Determination of Linalool in Different Hop Varieties Using a New Method Based on Fluidized-Bed Extraction with Gas Chromatographic–Mass Spectrometric Detection

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ABSTRACT

J. Am. Soc. Brew. Chem. 73(2):151-158, 2015

Linalool, an important substance in hoppy, aromatic beer, is one of the most aromatic components of essential oils in hops. Linalool concentration, among those of other substances, could be used to distinguish among hops varieties. As such, an effective, repeatable, and high-throughput method is required; a new method based on fluidized-bed extraction combined with detection by gas chromatography–mass spectrometry was developed and is presented in this study. This method is faster than the reference method, which uses steam distillation; it also reduces the possibility of thermal changes. Because this method saves organic solvent, energy, and sample amount, it can be considered a green method. Good repeatability of the method (8.0 mg/kg) was achieved with 3-hepten-1-ol as an internal standard. The detection limit of linalool is 1.0 mg/kg, and the limit of quantification is 3.5 mg/kg. Good agreement was achieved between the results of the new and steam-distillation methods. In addition, 59 samples of hops from four important Czech varieties (Agnus, Premiant, Saaz, and Sládek) from the 2013 harvest were analyzed, and the content of linalool was correlated with the content of bitter acids in *xyz* 3-D projection; four separate and clearly limited clusters that corresponded to the four tested varieties were obtained.

Hops, along with malt and water, are the basic raw materials used for beer production. The basic role of hops is to provide beer with a pleasantly bitter taste and a hoppy aroma. This result is achieved within the wort boiling, during which poorly soluble α-acids are isomerized into the more-soluble iso-α-acids. The content of bitter substances (mostly α-bitter acids), therefore, is one of the key indicators of the quality of hops used for beer production (6).

In addition to the bittering substances, hops contain other compounds that might affect the taste and flavor of beer. In particular, polyphenolic substances can modify the character of bitterness (25). Low-molecular-weight polyphenols can affect the sensory stability of beer indirectly due to their reducing capacity (8,26). Other very important ingredients are essential hop oils (0.5 to 3% w/w in hops), which are responsible for the smell of hops; the hop aroma is an important qualitative parameter that has a role in the selection of raw materials for brewing. The profile of hop essential oils is characteristic for each variety and can be used for variety identification (31). According to the chemical composition, compounds of essential oils can be divided into three basic groups: the hydrocarbon fraction, the oxygen fraction, and the fraction of sulfur compounds (5).

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http://dx.doi.org/10.1094/ASBCJ-2015-0406-01

Myrcene, α-pinene, farnesene, and humulene are the most important substances of the hydrocarbon fraction (70–80% essential oils) (5). The sulfur fraction contains only trace amounts of individual substances; however, due to their low flavor thresholds, they might significantly affect the taste and smell of beer, especially in a negative way. The oxygen fraction of essential oils is a mixture of alcohols, aldehydes, ketones, epoxides, esters, and acids. Due to their higher solubility in aqueous solutions, these substances might influence the flavor of beer in a significant way. Linalool is one of the major components of this group (5).

Linalool is one of the most aromatic flavor components of hop essential oils, and, along with myrcene, it is considered the most odor-active volatile in all analyzed hop varieties (11,34,35). Thus, linalool is a primary substance in hoppy aromatic beers (11,19). It is a very flavorful terpene alcohol, with a citrus- and bergamot-like odor. According to the literature (19,28), linalool is contained in hop essential oil in amounts up to 1.1% rel. The atlas of Czech hop varieties 2012 (7) states that this value in the current major Czech varieties (traditional Saaz and hybrid varieties Agnus, Premiant, and Sládek) is up to 0.7% rel. In addition to the flavor of hops, linalool is a significant part of several other aromas, such as fruit, spices, and chocolate, and it is widely used in the cosmetics industry. Its antiinflammatory properties also have a positive effect on human health (30).

Although the concentration of linalool in beer is low, it can contribute to the overall flavor of the beer due to its synergistic effect with other hop essential oils. Higher content levels of linalool can be achieved by adding hops in the last part of hopping or by using dry hopping (13,16). The content of linalool, as well as the contents of other components, varies during the fermentation process; linalool is formed from geraniol and nerol and is simultaneously transformed into α-terpineol. This process is shown in Figure 1 (20,36). Linalool can also affect the sensation of off-flavors in finished beer. For example, Hanke et al (15) found that linalool, at a concentration near its threshold (~25 µg/L), suppresses the perception of dimethyl sulfide (DMS) in beer. However, higher concentrations of linalool (~70 µg/L) highlight the perception of DMS. A similar effect has been reported for isovaleric acid and diacetyl. Moreover, Hanke et al (14) found that hop essential oils, such as linalool, decrease the gushing tendency of beer, especially when hops are added in the late stages of wort boiling.

Knowledge of the composition of hop secondary metabolites (essential oils, bitter acids, and polyphenols) is important not only for a qualitative description of the cultivar, but also because the spectrum of secondary metabolites can be used to differentiate among hop varieties. Several profiling methods of authenticating hop varieties using the polyphenol spectrum have been published (24,29). Linalool, along with a wide spectrum of hop compounds, can be used to identify the hop cultivar. In 1998,

Perpete et al (31) endeavored to differentiate hop pellets by essential oil analysis. Volatile compounds of five aroma cultivars (Styrie, Saaz, Lublin, Mount Hood, and Hallertau) and seven bitter cultivars (Northern Brewer, Nugget, Pride of Ringwood, Northdown, Galena, Target, and Challenger) were extracted with a Likens-Nickerson simultaneous solvent extractor, separated by gas chromatography (GC), and identified by mass spectrometry (MS). High levels of bergamotene and farnesene were found only in Saaz, Lublin, and Styrie samples. Quantification of 4-decenoic acid methyl ester and 3-methyl butyl isobutyrate proved to be a quick means of distinguishing between non-European and European bitter hops from aroma cultivars. Kovačevič et al (22) conducted statistical processing of the resulting fingerprints using the classical method, steam distillation with GC-MS, for the analysis. Based on the fact that the composition of essential oil in hops depends more on hop variety and less on growing, processing, and storage conditions, the fingerprints of 16 representative components of essential oils were correlated with a sample variety. Seventy-eight samples belonging to the five most important hop varieties (Aurora, Bobek, Celeia, Magnum, and Savinjski) grown in Slovenia were analyzed. The methods used, cluster analysis and principal component analysis, reliably differentiated the five varieties. Jelínek et al (17) compared the composition of the secondary metabolites α- and β-bitter acid analogues, essential oils, and polyphenols to perform a varietal differentiation of seven Czech hop varieties (dry Agnus, Bor, Harmonie, Premiant, Rubín, Sládek, and Saaz hop cones). They concluded that hop essential oil constituents contribute significantly to the individual hop varieties. They also established a dichotomous key for the authentication of Czech hop varieties based on some characteristic varietal markers. The authors used classical analysis with a steam distillation step in the sample preparation.

Several methods of determining linalool and other essential oils have been published; the methods differ mainly in the preparation step, which affects the recovery of the method. The last step of the method is GC with MS or flame ionization detection (FID). The reference methods, ASBC/Hops-17 and EBC 7.10 and 7.12 (3,9) are based on the steam distillation of essential oils from a mixture of ground hop material (more than 100 g of the sample) and a large volume of water. The oil floating on top of the mainly aqueous condensate is analyzed by GC-FID. The disadvantages of this method are the time-consuming nature of the procedure, which entails 3 to 4 h of distillation, and the tendency of essential oils (including linalool) to undergo changes in chemical composition during this long process (2,32).

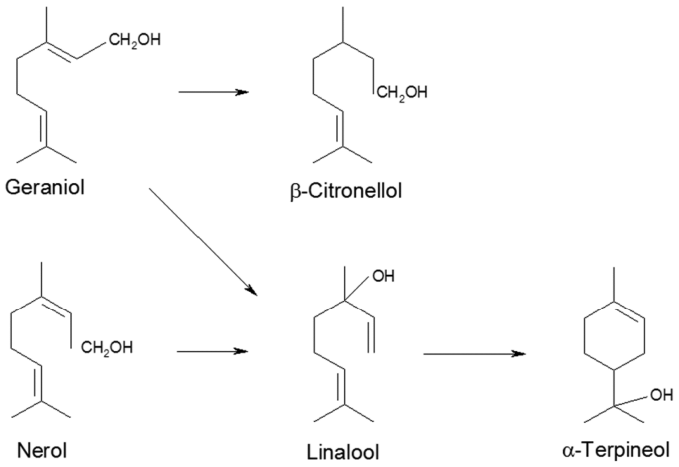

Fig. 1. Transformation of terpenic alcohols during fermentation (20,36).

One way to reduce the effects of oxygen and high temperatures on the chemical composition during extraction is through the use of supercritical hops extraction. In the present study, the sample was extracted by CO_2 (4) and subsequently extracted on a solid phase extraction (SPE) column (12). The disadvantages of this procedure are the limited extraction ability of polar compounds and an instrumental challenge (1).

Several authors have described methods based on a new principle, headspace solid-phase microextraction (SPME), which is a relatively simple procedure. The hops sample is milled and inserted into the headspace vial, and GC analysis is immediately performed (10,21). The disadvantage of this method is that the amount of analyte absorbed on a fiber depends on the extraction conditions, age of the fiber, and matrix effect (33). Another possible method of extracting the essential oils is the use of ultrasound. Aberl et al extracted oils from hops using ethanol during sonication of the sample, and the resultant extracts were analyzed by headspace GC-MS (2).

As mentioned previously, identification of the hop cultivar and the description of its qualitative parameters are very topical and important questions, both for hop producers, who need this information for distinguishing purposes and for qualitative descriptions of the cultivars, and for brewers, who must choose quality raw materials to maintain consistent beer quality. Therefore, the development of a high-throughput, reproducible, and effective method for this purpose is desired.

Thus, the aim of this study was to develop a method of determining linalool in hops using fluidized-bed extraction. This method was chosen because the principle behind it meets the requirements of an effective and high-throughput method, with its low sample and solvent consumption levels and short heating time of the sample at low temperatures, which leads to fewer thermal changes in the sample composition. The correlation between the new method and reference methods based on steam distillation was proven using eight samples (varieties Saaz, Sládek, Premiant, and Agnus, each from two different localities). In addition, the content levels of linalool in 59 hops samples from four important Czech varieties from the 2013 harvest were analyzed and correlated with the content levels of bitter acids. The probability of using linalool as a marker of hop variety was suggested and is discussed.

EXPERIMENTAL

Hop Samples

Four samples of Agnus, 10 samples each of Premiant and Sládek, and 35 samples of Saaz were analyzed. Samples were dried and randomly selected from approximately 150 harvested samples collected in the Czech Republic in 2013 to evaluate the content of bitter acids (27). The varieties were selected because of their commercial importance and genetic diversity. The Agnus variety (hybrid progenies include Bor, Saaz, Sládek, Northern Brewer, and Fuggle; registered in 2001) belongs, genetically, to hops of American origin; Sládek (hybrid progenies include Saaz and Northern Brewer; registered in 1994) belongs to hops of European origin of the Fuggle group; and Premiant (hybrid progeny, Saaz inzucht line; registered in 1996) and Saaz (fine aroma; registered in 1952) belong, genetically, to hops of European origin of the Saaz group (6).

Chemicals

The following chemicals were used to determine linalool content levels: (-)-Linalool 95% and 3-hepten-1-ol 95% (both Sigma Aldrich, Prague, Czech Republic); ethanol, reagent grade, 96% (Lachner, Czech Republic); dichloromethane and hexane (both Merck, Germany); anhydrous sodium sulfate, reagent grade, and ultrapure water prepared by MilliQ (Millipore, U.S.A.).

Sample Preparation for Linalool Analysis (Extraction on Fluidized Bed)

Internal standard 3-hepten-1-ol was prepared by dissolving 1,250 mg of a standard in 50 mL of ethanol (final concentration, 25 g/L). The linalool calibration curve was prepared in a hexane-dichloromethane mixture (1:1) at concentration levels of 1, 10, 50, and 100 mg/L. The concentration of 3-hepten-1-ol, 10 mg/L, was the same at all levels.

Fluidized-bed extraction, shown in Figure 2, was performed on a fexIKA vario control extractor (IKA-WERKE GmbH) connected to a recirculating cooler (FL 601; Julabo GmbH). Two grams of milled hops and 10 µL of internal standard (at a concentration of 25 g/L) were put on the filter of the extraction thimble, and the bottom vessel of the extractor was filled with 40 mL of ethanol. Extraction conditions were as follows: four extraction cycles; target temperature of the heating block, 117°C; extraction time, 6 min (7 min for the first cycle); cooling temperature of the heating block after the end of cycle, 60°C (40°C after the last cycle); waiting time after cooling, 1 min; temperature of cooling water, 5°C; PTFE filter, diameter 42 mm, pore size 10–20 µm. Ethanol was added to the obtained extract for a total volume of 50 mL.

A prepacked EXtrelut NT 20 column (Merck Millipore, U.S.A.) filled with diatomaceous earth-based solid phase was conditioned for 15 min with 10 mL of ultrapure water. Five milliliters of the ethanolic extract was loaded onto the column, and after 5 min, the column was rinsed stepwise with 5 mL of the hexane-dichloromethane mixture (1:1 v/v) (for most samples, five steps per 5 mL) until approximately 10 mL of eluate was obtained. The eluate was dried out with water-free sodium sulfate and analyzed by GC-MS.

Determination of Linalool by GC-MS

The GC analysis was performed with a Trace GC Ultra DSQ II GC/MS (Thermo Scientific, U.S.A.). The separation of linalool was made using a TG WAX MS column (30 m, inner diameter 0.25 mm, film thickness 0.25 µm). The oven temperature was programmed to go from 40 to 130°C at 8°C/min, with an initial holding time of 2 min. Subsequently, the temperature was increased from 130 to 230°C at 15°C/min, with a final holding time of 5 min. Injection was splitless; split valve open time was 1 min; split flow was 50 mL/min; temperature of injection was 250°C; injection volume was 1 µL; carrier gas (He, purity 5.0) flow rate was 1.2 mL/min; and temperature of the transfer line was 250°C. The GC-MS detection parameters—retention time (min), filter range (mass to charge ratio [*m*/*z*]), and qualifier ions (*m*/*z*) for linalool and internal standards—are shown in Table I. Total ion current (TIC)/selected ion monitoring (SIM) detection was used to improve the selectivity and sensitivity of detection.

Determination of Linalool by Reference Method (Extraction Using Steam Distillation)

Air-dried hop cones were coarsely milled and submitted to steam-distillation according to the EBC 7.10 method. The distillations were performed with 100 g of cones in 4,000 mL of water; the distillation period was 90 min. The amount of hop oil collected was measured gravimetrically. Oils were stored in glass vials at –18°C until the GC analysis was performed.

The GC analyses were made using a modified EBC 7.12 method on a Thermo-Focus gas chromatograph equipped with an autosampler, Thermo-DSQ II mass detector, and DB-5MS capillary column (Agilent Technologies, U.S.A.) with 30 m × 0.25 mm × 0.50 µm film thickness. The helium carrier gas was supplied at a head pressure of 60 kPa. The samples (1 µL) were applied by split injection (1:50) at 250°C. The oven temperature was programmed from 60 to 250°C at 1.5–5.0°C/min and held at 250°C for 5 min. The transfer line was kept at 250°C, and the ion source temperature was 210°C. Full-scan mass spectra were collected from 40 to 500 *m*/*z*. Linalool identity was assigned by comparing the mass

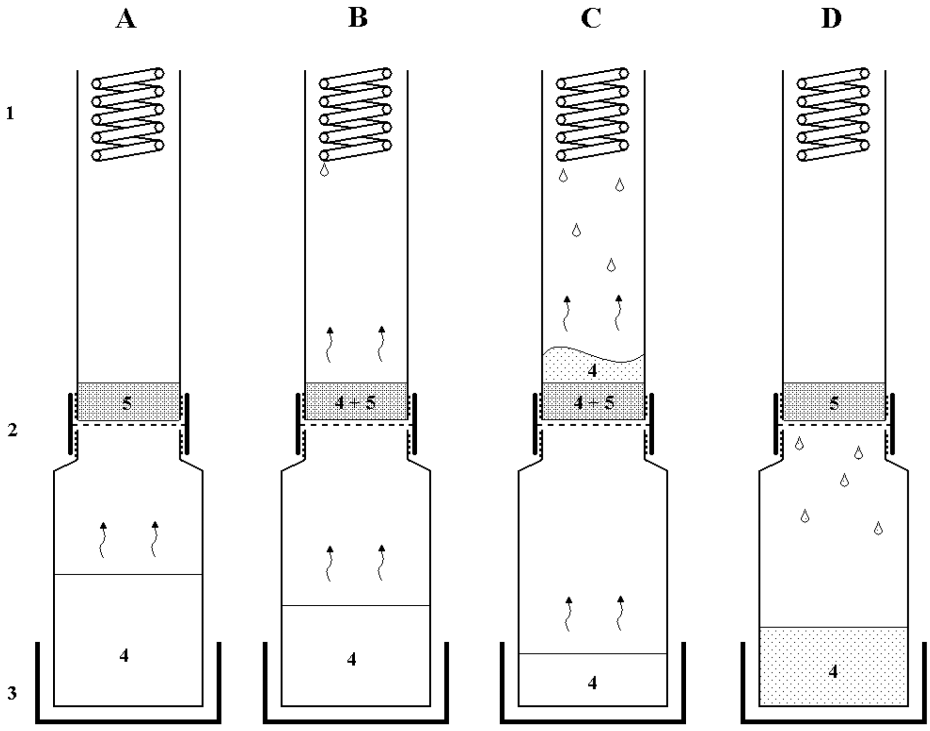

Fig. 2. Fluidized-bed extraction scheme: 1 = condenser; 2 = filter; 3 = heating/cooling; 4: solvent; and 5 = sample. **A,** Beginning of the cycle: heating of the lower vessel is started and the solvent begins to boil. **B,** The sample is moistened by solvent vapors and the solvent begins to condense in the condenser. **C,** Condensed solvent stays with the sample on the filter and is mixed by solvent vapors from the lower vessel, leading to intensive extraction. **D,** End of the cycle: solvent in the lower vessel is cooled; due to the decrease in pressure, the solvent and extracted analytes can flow down through the filter.

spectra and retention times of the analytical standard (Fluka). A GC chromatogram of Sládek hop oils with linalool position is shown in Figure 3.

Quantification of linalool was expressed on the basis of the external standard and linalool calibration curves in the range of 25–100 mg/100 mL. Results of linalool amounts in the hops samples are summarized in Table II.

Determination of α- and β-Acids

Analysis of α- and β-acids in the hop samples was performed according to EBC Method 7.7 (9).

RESULTS AND DISCUSSION

Optimization of Sample Preparation

Extraction. The extraction of linalool and the other essential oils was performed on a fluidized bed (Fig. 2). During the optimization of the extraction procedure (number of cycles and time of one cycle), the peak linalool area in crude ethanolic extract of oils after GC-MS analysis was assessed. The results obtained were normalized to the linalool peak using four cycles of 7 min each, set to 100% (Table III). It was found that the number of cycles was more important than the extraction time of one cycle. The results showed that three cycles provided a smaller area of linalool peak than four cycles did; there was minimal difference between four and five cycles. Thus, four cycles was established as optimal. The optimal extraction time of one cycle (i.e., endurance at maximum temperature) was found to be 6 min, whereas the first cycle needed 7 min to complete wetting of the sample with condensed solvent. A volume of 40 mL of solvent was determined to be the volume necessary to preserve the volume of the solvent at the bottom of the container. The target temperature of the heating block was set at a value 1.5 times higher than the boiling point of the solvent (i.e., 117°C for ethanol), to make the solvent boil, according to the manufacturer's recommendation; therefore, the temperature was not optimized further. In effect, the actual extraction temperature was equal to the boiling point of ethanol (78°C).

Purification. The crude linalool extract and essential oils contain many other substances (e.g., nonvolatile substances), which may negatively influence the GC-MS determination; therefore, it is necessary to purify the extract. EXtrelut columns filled with NT20 diatomaceous earth were used for purification; both manually filled columns and commercially obtained columns (filled by the manufacturer) were tested. Both types of columns provided comparable results, but the manually filled columns exhibited a greater dispersion of results. In other words, commercially filled column showed better repeatability, namely 1.28, while the repeatability of manually filled columns was 4.05 (expressed as percent relative standard deviation; data not shown).

Method Validation

The internal standard, 3-hepten-1-ol, was used for linalool quantification according to the method shown in Table IV. Determination of repeatability was obtained using eight individual samples with different concentration levels ranging from 15 to 90 mg/kg; each sample was measured four times. First, the standard devia-

TABLE I
Gas Chromatography–Mass Spectrometry Detection Parameters

Analyte	Retention time (min)	Filter range (*m*/*z*)	Qualifier ions (*m*/*z*)
3-Hepten-1-ol (ISTD 2)[a]	12.22	50–250	81; 96; 114
Linalool	13.18	50–250	93; 121; 136

[a] Internal standard 2.

TABLE II
Comparison Between the New Fluidized-Bed Extraction (FBE) Method and the Reference Steam Distillation (SDIS) Method

Variety	FBE	SDIS
Agnus (1)[a]	55.9	77.7
Agnus (2)	76.1	79.2
Premiant (1)	67.5	68.5
Premiant (2)	81.0	81.5
Saaz (1)	16.4	13.7
Saaz (2)	27.3	19.3
Sládek (1)	44.0	32.2
Sládek (2)	15.2	17.0

[a] Outlier result, not used for data processing. r_{95} (FBE) = 8.0 mg/kg. r_{95} (SDIS) = 7.1 mg/kg.

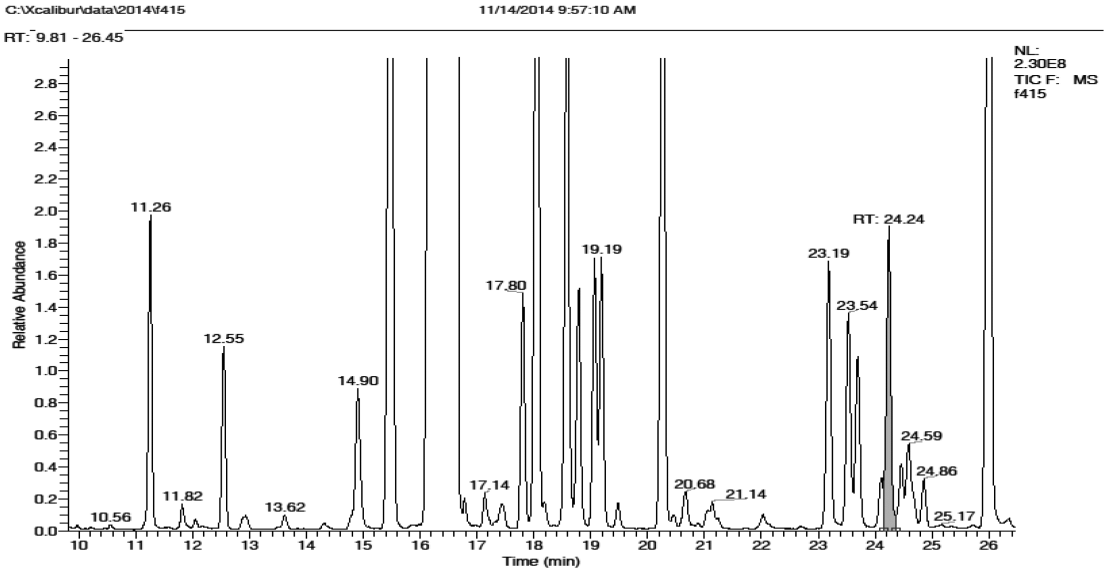

Fig. 3. Section of gas chromatography chromatogram of Sládek hop oils showing the linalool position (shaded peak, retention time 24.24 min); reference method based on fluidized-bed extraction.

tion for each level $s(1)$, $s(2)$, … $s(8)$ was calculated. From these results, the total standard deviation was calculated according to the following formula:

$$s_{tot} = \left[\frac{1}{k} \sum_{i=1}^{n} s(i)^2 \right]^{1/2} = 1.87,$$

where k is number of levels and n is number of repetitions. Finally, repeatability r_{95} was calculated according to the following formula:

$$r_{95} = t_{crit(n-1)} * \sqrt{2} * s = 8.0 \text{ mg/kg},$$

where $t_{crit(n-1)}$ is the critical value of t-distribution (3.18) for n–1 degrees of freedom.

The uncertainty estimate includes the total standard deviation of repeated measurement (s_{tot}), inaccuracy of sample preparation, and inaccuracy of instrument. The combined uncertainty was determined as a doubled standard deviation of presented factors (raw data are not shown) and was estimated as 10.0 mg/kg.

A four-level calibration curve was obtained for linalool determination; every level was a five-fold replicate (Fig. 4). The calibration curve was linear in the full range, from 1 to 100 mg/L, and linearity was verified by testing the quadratic term. The coefficient of determination for both internal standards was greater than 0.999.

The limit of detection (LOD) and limit of quantification (LOQ) were determined from 10 repeated low-concentration (1 mg/kg) measurements of the linalool calibration solution. The data processing was proved under SIM mode. The resulting LOD and LOQ were 1.0 and 3.5 mg/kg, respectively.

Comparison with Reference Method

The results of the compared methods (fluidized-bed extraction and steam distillation) are given in Table II; the repeatability values of the fluidized-bed extraction and steam distillation methods were 8.0 and 7.1 mg/kg, respectively. Based on the results of Student's paired t-test, it could be concluded that the results of the two methods were comparable. The higher results of the new method were negligible (average difference 2.3 mg/kg); however, the 95% confidence interval for this value (5.2 mg/kg) was rather high. In other words, the difference between the two methods was not statistically significant, but the variance was higher.

Determination of Linalool in Real Samples

As seen in a chromatogram obtained under TIC mode (Fig. 5), 19 compounds were separated within 25 min; the retention time of linalool was 12.86 min under the defined conditions. Although we have focused only on linalool, the chromatogram shows that the fluidized-bed method of extraction is suitable for a wide spectrum of essential oil constituents. In addition, the final GC analysis time was shorter than those of the other discussed methods (2,21,22,31). This factor, along with the shorter extraction time, good reproducibility, and sensitivity, rank this method as an effective and high-throughput method.

The average value of four linalool measurements was calculated. Every sample was double-extracted and every extract was double-analyzed by GC-MS. The linalool contents in the tested hop varietal samples ranged from 10 to 100 mg/kg; the results are shown in the box and whisker plots in Fig. 6A. The highest amount of linalool, ~90 mg/kg, was found in the Agnus variety; the Premiant variety also contained a high amount of linalool, ~80 mg/kg. The amount of linalool in Sládek ranged from 20 to 55 mg/kg and in Saaz, from 10 to 50 mg/kg. The total content of essential oils in a hop variety usually corresponds approximately to the contents of bitter acids. Varieties Saaz, Agnus, and Premiant had similar relative linalool contents in the total essential oils; therefore, linalool content lined up in accordance with the α-acids. The Sládek variety had a significantly lower relative content of linalool. In terms of an absolute amount, the linalool was virtually indistinguishable from that in the Saaz variety. Our results are in accordance with the varietal characteristics data recently published by hop breeders from the Hop Research Institute in Žatec (Saaz) (7), listed in Table V. They determined linalool content using the steam distillation preparation reference method (3,9). This agreement of values indicates a good correlation between the new and reference methods. Similar results were published by other Czech authors in 2010 (17), who again used the steam distillation method (3,9).

On the other hand, Vázquez-Araújo et al (37) compared the contents of volatile substances in hops using steam distillation and SPME. The final concentrations of linalool in variety Saaz were found to be 26.0 and 73.8 mg/kg for the distillation method and SPME, respectively. It is questionable which method is more accurate because no reference sample of linalool in hop was referenced. However, we can conclude that our results using fluidized-bed extraction are comparable to results obtained using the reference method (steam distillation).

TABLE III
Optimization of Extraction Process

Number of cycles	Time of extraction (min)	Relative peak area (%)
4	7	100.00
4	6	97.37
4	8	103.65
5	7	100.44
3	7	87.28

TABLE IV
Validation Parameters of the Method

Parameter	Value (mg/kg)
Repeatability, r_{95}	8.0
Combined uncertainty	10.0
Limit of detection	1.0
Limit of quantification	3.5

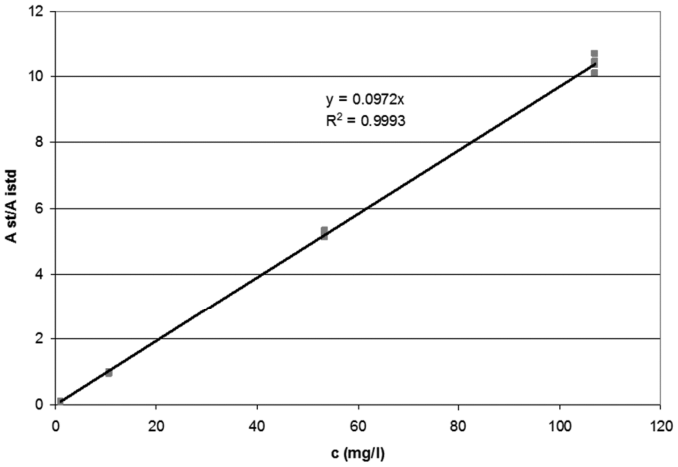

Fig. 4. Calibration curves of linalool. c = concentration; A st = peak area of standard; and A istd = peak area of internal standard.

Bitter Acid Content

The contents of α- and β-bitter acids in the samples of the tested variety are shown in Figs. 6B and C, respectively. The content of bitter acids corresponds to the character of the variety; α-bitter acid decreases in content from bitter variety Agnus to that found in fine aroma variety Saaz. The absolute content of bitter acids in the studied samples was in the middle of the range declared for the variety. The variation is caused by variability in climatic conditions in individual years. The content of α- and β-acids in the hops of a given variety greatly depends on the soil and climatic conditions, year, growing location, and age of the hop gardens (plants). The annual differences can be several tens of relative percent; variability within the varieties harvested is approximately ±15–18 relative percent (27). Comparisons of the tabulated results with the harvest values showed a good relationship between our results and the average value of the harvest; thus, the test samples can be considered a representative selection.

When the contents of α- and β-bitter acids were compared with linalool content, no significant correlation was obtained. This

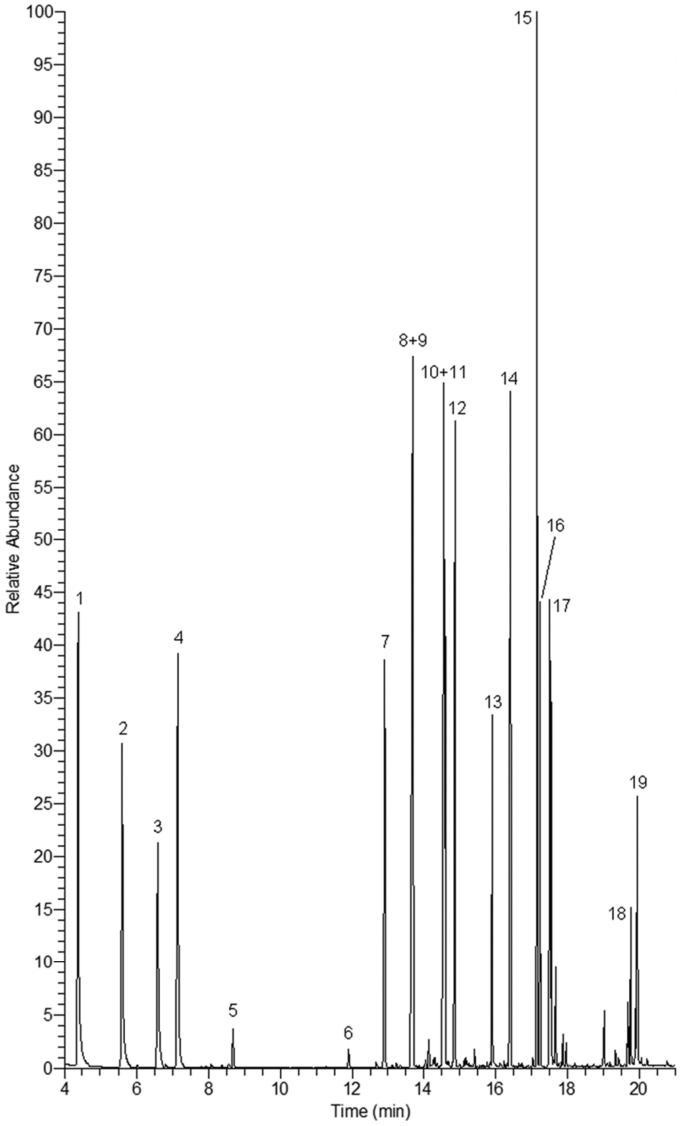

Fig. 5. Chromatogram of standard solution (new method). 1 = α-pinene (4.34 min); 2 = β-pinene (5.56 min); 3 = myrcene (6.55 min); 4 = limonene (7.10 min); 5 = methyl heptanoate (internal standard 1, 8.63 min); 6 = 3-hepten-1-ol (internal standard- 2, 11.84 min); 7 = linalool (12.86 min); 8 = β-caryophyllene (13.65 min); 9 = 4-terpineol (13.68 min); 10 = β-farnesene (14.53 min); 11 = α-humulene (14.57 min); 12 = α-terpineol (14.83 min); 13 = nerol (*cis*-geraniol, 15.88 min); 14 = α-ionone (16.39 min); 15 = β-ionone (17.13 min); 16 = α-irone (17.22 min); 17 = β-caryophyllene epoxide (17.54 min); 18 = farnesol 1 (19.76 min); 19 = farnesol 2 (19.93 min).

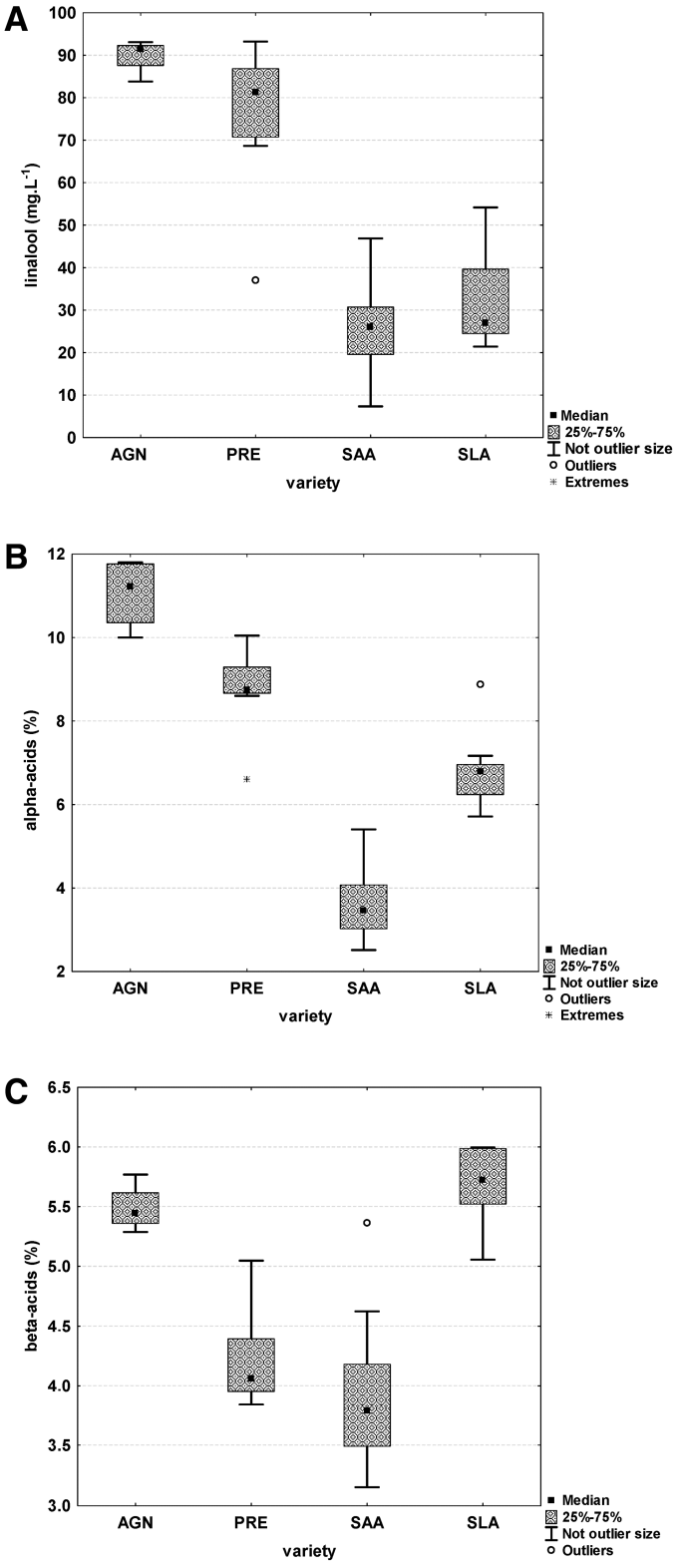

Fig. 6. Box and whisker plots: contents of linalool (**A**), α-acids (**B**), and β-acids (**C**) in analyzed hop samples of Czech varieties Agnus (AGN), Premiant (PRE), Saaz (SAA), and Sládek (SLA).

result might be explained by the influence of different factors during the hop cones' growth and maturing processes on metabolic pathways and the formation of bitter acids and linalool. The only correlation between the contents of linalool and α-bitter acids was found in variety Premiant ($r = 0.76$). However, no correlation was observed between the linalool and β-bitter acid contents. In addition, no dependence was found in varieties Saaz and Sládek ($r < 0.45$). Variety Agnus was not evaluated, due to the small number of samples.

Varietal Discrimination

The results of α- and β-bitter acids and linalool content were arranged to 3D projection, resulting in the distribution of tested varieties as shown in Figure 7. Except for one Saaz sample, four good separate clusters were obtained. Given the small number of samples in variety Agnus, it is questionable whether the resolution of its cluster is sufficient.

Based on the results, the newly developed and optimized method could be used not only for linalool determination ad hoc, but also (with a quantification of the rest of the detected hop oils or other metabolites) as a prescreening, high-throughput method of verifying hops varieties. The previously described methods for determining hops authenticity, based on a chemical profile of secondary metabolites, generally use a wider spectrum of essential oils in combination with the other metabolites (17).

Jelinek et al (18) devised a key to identifying eight Czech hop varieties using the contents of α- and β-bitter acids, selected hop oils (linalool was not determined), and polyphenols. The most reliable markers were selected indicators from the groups of essential oils and polyphenols, whereas the contents of hop resins were evaluated as the most dependent on external factors and therefore the least reliable for identification. Those findings are in accordance with our results; the content of α-bitter acids was determined to be below 4.5% w/w by those authors, while only five samples in our study had a higher content.

Other authors (31) developed a flowchart for distinguishing 12 hop cultivars. For this purpose, they separated and identified approximately 100 compounds after sample preparation using steam distillation. The flowchart was rebuilt, and only compounds remaining stable throughout aging were kept (23).

Finally, Kovačevič et al (22), who also used the reference method (9), obtained the profiles of 16 essential oils and processed them by cluster analysis and principal component analysis. The developed method was shown to be effective in all hop varieties important in Slovenia, and they provided excellent results in daily laboratory practice.

CONCLUSIONS

Compared to the other methods discussed, the new method uses a different principle of sample preparation, based on fluidized-bed extraction. This method does not require expensive instruments, as with SPME, and, compared with the steam distillation method, it exhibits low solvent and sample consumption and a short heating time of the sample at lower temperatures, which does not initiate changes in the composition of the sample. In addition, final GC analysis time was shorter compared with the other discussed methods (2,21,22,31). This factor, along with shorter extraction time and good reproducibility and sensitivity, ranks this method as an effective and high-throughput method. The method is also suitable for a wide spectrum of essential oil constituents. It provides results comparable to those of the steam distillation reference method but with a considerably lower consumption level of solvent and the sample (2 g).

Furthermore, we found that, based on the contents of linalool and hop bitter acids, the main Czech hop varieties can be distinguished relatively easily; however, for more reliable identification, it is necessary to use the other analytical characters as well.

Because no clear information about linalool transformation in aging hops under defined conditions was published, further work will be devoted to ascertaining the profile of aging and the possibility of distinguishing older hop varieties.

ACKNOWLEDGMENTS

This study was supported by the institutional support of the Ministry of Agriculture of the Czech Republic (No. RO1914), Institutional Research

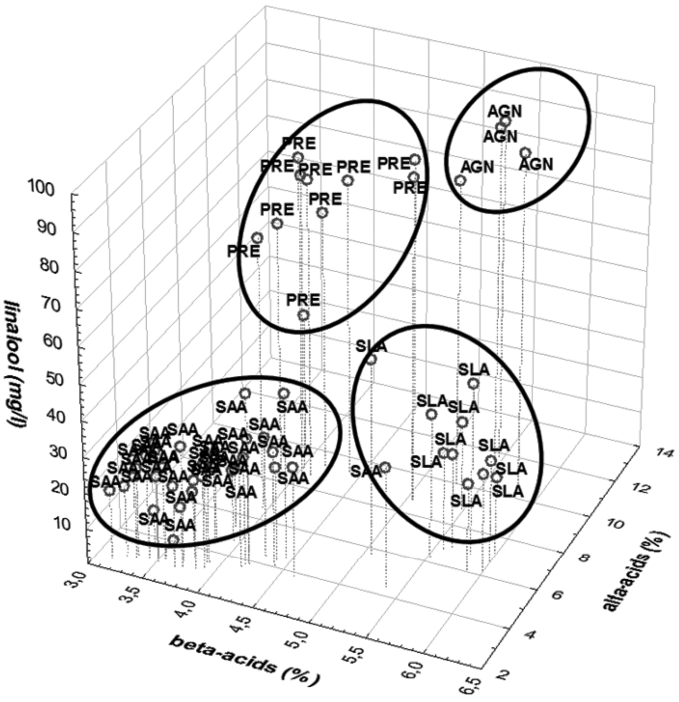

Fig. 7. *xyz* 3-D graph of 59 hop samples of four Czech cultivars presented in three-dimensional space. Concentrations of α-acids, β-acids (%), and linalool (mg/kg) are plotted on the *x*, *y*, and *z* axes, respectively. AGN = Agnus, PRE = Premiant, SAA = Saaz, SLA = Sládek.

TABLE V
Amount of Linalool in Czech Hop Varieties[a]

Variety	Total content of hop oils (g/100 g)	Relative content of linalool in hop oil (%)	Linalool content in hop (mg/kg)[b]	α-Bitter acids (% w/w)	β-Bitter acids (% w/w)
Agnus	2.0–3.0	0.3–0.5	60–150	9.0–12.0	4.0–6.5
Premiant	1.0–2.0	0.4–0.7	40–140	7.0–10.0	3.5–5.5
Saaz	0.4–0.8	0.4–0.6	16–48	2.5–4.5	4.0–6.0
Sládek	1.0–2.0	0.15–0.30	15–60	4.5–8.0	4.0–7.0

[a] Ref. (7).
[b] Linalool content was calculated from the total hop oil content and the relative content of linalool in hop oil.

Concept HRI Žatec MSM1486434701, and by project LLP-LDV-TOI-2013-1-SI1-LEO05-05341 "Micro-brewing learning and training program (LdV Beer School)" within the EU's financial scheme of Leonardo da Vinci. Finally, the authors thank colleagues Renata Mikulíková and Zdeněk Svoboda for their valuable advice in the development of the method.

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