA, and AA, and hence from the different mechanisms of their respective free radical scavenging reactions. Comparing the EAC values of the individual hop oils, analogous trends were observed within each dataset. Thus, independent of the free radical scavenger used, we could point out Ahhhroma, Azacca, and Chinook oils as those with the highest antioxidant capacity. Amarillo oil showed a perceptibly lower antioxidant capacity, and

Centennial oil showed a rather negligible antioxidant capacity. The antioxidant effect of the Cascade and Saaz oils was completely lacking. Summing up, the antioxidant capacity of the individual hop oils discussed in this study was quite diverse and yet, in general, the respective numerical values were in the range of 0–45 µmol of TR, GA, or AA per 100 mL hop oil sample (Table 3). This meant that, compared to other edible liquids, the antioxidant capacity of the commercial hop oils was lower than that of red wines [47,48] and comparable with the data obtained from EPR spectroscopy studies of beers [49] and white wines [47,48,50]. For example, the numerical values of TREAC reported in reference [49] for 63 commercial beers (pilsner, lager etc.) fell within the range of 100 to 1000 µmol TR/100 mL sample, while the average numerical value of TREAC reported in reference [47] for Slovak white wines was equal to 76 ± 27 µmol TR/100 mL sample, and that for Austrian Burgenland white wines was equal to 90 ± 19 µmol TR/100 mL sample.

Table 2. Contents of α-pinene, β-pinene, β-myrcene, methyl heptanoate, β-ocimene, limonene, β-caryophyllene, and α-humulene in samples of commercially available hop oil (c, white background) and homemade hop oils hydrodistilled from the commercial hop pellets (h, grey background) of the Amarillo, Azacca, Cascade, Chinook, Centennial, Saaz, and Ahhhroma varieties (n = 3). Means within a column followed by different letters are significantly different according to one-way ANOVA followed by Tukey's test ($p < 0.05$). An asterisk indicates that the mean concentration in a hydrodistilled (h) hop oil is significantly different from its commercially available (c) counterpart ($p < 0.05$, Student's *t*-test).

Hop Oil Variety	Source	Content in Hop Oil [µg mL^{-1}] (RSD [%])							
		α-Pinene	β-Pinene	β-Myrcene	Methyl Heptanoate	β-Ocimene	Limonene	β-Caryophyllene	α-Humulene
Amarillo	c	0.89 ^{b} (6.13)	1.07 ^{c} (4.24)	5.63 ^{b} (3.44)	0.92 ^{cd} (5.12)	0.65 ^{b} (2.89)	0.94 ^{b} (4.78)	0.63 ^{c} (3.17)	1.01 ^{c} (5.34)
Amarillo	h	0.91 (4.75)	1.03 (4.13)	1.17 (6.27)	0.88 (5.74)	0.63 (7.18)	0.95 (3.49)	0.58 (6.22)	3.43 * (2.88)
Azacca	c	0.89 ^{b} (4.44)	1.04 ^{c} (3.69)	4.21 ^{c} (4.21)	0.98 ^{bd} (5.02)	0.64 ^{b} (2.79)	1.09 ^{a} (3.97)	0.69 ^{c} (6.18)	0.94 ^{c} (4.34)
Azacca	h	0.90 (3.75)	1.01 (3.99)	2.52 * (4.17)	1.01 (6.02)	0.69 (4.86)	0.99 (6.68)	2.55 * (3.87)	5.65 * (4.91)
Cascade	c	1.08 ^{a} (4.28)	2.65 ^{a} (3.16)	6.39 ^{a} (5.27)	2.54 ^{a} (4.17)	1.09 ^{a} (3.99)	1.04 ^{ab} (4.12)	1.65 ^{a} (4.68)	2.71 ^{a} (5.38)
Cascade	h	0.90 (5.74)	1.08 * (3.34)	6.40 (4.92)	0.99 * (7.67)	0.70 * (4.83)	0.98 (6.01)	2.42 * (5.01)	5.65 * (2.98)
Chinook	c	0.89 ^{b} (3.19)	1.06 ^{c} (2.96)	4.15 ^{c} (3.99)	1.13 ^{b} (4.54)	0.64 ^{b} (4.99)	0.94 ^{b} (4.21)	0.73 ^{c} (5.27)	1.08 ^{bc} (4.96)
Chinook	h	0.92 (3.71)	1.07 (5.24)	0.90 * (4.89)	1.52 * (4.26)	0.66 (2.87)	1.00 (6.48)	0.68 (4.90)	1.36 * (3.73)
Centennial	c	0.91 ^{b} (4.12)	1.19 ^{b} (3.84)	6.40 ^{a} (4.92)	1.05 ^{bc} (3.91)	0.69 ^{b} (4.03)	0.94 ^{b} (3.76)	0.85 ^{b} (5.28)	1.22 ^{b} (5.69)
Centennial	h	1.02 (3.28)	1.03 (4.04)	1.47 * (4.89)	1.20 (5.74)	0.77 (6.03)	0.94 (4.27)	1.96 * (5.63)	4.88 * (3.99)
Saaz	c	0.89 ^{b} (3.77)	1.02 ^{c} (2.99)	2.74 ^{d} (4.02)	0.87 ^{d} (3.89)	0.67 ^{b} (4.11)	0.94 ^{b} (4.99)	0.49 ^{d} (5.01)	0.67 ^{d} (4.66)
Saaz	h	0.89 (4.88)	1.01 (5.17)	2.92 (6.11)	0.88 (4.73)	0.64 (5.12)	0.94 (4.81)	1.18 * (4.99)	3.52 * (5.08)
Ahhhroma	c	0.89 ^{b} (4.19)	1.01 ^{c} (3.67)	2.68 ^{d} (3.92)	0.93 ^{cd} (4.71)	0.63 ^{b} (4.44)	0.99 ^{ab} (3.79)	0.52 ^{d} (4.09)	0.61 ^{d} (5.11)

Table 3. Results of quantification of antioxidant capacity of 7 commercial hop oils, as established by EPR spectroscopy, against three different free radical scavengers, i.e., Trolox (TR), gallic acid (GA), and ascorbic acid (AA) (n = 3). Different letters indicate significantly different values within a column ($p < 0.05$).

Hop Oil Variety	TREAC * [µmol TR/100 mL Sample] (±SD)	GAEAC * [µmol GA/100 mL Sample] (±SD)	AAEAC * [µmol AA/100 mL Sample] (±SD)
Amarillo	27.51 (±0.50) ^{c}	12.02 (±0.20) ^{c}	28.55 (±0.47) ^{c}
Azacca	39.90 (±0.91) ^{b}	17.11 (±0.38) ^{b}	40.33 (±0.87) ^{b}
Cascade	0.00 ^{e}	0.00 ^{e}	0.00 ^{e}
Chinook	38.81 (±2.37) ^{b}	16.66 (±0.97) ^{b}	39.30 (±2.26) ^{b}
Centennial	3.31(±0.63) ^{d}	2.08 (±0.24) ^{d}	5.33 (±0.29) ^{d}
Saaz	0.00 ^{e}	0.00 ^{e}	0.00 ^{e}
Ahhhroma	44.74 (±1.95) ^{a}	19.10 (±0.80) ^{a}	44.99 (±1.97) ^{a}

* TREAC, Trolox equivalent of antioxidant capacity; GAEAC, gallic acid equivalent of antioxidant capacity; AAEAC, ascorbic acid equivalent antioxidant capacity.

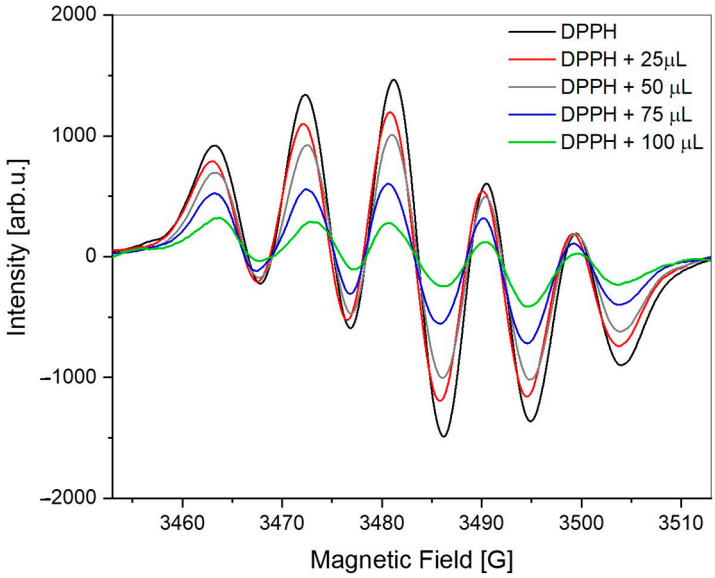

Figure 2. The effect of different volumes of hop oil on the EPR spectra of DPPH• for commercial hop oil sample no. 7 (Ahhhroma) characterized by the highest Trolox equivalent antioxidant capacity (TREAC) values (see Table 3).

3.3. Screening for Hop Oil Bioactive Components by HPTLC Hyphenations

The individual components of the commercial hop oils responsible for various bioactive effects were detected using in situ assays after separation by HPTLC. For this reason, two HPTLC methods were developed using *n*-hexane as the mobile phase for the separation of highly non-polar compounds, and chloroform was used for the separation of the other compounds. In the HPTLC–DPPH• assay, no active zones were revealed, which indicated that the oils had weak antioxidant activity, as observed in the EPR tests.

In this study, two HPTLC–EDA methods were used to detect hop oil compounds with potential anti-obesity activity, namely HPTLC–α-glucosidase and HPTLC–lipase assays. All four zones (at hR_F 45, 64, 73, and 87) separated with *n*-hexane and visualised with anisaldehyde possessed α-glucosidase inhibition activity (Figure 3a–c). However, upon employing the generally established method for the α-glucosidase assay, which used the 2-naphthyl-α-D-glucopyranoside substrate and Fast Blue B chromogenic reagent, a strange dark colour appeared in the middle of the zone at hR_F 64 and only the bright ring

implied inhibitory action of the present compound (s) (Figure 3b). This strange staining effect may have resulted from an interaction of the analyte with a chromogenic reagent. For this reason, elimination of the reagent was achieved by replacing the substrate with 4-methylumbelliferyl-α-D-glucopyranoside (Figure 3c). Providing a fluorescent signal, this improved the assay and confirmed the enzyme inhibitory effect of the compounds in all four zones, although none of them inhibited the lipase enzyme. Moreover, two out of four zones (at hR_F 73 and 87) also showed weak antibacterial activity against *A. fischeri* (Figure 3d). In this separation system, the β-farnesene standard migrated to the zone at hR_F 73 and inhibited both the α-glucosidase enzyme and, to a weaker extent, *A. fischeri*.

When screening the more polar components, six bioactive zones separated by chloroform were selected at hR_F 16, 35, 52, 61, 70, and 75 (Figure 3e–h). The hop oil samples showed α-glucosidase inhibition, but the responsible compounds could not be identified as no demarked inhibition zones were revealed and no compounds were visible in the darker areas even after derivatisation under UV light. Compounds in the six marked zones inhibited the lipase enzyme as well as the bioluminescence of Gram-negative *A. fischeri*, displaying an antibacterial effect. The β-farnesene, geraniol, d-limonene, and β-pinene standards (components of hop oils) were tested along with the samples. In HPTLC development with the use of chloroform, β-farnesene migrated to the front. No standard was active in the lipase assay, and β-farnesene was the only compound to inhibit the α-glucosidase enzyme. In the HPTLC–*A. fischeri* assay, β-farnesene, d-limonene, and β-pinene exhibited a weak antibacterial effect, while geraniol showed a strong antibacterial effect. Geraniol had the same hR_F value as the lowest marked zone. Gram-positive *B. subtilis* was not sensitive to the hop oil components.

3.4. Identification of Bioactive Compounds by SPME-GC-MS

Analysis of the compounds in the bioactive HPTLC zones was performed after their elution with ethanol. Only in the marked zones (H1, H2, H3, C1, and C2 in Figure 3) were the components detectable by SPME–GC–MS. A high amount of β-caryophyllene (about 55%) in the H1 zone was next to lower amounts of muurolene, eudesmene, and cadinene isomers. A comparison of the size of the H1 inhibition zone with the β-caryophyllene content of the oils suggested that the minor components could be responsible for the bioactivity of the zone. In the H2 zone of Saaz oil, β-farnesene and α-humulene were identified at a ratio of 1:1, but only α-humulene was detectable in the H2 zone of Azacca oil (β-farnesene was not detectable in any zone of Azacca oil). Therefore, it could be stated that the role of β-farnesene in the α-glucosidase inhibition and anti-*A. fischeri* activity in the H2 zone was more determinative than that of α-humulene. The hR_F value and the bio-effects of β-farnesene were also confirmed by the standard. Dimyrcene isomers, β-myrcene, and 2-methylbutyl isobutyrate dominated in the H3, C1, and C2 zones, respectively. Compounds separated with the use of chloroform at hR_F 35 and 52 were investigated by SPME–GC–MS and HPTLC–ESI-MS, but they did not give mass signals with the used ionization techniques.

The anti-obesity effect of a hop extract was previously established, demonstrating inhibition of the increase in body weight and liver lipids induced by a high-fat diet in mice and improvement of glucose tolerance [51]. Hop bitter compounds, such as prenylated flavonoids and phloroglucinols, were found to be α-glucosidase inhibitors [52,53] and effective in the prevention and treatment of hyperlipidaemia, diet-induced obesity, and type-2 diabetes [54–56]. However, naturally occurring mono- and sesquiterpenoids also showed promising anti-diabetic potential in vitro and in vivo [57,58]. Our results confirmed that both α-glucosidase and lipase inhibitors were present in the hop oils, which could contribute to their anti-obesity activity.

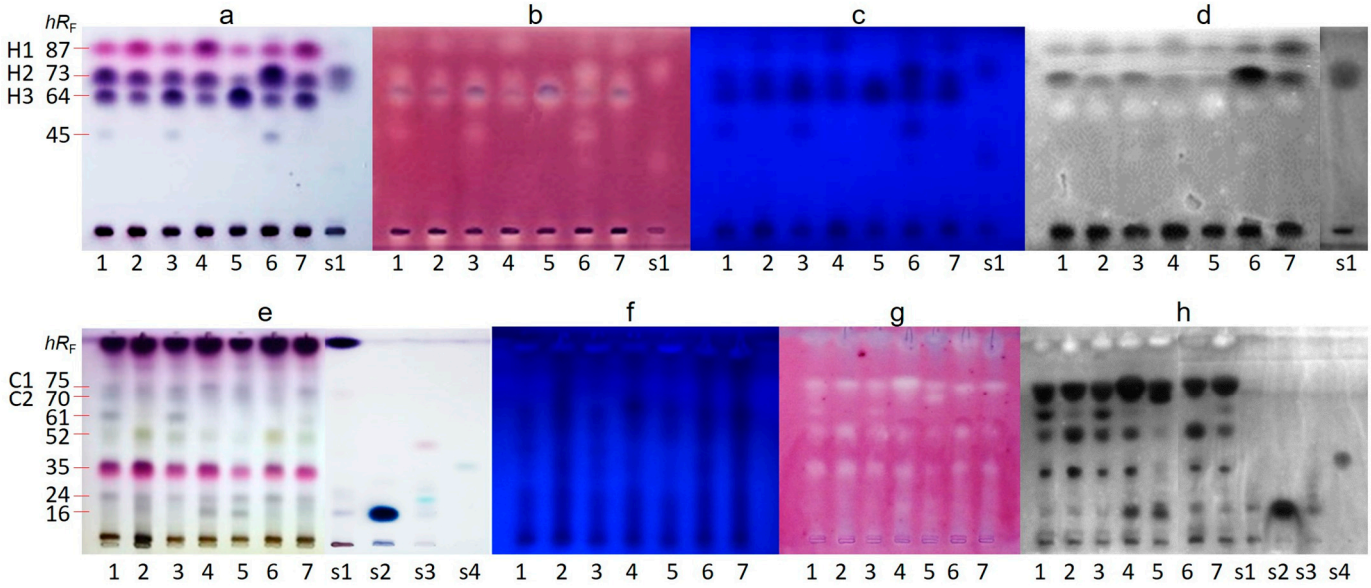

Figure 3. HPTLC chromatograms and bioautograms of seven commercial hop oils (Amarillo, Azacca, Cascade, Chinook, Centennial, Saaz, and Ahhhroma, 1–7, respectively): β-farnesene (s1), geraniol (s2), d-limonene (s3), and β-pinene (s4), developed with *n*-hexane (**a**–**d**) or chloroform (**e**–**h**), detected after anisaldehyde derivatisation and recorded under white light (**a**,**e**), the α-glucosidase assays with 2-naphthyl-α-D-glucopyranoside under white light (**b**), the α-glucosidase assay with 4-methylumbelliferyl-α-D-glucopyranoside substrate under UV 365 nm (**c**,**f**), the *A. fischeri* assay (**d**,**h**, greyscale images of bioluminescence), and the lipase assay under white light (**g**).

4. Conclusions

Seven hop oils commercially derived from American hop varieties (*Humulus lupulus* L.) Amarillo, Azacca, Cascade, Centennial, Chinook, Saaz, and Ahhhroma and six hop oils hydrodistilled in our laboratory from commercial hop pellets of the same varieties (except Ahhhroma) were compared with the aid of GC, HPTLC hyphenations, and electron paramagnetic resonance (EPR). To our best knowledge, this is the first reported quantification results with selected terpenes (i.e., α-pinene, β-pinene, β-myrcene, β-ocimene, limonene, β-caryophyllene, and α-humulene) and methyl heptanoate present in the discussed American hop oil varieties. Composition profiles of the commercial and homemade (hydrodistilled) hop oils considered in this study unequivocally showed that the commercial hop oils were richer in β-myrcene, while the contents of β-caryophyllene and α-humulene were usually higher in the hydrodistilled oils. Thus, the technology used in commercial hop oil production was not hydrodistillation. It was established that the antioxidant properties of the commercially available hop oils were comparable to those of white wines and beers. The results of the HPTLC assays indicated that several of the commercial hop oil components (e.g., β-myrcene, dimyrcenes, β-farnesene, 2-methylbutyl isobutyrate, and geraniol) had antibacterial activity or inhibited α-glucosidase and/or lipase enzymes, which could play a role in the anti-obesity activity of hops.

The combination of fast, high-throughput, inexpensive, and relatively simple hyphenated HPTLC with GC–MS enabled the comparison of the chemical and bio-profiles of hop oils. The developed streamlined workflow, including parallel HPTLC–bioassays and HPTLC—TLC–MS Interface—SPME–GC–MS, revealed the identities of the hop oil bioactive components.

Author Contributions: Conceptualization, Á.M.M., T.K. and M.S.; methodology, Á.M.M., T.K. and M.S.; investigation, Á.M.M., M.B., J.P., P.M., M.K., A.B. and M.S.; resources, Á.M.M. and M.S.; data curation, Á.M.M., M.B., J.P., P.M., M.K., A.B. and M.S.; formal analysis, J.F.; writing—original draft preparation, Á.M.M., M.B., J.P., P.M., M.K. and T.K.; writing—review and editing, Á.M.M. and T.K.; supervision, M.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work was partially funded by the National Research, Development and Innovation Office of Hungary (SNN139496).

Data Availability Statement: Data are available upon reasonable request.

Acknowledgments: The authors thank Péter G. Ott from the Plant Protection Institute, CAR, ELKH, for the preparation of the bacterial cell suspension.

Conflicts of Interest: The authors declare no conflict of interest.

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