C. Almaguer, F. Thiele, C. Schönberger and E. K. Arendt

Ultrastructure Studies of the Lupulin Glands of Different Hop (*Humulus lupulus* L.) Varieties Observed by Scanning Electron Microscope

The cones of the female hop (*Humulus lupulus* L.) plants are of importance to the brewing industry. Only the cones of the female plants are able to secrete the fine yellow resinous powder known as lupulin glands. The lupulin glands are technically termed glandular trichomes; secondary metabolites are synthesized in copious amounts by the glands. The resins and essential oils, synthesized and accumulated in the lupulin glands, impart the typical bitter taste and aroma to beer. To date, no clear distinctions have been made among the ultrastructure features of the glands in different hop varieties. In this study, the glandular trichomes of nine hop varieties, with different properties (e.g., hop class, country of origin), were observed using scanning electron microscopy (SEM). It is intended to compare the hop varieties and determine if the environmental factors have an effect on the ultrastructure of the gland. For each hop variety, 50 micrographs were visually assessed and compared based on their surface topography, degree of fullness, and volume. To determine if there are any similarities among the examined varieties, the gland features were correlated and clustered against the chemical properties of the corresponding variety. For the data analysis, mean centered values were used to maximize the variation between the clusters. The cluster data confirmed larger gland volumes in the bittering hop varieties independent of the country of origin. The collected data indicates that the ultrastructure characteristics of the lupulin glands are primarily associated with the hop class.

Descriptors: hops, lupulin glands, glandular trichomes, scanning electron microscope (SEM), *Humulus lupulus* L.

1 Introduction

The inflorescences of the female hop (*Humulus lupulus* L.) plants form the cones (strobile) [23] used by the brewing industry. The hop cone consists of stipular petal-like structures called 'bracts' and 'bracteoles' around a central axis. At the base of the bracteoles, the lupulin glands are formed as the hop ripens. Only the cones of the female plants are able to secrete the fine yellow resinous powder known as lupulin glands. The lupulin glands, which are technically termed glandular trichomes, are anatomical structures containing cells specialized for particular metabolic functions, usually the biosynthesis and secretion of copious amounts of particular secretory products, such as protective secondary metabolites [15]. The lupulin glands of hops synthesize the main brewing principles of hops, terpenophenolic resins (i.e., bitter acids: prenylated polyketides), essential oils (e.g., monoterpenes and sesquiterpenes), and prenylflavonoids (e.g., xanthohumol) [19, 26, 33, 38].

In 1821, Ives assigned the name 'lupulin' to the yellow powder; he was the first one to observe that it is in the lupulin where the bitter and aromatic substances of hops are stored [11]. Hops are of interest to the brewer since they impart the typical bitter taste and aroma to beer and are responsible for the perceived hop character. In addition to the comfortable bitterness and the refreshing hoppy aroma delivered by hops, the hop acids also contribute to the overall microbial stability of beer [9, 18]. Another benefit of the hops is that they help enhance and stabilize beer foam and promote foam lacing [6, 7, 29]. The hop secondary metabolites account for these traits in beer and these accumulate at high concentrations in the lupulin glands of the hop cones.

At its most simple, hop varieties have traditionally been classified, according to their flavoring properties and chemical composition, as "bittering hops" and "aroma hops" [2]. This distinction is important as the brewer may benefit from proper selection of particular hop varieties to add subtle tastes and flavors to beers. Most of the hop constituents present in the cone are characteristic of the hop variety. In some varieties, certain components are affected by the environmental conditions in which they were grown; in particular country of origin. Additionally, year to year variations in the hop chemical composition may be observed within the same variety, these can also be dependent on the harvesting time [12–14].

Authors

M. Sc. Cynthia Almaguer, Lehrstuhl für Brau- und Getränketechnologie, Technische Universität München Weihenstephan, Freising, Germany; Dr. Frithjof Thiele, Radeberger Gruppe KG, Frankfurt, Germany; Dr. Christina Schönberger, Joh. Barth & Sohn GmbH & Co. KG, Nuremberg, Germany; Prof. Dr. Elke K. Arendt, Department of Food and Nutritional Sciences, National University of Ireland, University College Cork, Cork, Ireland; corresponding author: Cynthia.Almaguer@tum.de

More than 60 % of the hop area under cultivation is located in Germany and the United States (USA) [25]. The largest hop growing areas include the Hallertau region in Germany and the states of Washington, Oregon, and Idaho in the USA. Other hop growing countries are England, Czech Republic, Poland, Slovenia, Ukraine, China, South Africa, Australia, and New Zealand. The farm structure varies from country to country; the average farm size in Germany and England is of 15 ha and 18 ha, respectively. The farms in the United States are significantly bigger, the average size is of 214 ha [5]. Another important aspect among the different growing areas is the cultivation of seeded hops. Seeded hops are commonly grown in England, but not in most countries. In most commercial hop growing areas worldwide the seed content in hops is regulated. Male hops are physically removed from the hop fields to avoid the fertilization of the female plants and, thus, the production of seeds. In many countries, seeds are considered by brewers to be undesirable. It is believed that oxidation of the seed fatty acids produce off-flavors in beer [8]. Several studies [20, 24, 37, 40] have considered the impact of hop seeds on beer quality in particular flavor, yet the results remain somewhat conflicting.

Early observations of the hop lupulin glands using scanning electron microscopy (SEM) were done by *Maeda* in 1976 [16]. Previous studies have looked into the ultrastructure of the hop lupulin glands [28, 39]. In these studies morphological changes of the trichomes in accordance with increasing hop acid content were monitored [10, 22, 30]; the ontogeny and histochemistry of the secretion have been characterized [21]; trichome structures have been compared [31]. The different phases of development have been successfully determined. It has been revealed that development of lupulin glands is strictly divided into a growth and a biosynthetic-secretory phase [34]. These studies, however, have failed to compare the impact of environmental factors on the ultrastructure of the glandular trichomes of hops. For this study three representative German hop varieties from the Hallertau region were chosen, Hallertauer Perle (H. Perle), H. Mittelfrüh, and H. Magnum. Four varieties from the United States were selected, Willamette, Cascade, Zeus, and Galena. Two seeded varieties from England were also used, East Kent Golding (E. K. Golding) and Fuggle.

In a scanning electron microscope (SEM) electromagnets are used to bend a very fine electron beam which scans the surface of the object, generating a 3-D image. In this study SEM was chosen over CLSM (confocal laser scanning microscopy) since it is possible to achieve a larger magnification of the lupulin glands and, thus, reveal the ultrastructure in detail. A good representation of the surface topography of the trichome is also retrieved [3]. The aim of this study is to provide a better understanding of the hop varieties from different growing regions. It is intended to compare the hop varieties and determine if the environmental factors have an effect on the ultrastructure of the gland. Further, the purpose of these observations is to clarify the relationship between the structural features of the glands and the valuable hop components. To do so, the homogeneity in size, structure, and appearance within each variety were examined from the SEM micrographs. Further, to determine if there are any similarities among the examined varieties, the gland features were correlated and clustered against the chemical properties of the corresponding variety.

2 Materials and Methods

2.1 Hop varieties

All hop cones from the same harvest year were generously donated by the Barth-Haas Group (Nuremberg, Germany). For this study nine hop varieties, listed in table 1, were observed using SEM. Three representative hop varieties from Germany and four varieties from the United States were selected, additionally two seeded varieties from England were used in this study.

Table 1 Chemical composition of the selected hop varieties

Country	Hop Variety	Hop Class	α-Acids [%]	Total Oils [mL/100 g]
Germany	H. Perle	Aroma	6.5	0.75
	H. Mittelfrüh	Aroma	4.3	1.00
	H. Magnum	Bittering	13.5	2.10
USA	Willamette	Aroma	5.0	1.25
	Cascade	Aroma	5.8	1.05
	Zeus	Bittering	15.5	2.50
	Galena	Bittering	13.0	1.05
England	East Kent Golding	Aroma	5.5	0.60
	Fuggle	Aroma	4.3	1.05

2.2 Chemical analyses of hop compounds

Hop compounds were determined according to the European Brewery Convention (EBC) published methods [36]. The α-acid content of all hop samples was determined by conductometry as described in EBC standard method 7.4. The hop oil was extracted by steam distillation and the total oil content was quantified following EBC method 7.10.

2.3 Scanning electron microscopy

Samples were mounted onto double-sided carbon tape fixed to an aluminium specimen stub. For each of the nine varieties examined, several hop cones were used to mount a large amount of lupulin glands (> 500) on the SEM stubs. These were immediately freeze-dried and placed in a desiccator for better preservation. A JEOL JSM-5510 (JEOL, Tokyo, Japan) scanning electron microscope was used to take the detailed micrographs of the hop lupulin glands at an accelerating voltage of 5 kV (400–500x magnification).

2.4 Estimation of dimensions of glands

To calculate the volume of the glands, it was first necessary to do a visual assessment of the SEM micrographs. The lupulin glands were classified, according to their degree of fullness, into four categories: “empty”, “half full”, “partially full”, and “full”. A correction factor (CF) was assigned to each category, the factors range from 0.25 for the empty glands to 1 for the full ones. The dimensions of the glands for each of the varieties were calculated from the micrographs. The diameter of the lupulin glands was determined with the measuring tool of the image producing software. For each

of the nine varieties, the diameter of 50 different trichomes was measured and the size (i.e., volume) of the lupulin glands was calculated. The estimated volume of the glands was calculated by following the equation for spheroid bodies (Equation 1) and adjusting it with the correction factor (CF = 0.25, 0.50, 0.75, or 1).

$$V = \frac{3}{4}\pi \, r^3 \cdot C \, F \qquad \textbf{(Eq. 1)}$$

3 Results and Discussion

3.1 Scanning electron microscope observations of the lupulin glands

The lupulin glands of different hop varieties were observed using scanning electron microscopy. The homogeneity in size, structure, and appearance within each variety were evaluated. In figure 1 more than sixty glandular trichomes of the seeded aroma variety East Kent Golding can be seen. On the same micrograph a diversity of shapes and forms appears, these are the hop glands in different stages of morphogenesis. In previous studies [10, 30], the phases of morphogenesis were termed as “initiation”, “differentiation”, “determination”, “intensive growth”, and “maturity”.

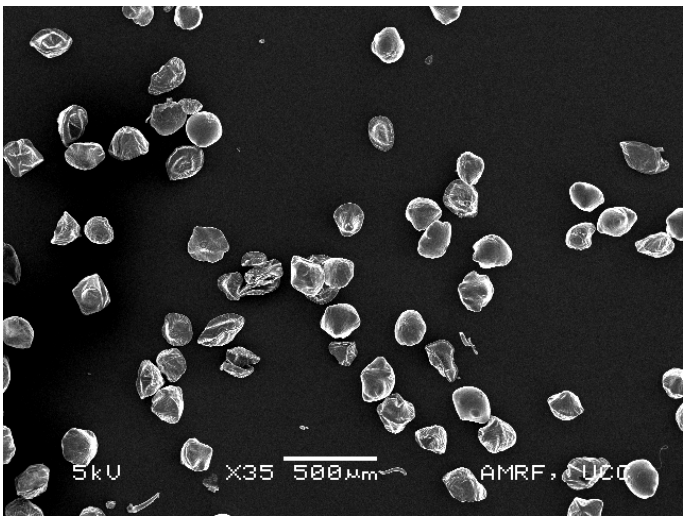

Fig. 1 Scanning electron micrograph of the lupulin glands of the seeded aroma hop variety East Kent Golding

Some hop cone shapes are characteristic of a particular hop variety and these can be identified by visual assessment. Seeded hop cones (e.g., East Kent Golding) tend to be heavier and less compact than seedless ones. The hop cones of Cascade and H. Magnum are long and compact while Zeus and H. Perle cones are medium sized. The physical characteristics of the hop cones do not seem to be hop class nor country of origin dependent, rather they are primarily associated with the hop variety. When Cascade hop cones grown in the United Stated and Germany are compared, slight differences can be observed but the general appearance remains constant. Thus, indicating that variety is the dominant factor determining the hop cone appearance. It is of interest to determine whether varietal characteristics are revealed and can be identified on the lupulin glands.

By using SEM it is possible to study the ultrastructure of the lupulin glands in more detail. In figure 2, three glandular trichomes are shown from three different hop varieties with different origins. Figure 2 **A** is a lupulin gland from the German aroma variety H. Mittelfrüh. In figure 2 **B** a Zeus trichome is shown, this is an American bittering hop. In figure 2 **C** the lupulin gland of the English seeded aroma variety Fuggle is shown. Although these varieties are chemically different, have different origins, and are categorized in different hop classes; by simple visual assessment of the micrographs no noticeable varietal differences can be appreciated. Therefore detailed analysis of the SEM images is necessary to determine if it is possible to classify the glandular trichomes into categories. It is ultimately intended to establish if relevant conclusions may be drawn from the evaluated ultrastructure properties (e.g., volume) of the lupulin glands.

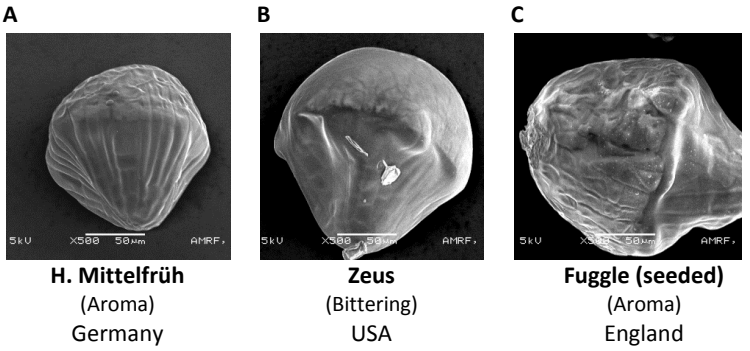

Fig. 2 Scanning electron microscopy micrographs of the lupulin glands of H. Mittelfrüh (Fig. 2 A), Zeus (Fig. 2 B), and Fuggle (Fig. 2 C)

As mentioned previously, it is in the lupulin glands of the female hop cone that the main brewing principles of hops, the resins and essential oils, are synthesized and accumulated. In the hop growing regions located in the northern hemisphere, by the end of July or early in August the glands appear as small cup-shaped structures. These become more and more filled with oily, resinous content as the season advances. Once the hop cone is fully ripe, the lupulin glands have a bulbous shape. *Menary* and *Doe* [17] observed the morphological changes of the lupulin glands in hop samples from the 1975, 1976, 1977, and 1978 harvests. Each year the maximum α-acid content coincided with the maximum gland filling. Twenty years later, *Hirosawa* et al. [10] concluded that the morphological changes of the lupulin glands occur in accordance with increasing α-acid content. Recent studies [22, 30] continue to link the morphology of the glands with the accumulation of α-acids.

After SEM observations the morphogenesis of the glandular trichomes was divided into four categories. In figure 3 **A–D** scanning electron microscopy images of the lupulin glands of Cascade hops are shown. In these micrographs, the morphological changes of the lupulin gland during the ripening period can be appreciated. When the gland is empty, it appears as a flat disc (Figure 3 **A**), as the secondary metabolites are being synthesized a cup-shaped gland (Figure 3 **B**) begins to form. As development continues the resins and essential oils accumulate, causing the glands to become more and more elevated in their central region (Figure 3 **C**) until maturity. The bulbous shape is characteristic of a mature hop gland (Figure 3 **D**).

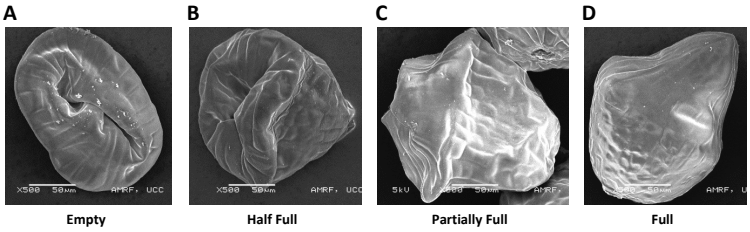

Fig. 3 **Scanning electron microscopy images of the morphology of the lupulin glands of the aroma hop variety Cascade at different stages of maturity. The images are classified based on their degree of fullness. In Fig. 3 A an empty gland can be observed. As the secondary metabolites are being synthesized the glands become more full (Fig. 3 B). Fig. 3 C appears to be completed however this gland is only partially full. In Fig. 3 D a full bulbous shaped gland is shown**

With SEM, high magnifications can be achieved, this makes scanning electron microscopy a powerful tool for detailed research on the ultrastructure of the surface topography of the lupulin gland. From the collected images it was revealed that the size, shape, form, and degree of fullness varied significantly between the observed glands. The surface structure of mature and immature lupulin glands is shown in figure 4 **A–D**. From the micrographs it can be concluded that the glands within the individual varieties cannot be considered homogeneous. Some differences on the surface topography can be seen among the different hop classes (aroma and bittering) and among the different hop varieties. In particular, the bittering variety Galena (Figure 4 **D**) had a papillary (i.e., bumpy) surface while the other varieties showed no bumps. It has been suggested that during biosynthesis of the hop secondary metabolites partial pressures of liquids and gasses increase this, in turn, causes breakage of the cell walls; consequently, the papillary texture on the glands is formed [31]. However, since secondary metabolites were synthesized in all hop varieties the papillary surface could suggest that either biosynthesis was more intensive in Galena or there is a compound (or compounds) present in Galena, but not in the other varieties, which causes the bumps to form.

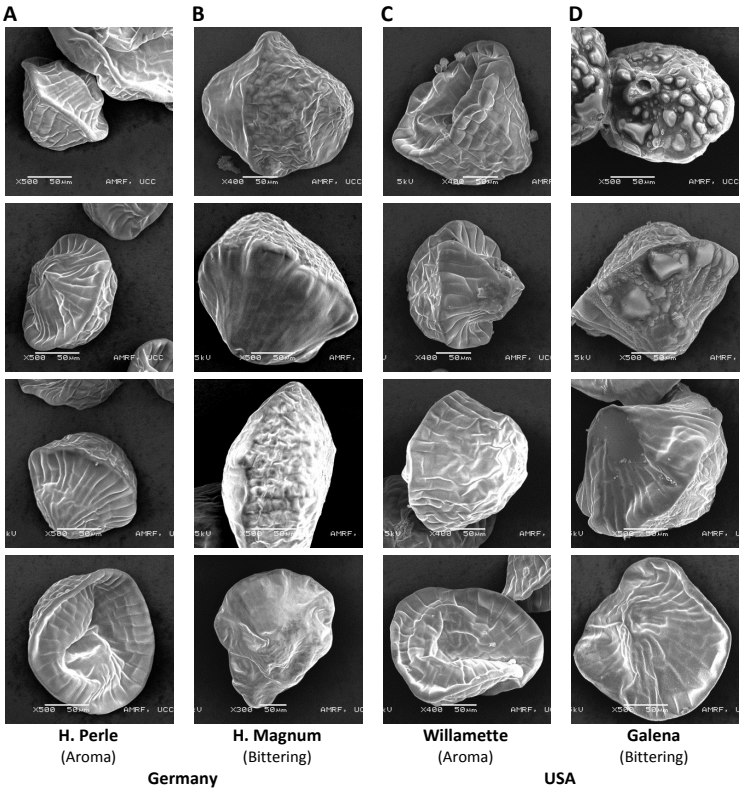

Fig. 4 **Scanning electron microscopy images of the lupulin glands of H. Perle (Fig. 4 A), H. Magnum (Fig. 4 B), Willamette (Fig. 4 C), and Galena (Fig. 4 D)**

3.2 Size distribution of the observed lupulin glands

As mentioned above, the lupulin glands were classified into four groups according to their degree of fullness. From the results shown in table 2 it is possible to see that the degree to which the glands are filled varies greatly among the hop varieties [35].

Table 2 **Lupulin gland (*n* = 50) percentage distribution belonging to the individual categories [35]**

Country	Hop Variety	Hop Class	Full	Partially Full	Half Full	Empty
Germany	H. Perle	Aroma	6 %	2 %	38 %	54 %
	H. Mittelfrüh	Aroma	54 %	32 %	10 %	4 %
	H. Magnum	Bittering	36 %	28 %	22 %	14 %
USA	Willamette	Aroma	2 %	10 %	32 %	56 %
	Cascade	Aroma	50 %	24 %	24 %	2 %
	Zeus	Bittering	36 %	40 %	24 %	0 %
	Galena	Bittering	72 %	20 %	8 %	0 %
England	East Kent Golding	Aroma	52 %	24 %	14 %	10 %
	Fuggle	Aroma	78 %	14 %	6 %	2 %

From the collected data it is not possible to establish a strong correlation between the hop class and the gland percentage distribution. It is also not possible to ascribe the differences to the country of origin. While H. Perle had a very low amount of full glands, over 50 % of the glands of the other examined German aroma variety, H. Mittelfrüh, were full. Moreover, the gland distribution of H. Perle is comparable to that of Willamette but not to that of Cascade. The last two varieties mentioned represent aroma hops grown in the United States. It is also not comparable to the distribution of both English aroma hop varieties. However, the percentage distribution of three of the six examined aroma hop varieties is similar. These are H. Mittelfrüh, Cascade, and East Kent Golding which are grown in Germany, United States, and England, respectively. More than 50 % of the observed glands of H. Perle and Willamette were empty. The data could suggest that both varieties were harvested before maturity. However, if the chemical data (see Table 1) is compared to the long-term average content of the variety, it may be concluded that the measured values are within the expected limits. The long-term average α-acid contents in H. Perle and Willamette are in the range of 4.0–9.0 % and 4.0–6.0 %, respectively [4]. The measured α-acid content for H. Perle and Willamette were 6.5 % and 5.0 %, respectively. Similarly, the long-term average oil contents in H. Perle and Willamette are in the range of 0.50–1.50 mL/100 g and 1.00–1.50 mL/100 g, respectively [4]. The measured total oil content for H. Perle and Willamette were 0.75 mL/100 g and 1.25 mL/100 g, respectively.

The gland distribution of the German bittering variety H. Magnum is almost evenly distributed among the four categories. Over 70 % of the observed lupulin glands in Galena and Fuggle were full. Interestingly, these two varieties differ in hop class and country of origin. While Galena is a bittering hop variety grown in the United States, Fuggle is a seeded aroma hop variety grown in England. Out of the nine varieties examined, only two bittering varieties had no empty glands, Zeus and Galena. In general, the data could suggest that some of the hops were picked before they were fully ripe. However, since the measured α-acid and total oil content, for all varieties, is within the long-term average content range it may be concluded that all hop varieties were picked at maturity. Another possibility is that the gland content decreased during handling (i.e., kilning and packaging). Unfortunately, it is also not possible to determine whether this was their original appearance or if the glands were damaged while mounting them on the stubs, during the freeze drying process, and/or by the electron irradiation [3].

Fifty lupulin glands of each hop variety were observed by SEM for diameter analysis. The gland diameter distribution of the examined hop varieties is shown in figure 5. The measured diameter of the lupulin glands ranged from 103 to 264 µm. The lower and higher range limits were measured in East Kent Golding and Willamette, respectively. H. Mittelfrüh (153 µm) had the smallest mean diameter whereas Willamette (210 µm) had the largest. The measured diameter correlates well with the degree of fullness. The flat, empty trichomes tend to have a large diameter; as secondary metabolite biosynthesis takes place, the disk-shaped trichomes gradually fill up and expand, thus elongating and consequently, the diameter decreases. As mentioned above, Willamette was the variety with the highest percentage of empty glands; this could account for the large mean diameter. Volume analysis is necessary to determine the overall size and appearance of the lupulin glands.

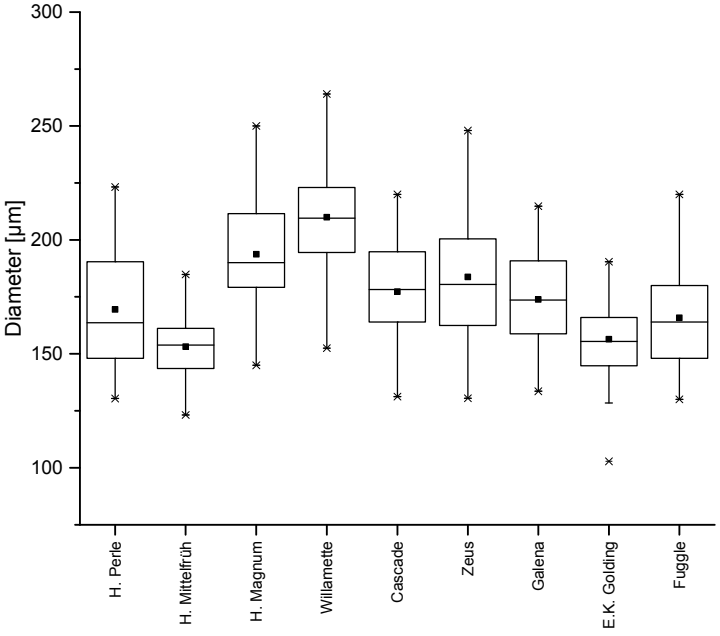

Fig. 5 Box and whisker plot of the measured diameter [µm] of the lupulin glands (*n* = 50) of 9 different hop varieties. Median (—); box = 25th and 75th percentiles; (■) = mean values; bars = min and max values

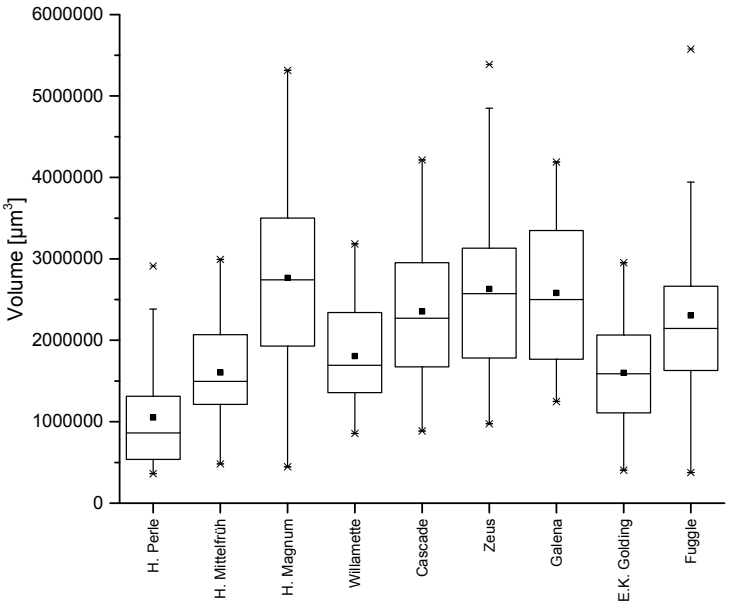

Fig. 6 Box and whisker plot of the calculated volume [µm³] of the lupulin glands (*n* = 50) of 9 different hop varieties. Median (—); box = 25th and 75th percentiles; (■) = mean values; bars = min and max values

A comparison between the calculated volume of the lupulin glands of hop varieties from Germany, United States, and England was made. In figure 6 the distribution of the data for each hop variety may be readily examined. The dimensions of the trichomes were calculated from the SEM images. The calculated volume of the glandular trichomes during the different phases of morphogenesis varied from 362,276 µm³ to 5,575,280 µm³. The smallest gland was found in H. Perle and the biggest in Fuggle, these glands were an empty and a full one, respectively. H. Perle had the lowest mean volume (1,051,314 µm³) and generally the H. Perle trichomes were small. When the measured diameter values were analyzed it was concluded that for H. Perle even the empty glands had a small diameter; suggesting that glandular trichomes of H. Perle are characteristically smaller. Larger volumes were calculated for the bittering hop varieties, H. Magnum, Zeus, and Galena. Of the three varieties, the highest mean volume was calculated for the German variety H. Magnum (2,765,685 µm³).

3.3 Clustering of the examined lupulin glands

For a robust and redundant based classifier it is essential to have more than one variable for clustering. To select the variables it was first necessary to test the data for overlapping. In figure 7–figure 10 the graphical presentation of the main variables: α-acids, oil content, mean diameter, and mean volume, is obtained and investigated for the Euclidean-based overlapping conditions. The mean centered values for α-acids (Equation 2), oil content (Equation 3), diameter (Equation 4), and volume (Equation 5) were calculated and used. This is a common practice for data pre-treatment which helps eliminate offsets and express the differences in the data set rather than similarities.

$$\alpha^* = \alpha - \alpha_\mu \quad \text{(Eq. 2)}$$

$$Oil^* = Oil - Oil_\mu \quad \text{(Eq. 3)}$$

$$\Phi^* = \Phi - \Phi_\mu \quad \text{(Eq. 4)}$$

$$\forall^* = \forall - \forall_\mu \quad \text{(Eq. 5)}$$

After mean-centering, the data was investigated for overlapping regions hashed diagonally in figure 7–figure 10. The figures were sorted assigning highest clustering ability according to minimal overlapping regions, with the highest labeled as ***. As expected the highest clustering ability (*s****) was obtained in figure 7 in which the hop varieties are grouped based on their α-acid content. As previously mentioned, hop varieties have traditionally been classified as bittering hops and aroma hops. At the time of harvest, the long-term average α-acid contents in the bittering and aroma varieties are in the range of 10 to 20 % and 2 to 10 %, respectively [32]. When the varieties were tested based on their oil content no clustering could be found. This result is not surprising since the quality of the oil is primarily assessed by its composition and not the total content [1]. When the diameter data was tested (Figure 9) only weak clustering (*s**) is seen due to high overlapping areas. The calculated volume was also tested, there was strong clustering (*s***) of the data. By inspecting the overlapping regions of the figures it was possible to select the two variables with highest clustering abilities, α-acids and volume, to plot and cluster the data.

The two variables scoring the highest clustering abilities, α-acids (*s****) and volume (*s***), were taken as the main components for the cluster. When both variables are plotted together, as in figure 11, a fully partitioned cluster is retrieved. Two main groups within the hop varieties were identified, the clusters coincided with the hop classes (i.e., bittering and aroma). The closest fit envelope (Euclidean-based), for both clusters, obeys an elliptic equation (see Equation 6 and 7).

$$\frac{(x-a)^2}{a^2}+\frac{(y-b)^2}{b^2}=1 \qquad \textbf{(Eq. 6)}$$

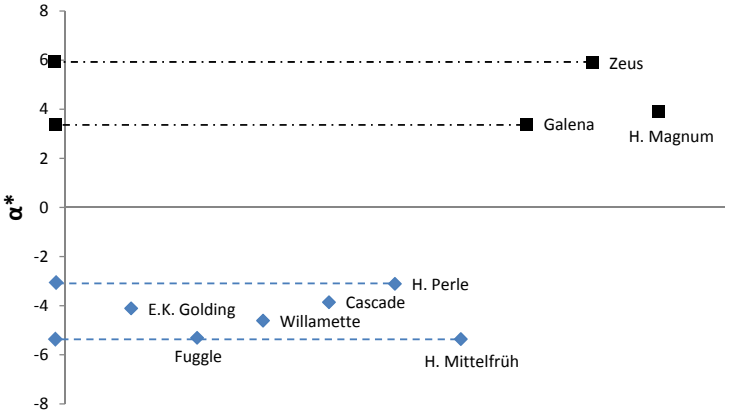

Fig. 7 **Distribution of the mean centered α-acids (α*) of bittering varieties (■) and aroma varieties (♦). The plot shows a clear distinction between the two hop classes with no overlapping regions. The plot is therefore assigned with a clustering ability of *s****

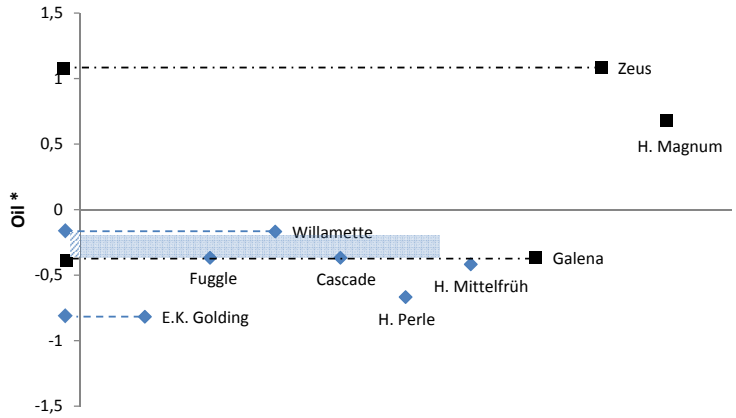

Fig. 8 **Distribution of the mean centered oil content (Oil*) of bittering varieties (■) and aroma varieties (♦). The two hop classes overlap in the centered region of –0.37 to –0.17; no clustering could be assigned to the Oil* plot**

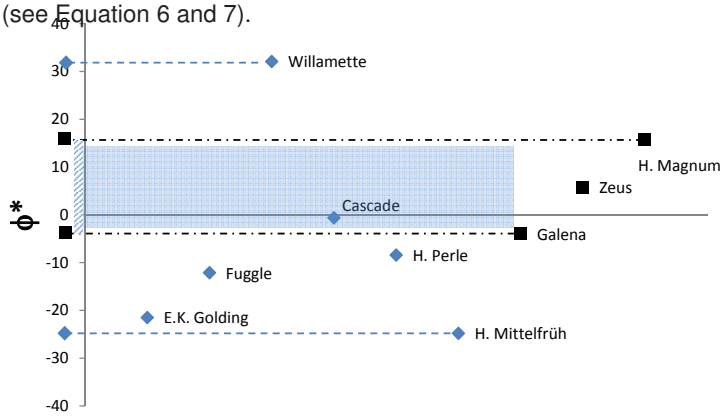

Fig. 9 **Distribution of the mean centered diameter (ϕ*) of bittering varieties (■) and aroma varieties (♦). The two hop classes overlap in the centered region of –3.99 to 15.81; therefore the ϕ* plot is assigned a clustering ability of *s****

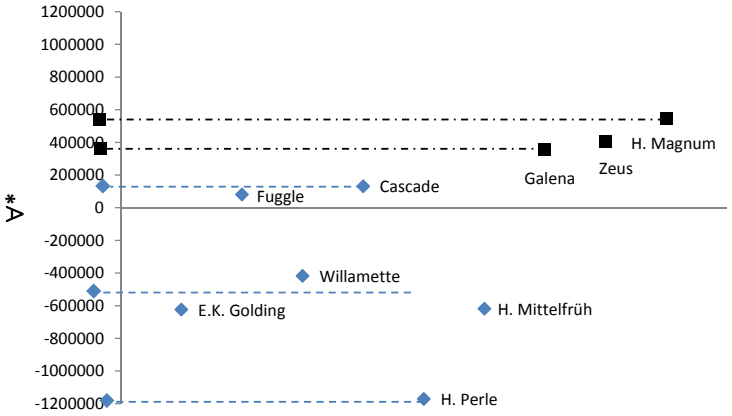

Fig. 10 **Distribution of the mean centered volume (∀*) of bittering varieties (■) and aroma varieties (♦). The plot shows a clear distinction between the two hop classes with no overlapping regions. The plot scored a high clustering ability of *s****

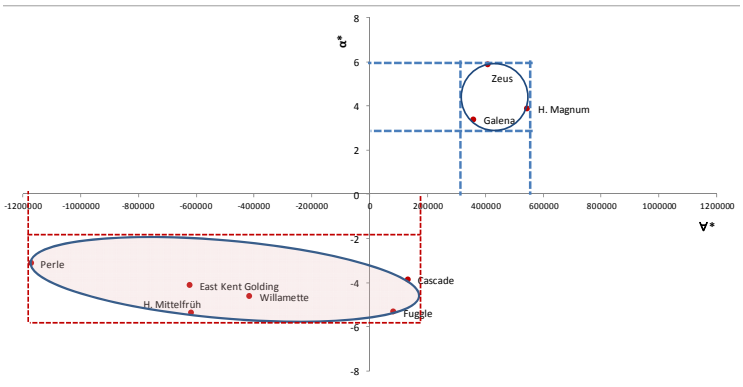

Fig. 11 **The two main variables scoring the highest clustering abilities, α-acids (*s****) and volume (*s***), are plotted orthogonal to each other, showing clear independent clusters of elliptic nature. The equations are deduced in equation 6 and equation 7**

a = 1853214
b = 2.5

The closest fit envelope equation for the cluster in which the aroma varieties are categorized was calculated by the equation below (Equation 7).

$$\frac{((x-a)\cos\alpha+(y-b)\sin\alpha)^2}{a^2}+\frac{((x-a)\cos\alpha+(y-b)\sin\alpha)^2}{b^2}=1$$

a = 1307430
b = 25.8 **(Eq. 7)**
$\alpha = 5°$

Each cluster was subsequently tested by a silhouette, which is based on the comparison of its tightness and separation. The silhouette pattern provides an evaluation tool for clustering validity [27]. Figure 12 helps interpret the dissimilarity among both clusters and confirm whether there are significant differences among them. Except for one, all values are above 0.8 thus confirming that the envelope cluster equation is correct and very limited overlapping is, in fact, occurring.

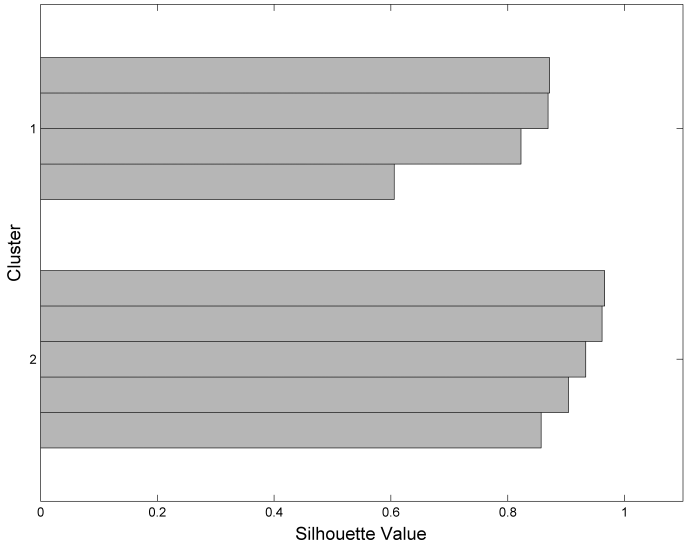

Fig. 12 Silhouettes of a clustering with k = 2 of nine hop varieties. The silhouettes further validate the previous result in figure 11, showing two distinct consistent classes within the cluster data

No satisfactory conclusions could be reached from the box and whiskers plots (Figure 5 and Figure 6) regarding the correlation between either the diameter or calculated volume and the hop variety. It was also not possible to establish whether the country of origin, the chemical composition, or the hop seeds had an effect on the morphology of the lupulin glands. The mean centered values of the data were calculated in an attempt to minimize the variation within the clusters and, thus, maximize the variation between the clusters. This is an important aspect as it constraints the resulting groups to have similar properties. It was possible to categorize the data into two clusters; these correspond to the hop classes (bittering and aroma). From the plotted data it was concluded that generally the bittering varieties have larger volumes than the aroma ones. This is due to the higher secondary metabolite, bitter acids and essential oil, contents in the bittering varieties. The country of origin does not seem to have an effect on the volume of the glandular trichomes. No trend regarding the volume of the glands could be established within the countries. It has been proven that seedless hops are generally richer in essential oils and resins than seeded ones [33]. Both seeded varieties did not show a negative effect on the size and volume of the lupulin glands. However, based solely on the physical properties of the gland it is no possible to reach any conclusions on the quality of the secondary metabolites.

4 Conclusions

In this study, nine hop varieties were observed using scanning electron microscopy; 50 micrographs for each variety were analyzed. The selected varieties were representative of their country of origin: Germany, USA, or England. Varieties from both hop classes, bittering and aroma, were examined; two seeded varieties were also included in the sample. Through the selection of the hop varieties it was possible to investigate several variables. By doing so it was intended to determine the influencing factor on the morphogenesis of the lupulin glands. The collected data provides an insight on the ultrastructure of the hop lupulin glands. The homogeneity in size, structure, and appearance within each variety were evaluated from the micrographs. The obtained results provide a better understanding of the factors influencing the morphology of the lupulin glands. The collected data in this study suggest the environmental conditions (i.e., country of origin) is not the only factor influencing the secondary metabolite biosynthesis. Although, the handling conditions vary between countries, these also do not seem to have an effect on the gland structure. While the climatic conditions have an effect on the hop brewing principles, they do not have the same impact on all varieties. Through data clustering two groups were obtained, both relate directly to the hop class and appear to be independent of the other variables. This could suggest that the genetic coding of the hop variety ultimately determines the rate and extent of accumulation of secondary metabolites. In further research it would be valuable to investigate the same hop variety grown in different countries and to conclusively determine the effect of environmental and handling conditions on the structural features of the lupulin glands.

Acknowledgment

This work was supported by the Barth-Haas Grant.

5 References

1. Almaguer, C.; Gastl, M.; Arendt, E. K. and Becker, T.: Comparative study of the contribution of hop (*Humulus lupulus* L.) hard resins extracted from different hop varieties to beer quality parameters, Journal of the American Society of Brewing Chemists **73** (2015), no. 2, pp. 115-123.
2. Almaguer, C.; Schönberger, C.; Gastl, M.; Arendt, E. K. and Becker, T.: *Humulus lupulus* – a story that begs to be told. A review, Journal of the Institute of Brewing **120** (2014), no. 4, pp. 289-314.

3. Almaguer, C.; Thiele, F.; Schönberger, C. and Arendt, E. K.: Scanning electron microscope (SEM) observation of the lupulin glands of different hop varieties, 2nd International Symposium for Young Scientists and Technologists in Malting, Brewing and Distilling, 2010, Freising, Germany.
4. Barth-Haas-Group: Hop varieties – From Australia to USA.
5. The Barth Report (2014/2015) 2015.
6. Diffor, D. W.; Likens, S. T.; Rehberger, A. J. and Burkhardt, R. J.: The effect of isohumulone/isocohumulone ratio on beer head retention Journal of the American Society of Brewing Chemists **36** (1978), no. 2, pp. 63-65.
7. Fly, W. H. and Chicoye, E.: The effect of countercurrent distribution fractions of hop extracts on beer foam Journal of the American Society of Brewing Chemists **35** (1977), no. 2, pp. 69-72.
8. Harrison, J.: Effect of hop seeds on beer quality, Journal of the Institute of Brewing **77** (1971), no. 4, pp. 350-352.
9. Hayduck, M.: Über die bitteren und harzigen Bestandteile des Hopfens, Wochenschrift für Brauerei **5** (1888), no. 47, pp. 937-947.
10. Hirosawa, T.; Saito, T.; Tanaka, T. and Matsushima, H.: SEM observation and HPLC analysis of the accumulation of alpha-and beta-acids in the fresh developing hop (*Humulus lupulus* L.) peltate glandular trichomes, Journal of Electron Microscopy **44** (1995), no. 3, pp. 145-147.
11. Ives, A. W.: An experimental inquiry in the chemical properties and economical and medicinal virtues of the *Humulus lupulus*, or common hop, Annals of Philosophy **17** (1821), no. pp. 194-202.
12. Kishimoto, T.; Kobayashi, M.; Yako, N.; Iida, A. and Wanikawa, A.: Comparison of 4-mercapto-4-methylpentan-2-one contents in hop cultivars from different growing regions, Journal of Agricultural and Food Chemistry **56** (2008), no. 3, pp. 1051-1057.
13. Kovačevič, M. and Kač, M.: Solid-phase microextraction of hop volatiles: Potential use for determination and verification of hop varieties, Journal of Chromatography A **918** (2001), no. 1, pp. 159-167.
14. Kralj, D.; Zupanec, J.; Vasilj, D.; Kralj, S. and Pšeničnik, J.: Variability of essential oils of hops, *Humulus lupulus* L., Journal of the Institute of Brewing **97** (1991), no. 3, pp. 197-206.
15. Lange, M. and Turner, G. W.: Terpenoid biosynthesis in trichomes – current status and future opportunities, Plant Biotechnology Journal **11** (2013), no. 1, pp. 2-22.
16. Maeda, E.: Scanning electron microscope studies on lupulin glands in *Humulus lupulus* L., Japanese Journal of Crop Science **46** (1977), no. 2, pp. 249-253.
17. Menary, R. C. and Doe, P. E.: Some morphological and chemical changes in hops during maturation, Journal of the Science of Food and Agriculture **34** (1983), no. 9, pp. 921-929.
18. Moritz, E. R. and Morris, G. H.: A text-book of the science of brewing, Auflage: E. & F. N. Spon, 1891.
19. Nagel, J.; Culley, L. K.; Lu, Y.; Liu, E.; Mathews, P. D.; Stevens, J. F. and Pege, J. E.: EST Analysis of hop glandular trichomes identifies an O-methyltransferase that catalyzes the biosynthesis of xanthohumol, The Plant Cell **20** (2008), no. 1, pp. 186-200.
20. Neame, C. R. B.; Ely, G. and Laws, D. R. J.: Lager brewing on the production scale using enriched pellets made from seeded hops, Journal of the Institute of Brewing **86** (1980), no. 2, pp. 84-86.
21. Oliveira, M. M. and Pais, M. S. S.: Glandular trichomes of *Humulus lupulus* var. Brewer's Gold: Ontogeny and histochemical characterization of the secretion, Nordic Journal of Botany **8** (1988), no. 4, pp. 349-359.
22. Patzak, J.; Krofta, K.; Henychová, A. and Nesvadba, V.: Number and size of lupulin glands, glandular trichomes of hop (*Humulus lupulus* L.), play a key role in contents of bitter acids and polyphenols in hop cone, International Journal of Food Science and Technology **50** (2015), no. 5, pp. 1864-1872.
23. Percival, J.: The Hop Plant In: Chapman, A. C.: The Hop and its Constituents – A Monograph on the Hop Plant, 1905.
24. Pfenninger, H. B.; Hug, H.; Ault, R. G. and Kenber, R. M. J.: Effects of products from seedless and seeded hops on beer quality, Journal of the Institute of Brewing **84** (1978), no. 5, pp. 276-2277.
25. Raiser, T. C.: Commercial aspects of hops, 1st Hops Academy, 2011, Nuremberg, Germany, 1-28,
26. Roberts, T. R. and Wilson, R. J. H.: Hops In: Priest, F. J. and Stewart, G. G.: Handbook of Brewing, 2006.
27. Rousseeuw, P. J.: Silhouettes: a graphical aid to the interpretation an validation of cluster analysis, Journal of Computational and Applied Mathematics **20** (1987), no. pp. 53-95.
28. Saito, T.; Hirosawa, T.; Horiuchi, S.; Murakami, A. and Matsushima, H.: A study of SEM examination on fresh hop (*Humulus lupulus* L.) peltate glandular trichomes, Journal of Electron Microscopy (Tokyo) **44** (1995), no. 1, pp. 39-44.
29. Smith, R. J.; Davidson, D. and Wilson, R. J. H.: Natural foam stabilizing and bittering compounds derived from hops Journal of the American Society of Brewing Chemists **56** (1998), no. 2, pp. 52-57.
30. Srečec, S.; Zechner-Krpan, V.; Marag, S.; Špoljarić, I.; Kvaternjak, I. and Mršić, G.: Morphogenesis, volume and number of hop (*Humulus lupulus* L.) glandular trichomes, and their influence on alpha-acid accumulation in fresh bracts of hop cones, Acta Botanica Croatica **70** (2011), no. 1, pp. 1-8.
31. Srečec, S.; Zechner-Krpan, V.; Petravić-Tominac, V.; Mršić, G.; Špoljarić, I. and Marag, S.: ESEM comparative studies of hop (*Humulus lupulus* L.) peltate and bulbous glandular trichomes structure, Agriculturae Conspectus Scientificus **75** (2010), no. 3, pp. 145-148.
32. Steinberg, A. S.: Hops, 1st Hops Academy, 2011, Nuremberg, Germany, 1-40,
33. Stevens, R.: The chemistry of hop constituents, Chemical Reviews **67** (1967), no. 1, pp. 19-71.
34. Sugiyama, R.; Oda, H. and Kurosaki, F.: Two distinct phases of glandular trichome development in hop (*Humulus lupulus* L.), Plant Biotechnology **23** (2006), no. 5, pp. 493-496.
35. Thiele, F.; Schönberger, C. and Arendt, E. K.: Scanning electron microscope (SEM) examination of lupulin glands of different hop varieties, 32nd European Brewery Convention, 2009, Hamburg, Germany,
36. Van Erde, P.: Analytica-EBC, Auflage: Fachverlag Hans Carl, 1998.
37. Virden, J.: Brewing trials of seeded and seedless hops from the 1969 crop, Journal of the Institute of Brewing **78** (1972), no. 5, pp. 399-403.
38. Wang, G.; Tian, L.; Aziz, N.; Broun, P.; Dai, X.; He, J.; King, A.; Zhao, P. X. and Dixon, R. A.: Terpene biosynthesis in glandular trichomes of hop, Plant Physiology **148** (2008), no. 3, pp. 1254-1266.
39. Yamada, M.; Suzuki, T.; Oho, E. and Matsushima, H.: Colour scanning electron microscopy of peltate glandular trichomes of fresh developing hops (*Humulus lupulus* L.), Journal of Electron Microscopy **47** (1998), no. 5, pp. 539-542.
40. Zarnkow, M.: Influence of seeded hops on glycosidic bound hop aroma compounds, 35th European Brewery Convention, 2015, Porto, Portugal,

Received 9 November 2015, accepted 7 December 2015