K. Krofta, J. Patzak, P. Vondráčková and L. Mravcová

Sulfur substances of hop oils and identification of hop varieties – advantages and limitations

Sulfur compounds of hop oils are important components of hop oils although their share is relatively very small, approximately 1 %. A collection of 30 sulfur-containing volatiles was detected by gas chromatographic analysis of hop oils which were isolated from the hops by steam distillation. Thioesters S-methylthio-hexanoate, S-methylthio-isovalerate, S-methylthio-butyrate have been identified as the dominant components. It was found out that the composition of sulfur compounds of hop oils, examined on a set of 14 Czech hop cultivars, is varietally characteristic. Statistical evaluation using multivariate statistical methods has shown that the spectrum of sulfur substances can be used to determine the variety authenticity for only a few cultivars (Boomerang, Kazbek, Gaia, Vital). For other varieties it is not sufficient to distinguish them reliably. The analysis of sulfur compounds of hop oils must therefore be considered as an auxiliary chemotaxonomic tool. To determine the identity of the variety with greater reliability, it is necessary to use other chemotaxonomic tools (terpenic and oxygenated substances of hop essential oils, bitter substances, prenylfavononoids and polyphenols). Genetic methods are also an important approach at determining and identifying the purity of hop varieties.

Descriptors: hop oils, sulfur compounds, gas chromatography, statistical analysis

1 Introduction

Hop chemists often face the task of determining the identity of a hop variety. This is usually a requirement of brewers that want to verify whether the hops used have the required brewing properties. Confirmation of the authenticity of hops cultivars (and many other agricultural products) is also an important tool for proving their falsification.

The content and composition of secondary metabolites of hops offers several tools for this purpose, which can be used to successfully complete this task. Analysis of alpha and beta acids is a common analysis in many hop laboratories. The bitter acid content itself is subject to significant year-on-year fluctuations in many growing areas due to the weather conditions during the growing season [1, 2]. Thus the predictive power of this chemotaxonomic parameter is limited. The cohumulone and colupulone ratio is a much more stable parameter, but the typical ranges for individual varieties often overlap and thus allow only group classification of an unknown variety [3]. A useful additional criterion for the specification of certain varieties is the content of prenylflavonoids. The high content of xanthohumol (1 – 1,8 % by weight) is typical for the German variety Taurus and Xantia, the English varieties Admiral and Target or the Czech variety Agnus [4, 5] High content of desmethylxanthohumol (0.3 – 0.4 % w/w) is typical for the Czech variety Vital [6]. Number of identification tools provide composition of hop polyphenols, primarily from the group of flavonols and proanthocyanidins. The flavonols quercetin and kaempherol, which are found in hops in the form of glycosides, were used for hop varieties chemotaxonomy by *Van Sumere* [7]. Kammhuber analyzed the same compounds by liquid chromatography as aglycones after hydrolytic cleavage of the sugar component [8]. Glycosidically bound polyphenols are relatively stable and can be identified not only in raw hops but also in hop pellets and beer [9]. Several authors used the composition of proanthocyanidins to identify hops using UHPLC in combination with high-resolution mass detectors [10]. Profile of proanthocyanidins is variety-specific, but it is also influenced by geographical origin [11].

Content and composition of several hundred terpenic and oxygenated substances of hop essential oils is a very effective tool for the identification of hop varieties. The group of sesquiterpenes (caryophyllene, humulene, farnesene, selinenes) and their relative proportions are often variety-specific. The same holds true for geraniol compounds (esters, geranic acid, methyl geranate) and isobutyl esters. The composition of hop essential oils was used for variety classification by a number of authors [12, 13, 14, 15]. Harvest maturity plays an important role in the composition of essential oils. Late harvested hops contain more oils and were better rated in aroma quality, which was positively reflected in beers [16]. Genetic methods can be also used for identification and authentic-

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Authors

Karel Krofta, Josef Patzak, Petra Vondráčková, Lenka Mravcová, Hop Research Institute Co., Ltd., Zatec, Czech Republic; corresponding author: krofta@chizatec.cz

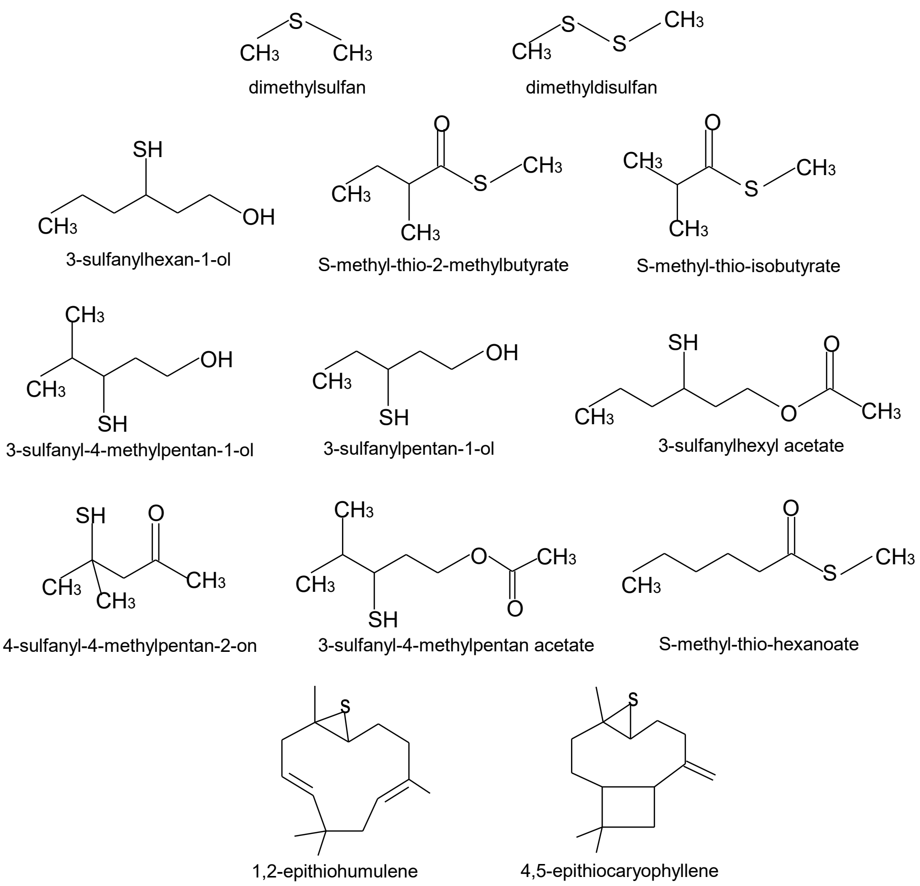

Fig. 1 Chemical structure of selected sulfur compounds of hop oils

ity control of hop cultivars. Molecular markers are independent on environmental, processing and storage conditions [17].

Hop oils contain a very small amount of sulfur compounds (~ 1 %). However, these are mostly sensory active substances with very low perception thresholds, which can affect the overall aroma of hops and beer. Tens of volatile sulfur compounds from the group of thiols, polysulfides, S-methyl thioesters, sesquiterpene episulfides have been identified in hop oils [18]. Polyfunctional thiols have recently been referred to as important hop-derived flavoring compounds of hops and beers [19, 20]. Blackcurrant-like 4-sulfanyl-4methylpentan-2-one constitute key compound of Nelson Sauvin variety [21]. Floral-like 3-sulfanyl-2-ethylpropyl acetate distinguish Tomahawk from other thiol-rich varieties. Most low-bitter varieties, including Saaz, are greatly lacking in polyfunctiolal thiols [22]. Isolation of polyfuntional thiols from hops requires special approach based on formation of p-hydroxymercury-benzoate conjugates or silver ion solid phase extraction preconcentraction [23, 24]. The structural formulas of selected sensory active sulfur compounds of hop oils are shown in figure 1.

Until the 1980s, the composition of the sulfur substances of hop oils was greatly influenced by external factors during cultivation and processing. Increased content of sulfur substances, especially sulfur derivatives of sequiterpenes, was found in hops preserved by sulfur combustion or hops treated with sulfur pesticides [25, 26]. Nowadays, when sulfurization has not been carried out for many years, the composition of sulfur compounds is less affected by growing history and post-harvest processing. This creates a precondition for using the composition of sulfur substances in essential oils to identification of hop varieties. *Kammhuber* et al. [18] have already pointed out that the spectrum of sulfur substances of hop oils is varietally specific, but the utilisaion of these substances for hop varieties discrimination has not yet been published.

This work is therefore focused on the use of a specific spectrum of sulfur components of essential oils to identify Czech hop varieties. Therefore, advantages and limitations of this approach was investigated on a set of 14 Czech hop cultivars.

2 Materials and methods

2.1 Hop samples

Samples of 14 Czech hop varieties in the form of raw cones were obtained directly from farmers and commercial companies (Saaz, Premiant, Sladek, Premiant, Kazbek, Saaz Late, Saaz Special, Agnus, Vital, Harmonie). Less widespread varieties (Gaia, Boomerang, Bor, Bohemia) came from the field experiments of ÚKZÚZ (Central Control and Testing Institute for Agriculture). Detail information on harvest maturity were not known (farmers samples), but it can be assumed that hops were harvested at the optimal/typical stage of ripeness. Farmers decide on the term of the harvest on the basis of an assessment of the smell and appearance of the cones and the content of alpha acids.

During the three harvests of 2017, 2018, and 2019, at least 3 independent samples from all hop-growing regions in the Czech Republic were obtained from each variety. The samples were stored in an air-conditioned warehouse at a temperature of up to + 5 °C until processing within 2 months after harvesting.

2.2 Isolation of hop oils

Hop oils were isolated from the cones by the steam distillation method according to the ASBC Hops-13 method [27]. Immediately before processing, the cones were ground in a grinder to a particle size of max. 3 mm. The suspension prepared from 100 g of hops and 2000 ml of water was poured into a 4000 ml ground glass flask and placed in a heating nest. A collimator with a two-circuit water cooling was fitted to the flask. After 90 minutes of boiling, the essential oils were poured into a 10 ml glass vial and placed in the freezer where they were stored at –20 °C until analysis. The method is still widely used, although it has some drawbacks. Steam distillation can lead to the formation of artifacts [28]. *Rettberg* el al. [29] refer about the isomerization and oxidation of terpene alcohols. The change in the composition of hop essential oils during distillation was minimized by reducing the boiling time from 4 hours to 90 minutes, during which the majority of essential oils flowed. The method is unsuitable for isolation of polyfunctional thiols.

2.3 Chromatography analyses

The analysis of hop oils were performed by gas chromatography on a Shimadzu GC 2010 Plus gas chromatograph on a Rxi-5Sil-MS column (30 m x 0.25 mm x 0.50 µm, Restek). The temperature program began with a 5-minute delay at 60 °C, followed by successive temperature gradients at 170 °C at 3.0 °C/min, 220 °C (5.0 °C/min), and 300 °C (25 °C/min). min) with a delay of 5 minutes at the final temperature. The carrier gas was pure helium with a constant flow rate of 3.1 ml/min. The injector temperature was 250 °C. Sulfur compounds were detected using a specific flame-photometric detector (FPD) operating in a sulfur mode at 394 nm and a constant temperature of 330 °C. The combustion gas flow rates for the FPD detector were set to 62.5 ml/min (hydrogen) and 90 ml/min (air). The injection volume with the help of autoinjector was 0.3 µl at a split ratio of 1 : 5. The essential oils were diluted 1 : 1 with n-hexane prior to injection.

Some sulfur compounds were identified using analytical standards. Dimethyldisulfan, dimethyltrisulfan were purchased through Sigma-Aldrich, S-methylthio-isovalerate and S-methylthio-hexanoate were purchased from Penta (USA). Other sulfur compounds have not been identified yet. For the purposes of chemotaxonomics of hop varieties, a sufficient semi-quantitative evaluation was carried out as a ratio of the integrated area of the component to the total integrated area of all sulfur substances, and expressed as a relative percentage. Substances with a signal to noise ratio (S/N) greater than 1.10^4 were included in the statistical evaluation [18].

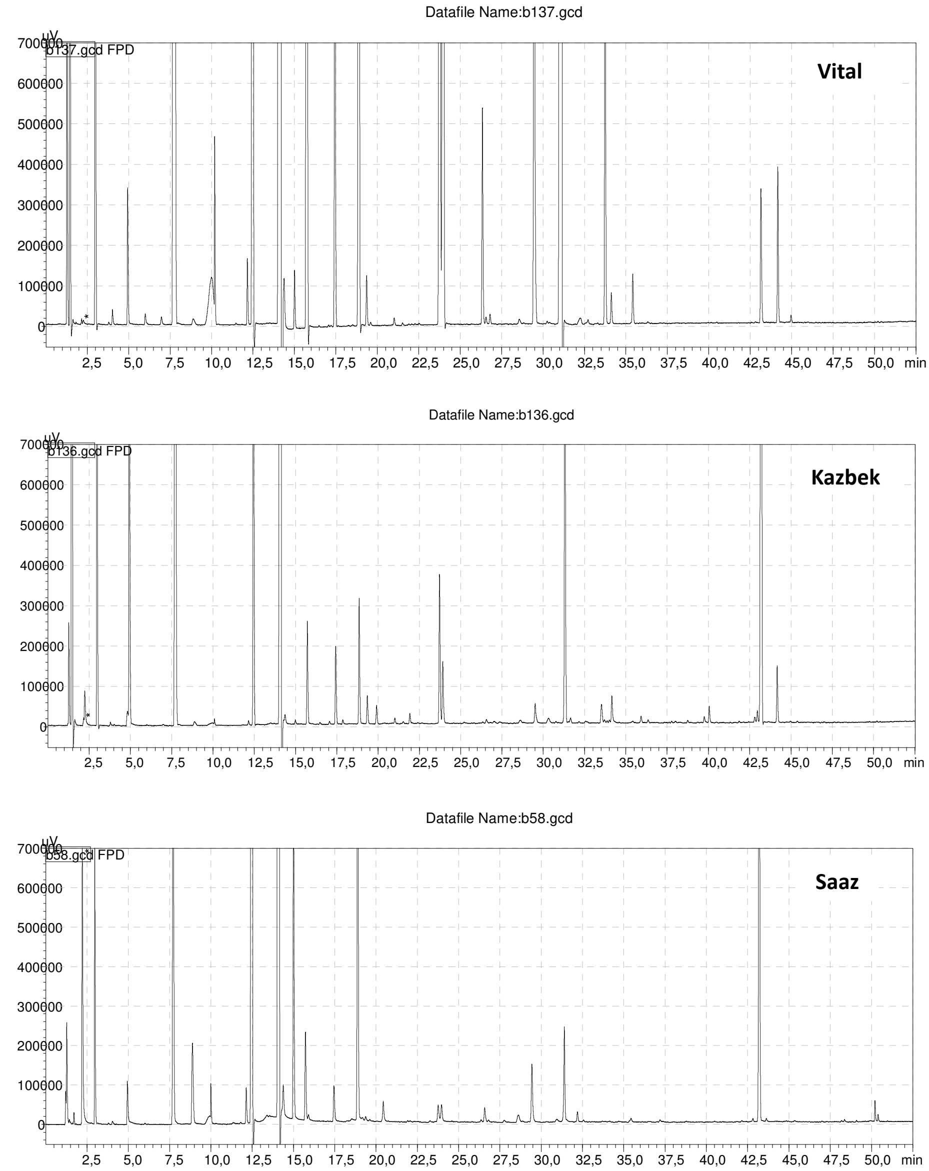

Fig. 2 Chromatograms of sulfur compounds of hop essential oils of varieties Vital, Kazbek and Saaz (GC/FPD, Rxi-5SIL-MS, 30 m x 0,25 mm x 0.50 µm, temperature program 60 °C – 300 °C)

2.4 Statistical evaluation

Since the analytical data of the contents of the individual components had a varied range of values, it was first standardized (min-max normalization) in the range of values from 0 to 1, for individual years and for summary data as well. The statistical similarity of individual samples was performed on the basis of the Euclidian distance, which were used for the multivariate hierarchical cluster analysis by Ward algorithm, the principal component analysis (PCA) and the discriminant analysis (DA) in STATISTICA 8.0 CZ (StatSoft), Tulsa, OK, and DARwin v. 5.0.155 (Dissimilarity Analysis and Representation for Windows, http://darwin.cirad.fr/darwin). Three-dimensional graphs were visualized with SigmaPlot for Windows v.10.0.0.54 (Systat Software Inc., San Jose, CA). GenEx v.6.0.1.612 (MultiD Analyzes AB, Gothenburg, Sweden) was used to create the heatmap.

3 Results and discussion

Figure 2 displays the chromatograms of the sulfur compounds of hop oils of the three varieties Vital, Kazbek and Saaz. It clearly shows that the composition of the sulfur compounds is varietal-specific and thus confirms the findings already made by Kammhuber et al. [18]. Unlike in the earlier studies [26] the spectrum of sulfur compounds of all varieties during 3 harvests and from different locations was consistent and did not show significant year-on-year differences, due to the growing history, i.e. the application of sulfur pesticides (e.g. propagrite) or elemental sulfur in chemical treatment against hop powdery mildew. During 1960s and 1980s it led to a substantial increase in the amount of certain sulfur compounds such as dimethyltrisulfan, thiofene derivatives,

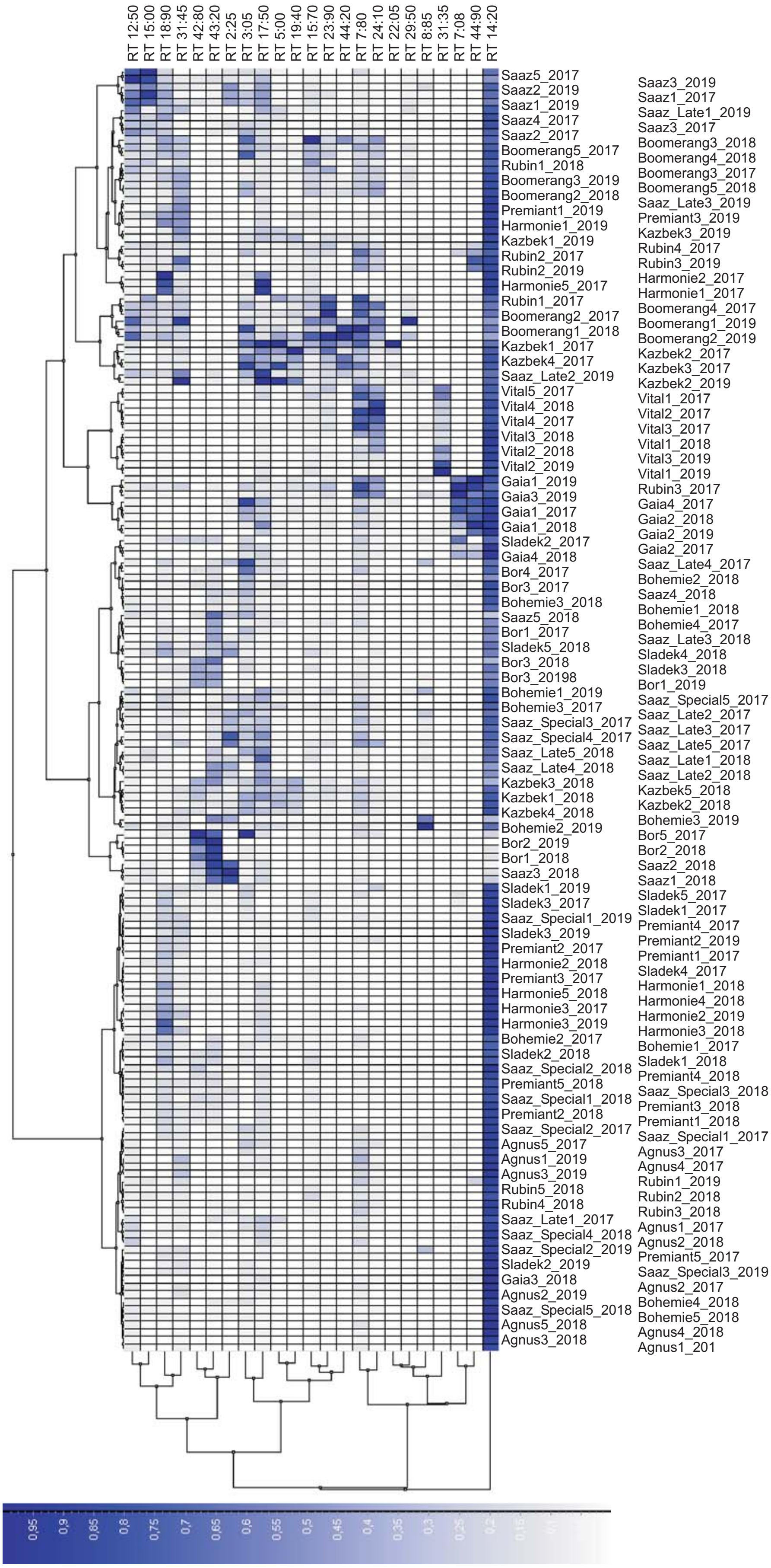

Fig. 3 **Heat map of sulfur compounds in hop oils of 14 Czech hop varieties (harvest 2017, 2018, 2019)**

and caryophyllene and humulene episulfides in the hops cultivation and preservation [25]. Sugget [26] even states that, owing to the growing history, the composition of the sulfur compounds of hop oils, unlike the oxygenated and terpenic ones, cannot be regarded varietal specific. The situation has changed since that time, the use of sulfur-free fungicides minimizes the effect of chemical protection of hop on the compositiion of essential oils.

When using a limiting signal to noise ratio, 30 sulfur compounds were identified in hop oils. At higher resolution, a large amount of minor sulfur substances appears in the chromatogram, which are difficult to identify. Since only a small part of the whole sulfur spectrum has been identified using the available analytical standards, the whole analysis can be considered as a varietal fingerprint sufficient for the purpose of varieties discrimination. Methyl thioesters are prominent in the FPD chromatograms of all steam-distilled hop oils. They are not artifacts of the boil in the extraction procedure since they are present in cold extracts of hops [30]. These are mainly S-methyl thiohexanoate (Fig. 2, elution band RT 14:20 min), only Bor variety contains lesser amounts, and S-methylthio-isovalerate (Fig. 2, elution band RT 7:80 min) was also identified in all hops, a large amount was found in the varieties Boomerang, Gaia, Kazbek, Rubin, and Vital. Dimethyltrisulfan has been identified in small quantities only in Saaz and genetically related varieties Saaz Late and Saaz Special. The aroma of alkyl sulfides and polysulfides are generally chraracterized as "cooked vegetables or onion", but these compounds are largely evaporated during the wort boiling. It was shown to originate from a labile precursors (S-methylcystein sulfoxide) during steam distillation at 100 °C [31]. Still unknown substances eluting at RT 44:20 and 44:90 minutes of chromatographic analysis have great discriminatory potential.

A very effective tool of visualization of analytical data is a two-dimensional analysis ("heatmap") which shows the relative contents of the individual components expressed as a Z-score (Fig. 3). The shades of blue colour correspond to the standardized concentrations of individual sulfur compounds in the range of 0 – 1.This evaluation method has already been used for hop bitter compounds [32] and polyphenols [33]. The analysis shows that some components have the same precursors or are located on the same biosynthetic pathway. These are RT components 12:50 and 15:00, 18:90 and 31:45, 42:80 and 43:20, 3:05 and 17:50, 5:00 and 19:40, 15:70 and 23: 90, 7:80 and 24:10, 7:08 and 44:90

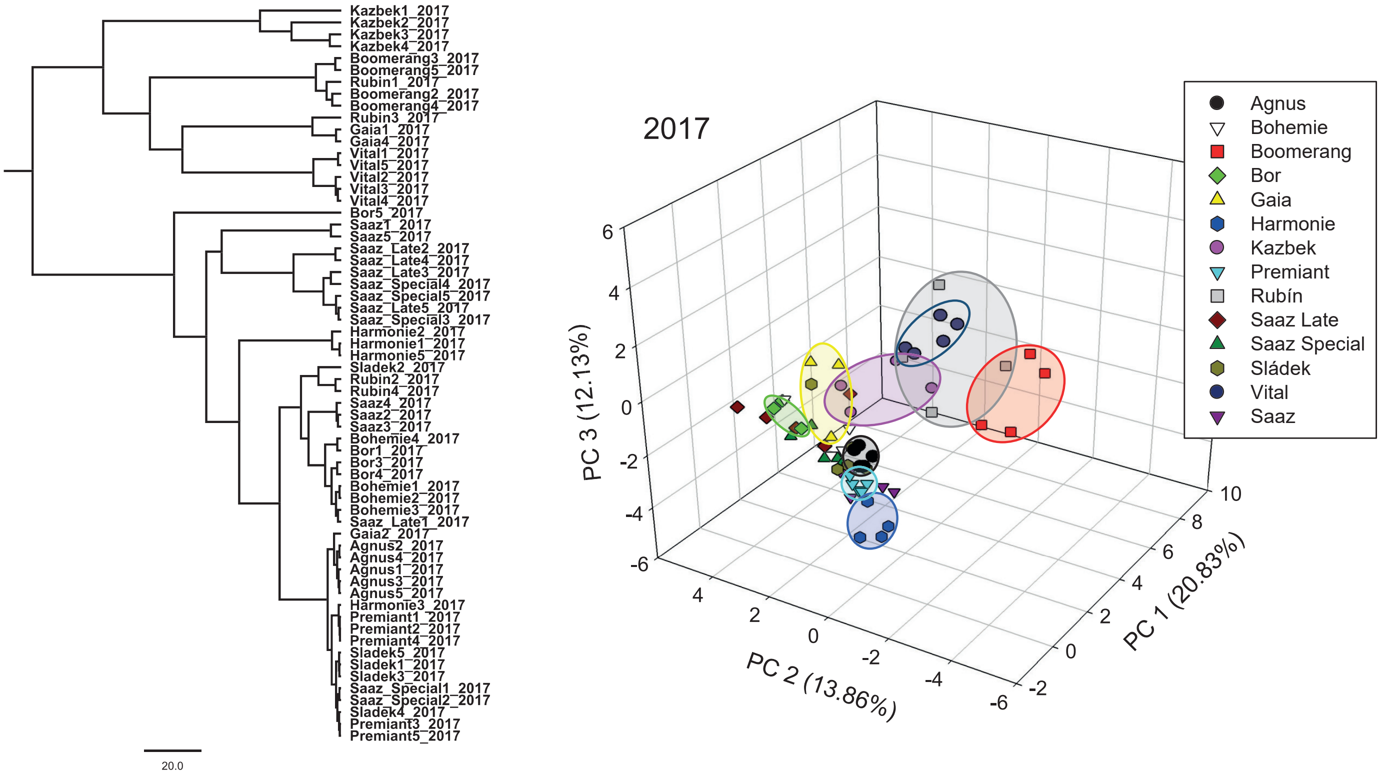

Fig. 4a Statistical evaluation of sulfur compounds in hop oils of 14 Czech hop varieties by multidimensional PCA analysis (harvest 2017)

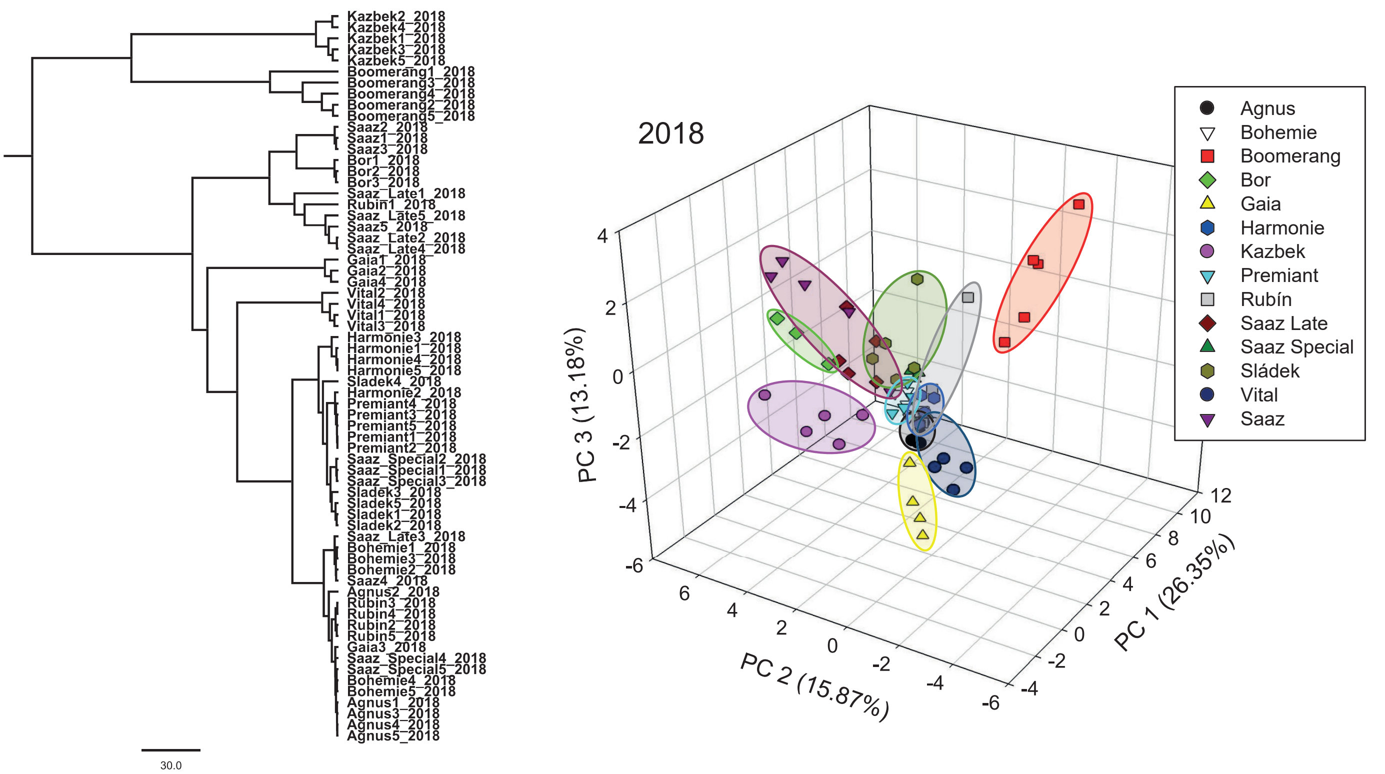

Fig. 4b Statistical evaluation of sulfur compounds in hop oils of 14 Czech hop varieties by multidimensional PCA analysis (harvest 2018)

min. Samples of individual varieties show that the contents of the sulfur compounds can be significantly influenced by locality and year of harvest.

Principal components analysis (PCA) and hierarchical cluster analysis (HA) were used to evaluate the similarity and variability of individual samples. Figures 4a, 4b and 4c (see page 31) show the results of statistical analysis of sulfur compounds from harvests 2017, 2018 and 2019 by the PCA method in a three-dimensional design and hierarchical cluster analysis. Principal component

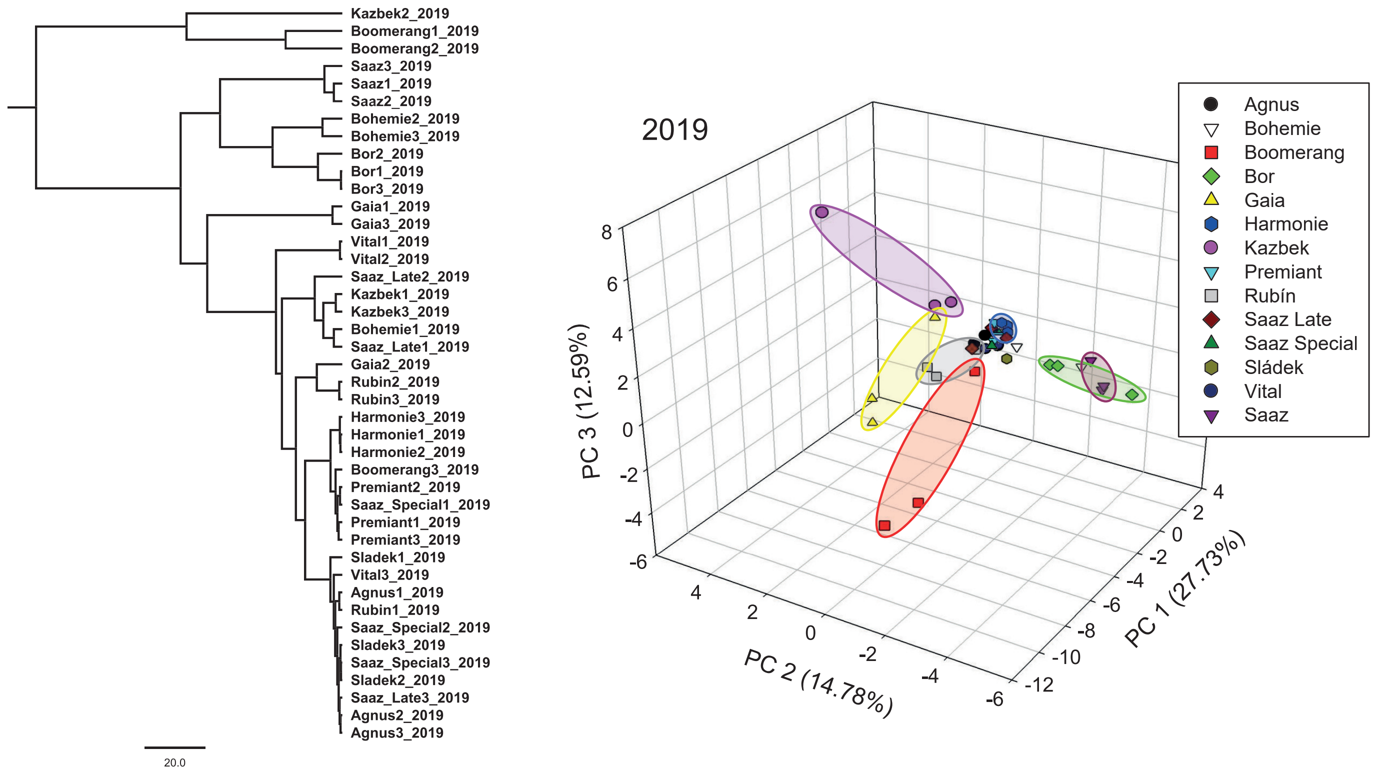

Fig. 4c Statistical evaluation of sulfur compounds in hop oils of 14 Czech hop varieties by multidimensional PCA analysis (harvest 2019)

analysis explained 47 % (2017) and 55 % (2018, 2019) of the total variability. Another source of variability may be the growing locality [34], although all tested hops in this study were grown in comparable climatic conditions. Next contribution to the overall variability may be harvesting of hops at different stages of maturity. The amount and composition of essential oils varies significantly in the course of hops ripening and maturation, therefore the harvesting date significantly impacts their final profile [35, 36].

Classification of tested varieties from the point of their discrimination on the basis of sulfur compounds in hop oils are summarized in table 1. Classification into individual categories (good, partial and poor) in each year is carried out on the basis of clustering using PCA and hierarchical cluster analysis. The individual years differ considerably in the distinction among varieties. While in 2018 it was possible to distinguish five varieties well by both statistical approaches (Boomerang, Bor, Gaia, Kazbek, Vital), in 2017 and 2019 it was achieved only for three varieties. Vital varietiy don´t show good results in 2019, but a good discrimination was achieved in previous years. During the evaluated period varieties Agnus, Rubin, Premiant, Saaz, Sladek and Harmonie were classified at different levels of discrimination. Their identification on the basis of the sulfur composition is therefore not reliable. The remaining genetically related varieties Saaz Late and Saaz Special, whose samples in figures 4 form a dense cluster of experimental points, cannot be identified solely on the basis of the sulfur compounds fingerprint.

4 Conclusion

Pattern recognition approach based of sulfur compounds profile of hop oils was used for classification of 14 Czech hop cultivars. It was proved that the composition of sulfur substances is varietally

Table 1 Distribution of varieties according to the degree of differentiation based on the composition of sulfur substances in essential oils

Year	Good discrimination		Partial differentiation		Poor discrimination	
	PCA	HA	PCA	HA	PCA	HA
2017	Boomerang Harmonie **Vital**	Agnus Kazbek **Vital**	Agnus Bor Gaia Kazbek Rubín Saaz Late	Boomerang Bor Gaia Saaz Late Sladek	Bohemie Premiant Saaz Saaz Special Sladek	Gaia Premiant Rubín Saaz Saaz Late Saaz Special
2018	**Boomerang** **Bor** **Gaia** **Kazbek** **Vital**	**Boomerang** **Bor** **Gaia** **Kazbek** Premiant **Vital**	Saaz Saaz Late Sladek	Agnus Harmonie Rubín Saaz Sladek	Agnus Bohemie Harmonie Premiant Rubín Saaz Special	Bohemie Saaz Late Saaz Special
2019	Boomerang Gaia Kazbek	Bor Harmonie Saaz	Bor Rubín Saaz	Boomerang Gaia Kazbek Premiant Rubín Vital	Agnus Bohemie Harmonie Premiant Saaz Late Saaz Special Sladek Vital	Agnus Bohemie Saaz Late Saaz Special Sladek

PCA-principal component analysis HA - hierarchical cluster analysis characteristic. The spectrum of sulfurous substances of all varieties during 3 harvests and from various locations was consistent and didn´t show significant year-on-year differences. Statistical evaluation has shown that the spectrum of sulfur substances can be used to determine the variety's authenticity for only a few of them (Boomerang, Kazbek, Gaia, Vital). For other varieties it is not sufficient to distinguish them reliably. Other tools, the content and composition of hop resins, polyphenols and other (terpene and oxygen) components of hop essential oils, can be used for improvement of varietal identification. In addition, genetic methods can also be used. The analysis of sulfurous substances of hop oils must be considered as an auxiliary chemotaxonomic tool. The identification of so-far unknown sulfur compounds using high-resolution mass detectors as well as the changes during hops processing will be the subject of further investigation.

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