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Determination and verification of hop varieties by analysis of essential oils

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Abstract

The composition of essential oil in hops depends mainly on hop variety and less on growing, processing and storage conditions. Therefore the data on the composition can be used to distinguish between hop varieties. During the years 1998–2000, 78 samples of the commonly grown varieties in Slovenia were analyzed by gas chromatography. Research was focused on the selection of suitable components characteristic of the varieties in question. Since the changes in essential oil composition due to ageing are the most important interferences for determination of the varieties, these influences were successfully suppressed using the so-called indexes. Sixteen representative peaks were selected, indexes were calculated and processed by cluster analysis and principal component analysis. The developed method was shown to be effective on all hop varieties important in Slovenia and gave excellent results in daily laboratory practice.

Keywords: Hops; Essential oil; Cluster analysis; Principal component analysis

1. Introduction

Hops (*Humulus lupulus* L.) became a widely grown agricultural plant because of its use to give bitterness and aroma to beer, the beer quality depending partly on the hops used in the brewing process. Since quality is best maintained by defining raw materials, many, especially traditionally oriented, brewers tend to buy hops of known variety and origin. As each hop variety has a typical essential oil pattern, hop oil analyses can be used to identify hop varieties (Sharpe & Laws, 1981). Dry hops contain from 0.5 to 2% of essential oil, which consists mainly of terpene hydrocarbons and their oxidation products. The routine method for their analysis is based on isolation by steam distillation (Howard, 1970) followed by gas chromatographic separation. The relative areas of the chromatographic peaks for the components of the essential oil are used to give an approximate mass fraction of each component.

It is common to all the methods used for the identification of hop varieties that they compare the composition of the essential oil(s) of unknown sample(s) with that of reference samples. The most straightforward methods involve the simple comparison of a sample chromatogram with chromatograms of reference samples (Likens & Nickerson, 1967). Green (1997) used a chromatography data system for arithmetic manipulation of chromatograms, thus obtaining theoretical chromatograms for mixtures, using them for comparison with real samples of supposed mixtures.

Another way of processing chromatography data is to compare concentrations of characteristic components and/or their ratios. These can be compared successively or all in one step. In the first case, a flowchart is constructed from the reference database (Buttery & Ling, 1967; Kenny, 1990; Lermusieau & Collin, 2001; Perpete, Melotte, Dupire, & Collin, 1998), the most important part being determination of critical values for concentrations and/or ratios, which are used for decision making in the flowchart. As a result, the sample characterized by those critical values ends up in one of the groups, each representing a certain variety. In the case of parallel comparison of essential oil compositions, concentrations of some components and/or ratios between components are represented graphically, a typical representative of these being the so-called MIN-MAX

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model (Kač & Kralj, 1998; Kač & Kovačevič, 2000). Different essential oil components are listed on the abscissa and their relative contents on the ordinate. When several reference samples of the same variety are charted, a "corridor" for that variety is obtained. If graphical presentation of an unknown sample falls into that corridor, it belongs to that variety.

The problem of verifying and determining varieties can also be solved with statistical multivariate methods. They differ according to the type of transformation of the measuring space. The sample is grouped together with reference samples and its position among them gives information on sample identity. This type of method has been used to classify essential oils in several cases. Principal component analysis (PCA) has been used for chemometric processing of data obtained by solid-phase microextraction of hop volatiles (Kovačevič & Kač, 2001) and for distinguishing three different hop varieties on the basis of flavonoids, essential oil and hop acids (De Cooman, Everaert, & De Keukeleire, 1998). Freundorfer (1988) demonstrated the use of discriminant analysis to distinguish between 10, mostly German, hop varieties. The possibilities of these methods for hop taxonomy have been described in detail by Stenroos and Siebert (1984).

In this contribution, a new and reliable method for determination and verification of the most important hop varieties grown in Slovenia (Aurora, Bobek, Celeia, Magnum, and Savinjski golding) is presented. Since the changes in essential oil composition due to ageing are the most important interference for determination of varieties, these influences were successfully suppressed using the so-called indexes. In the final step data were chemometrically processed by cluster analysis and principal component analysis. The developed procedure was successfully tested on a group of Slovenian commercial hop samples. Additionally, the possibility of determining binary mixtures was also studied.

2. Experimental

2.1. Materials

The procedure described was developed by first establishing a reference database by analysing 78 hop samples belonging to the five most important hop varieties grown in Slovenia. The reference set included data on the following varieties: Aurora (23 samples), Bobek (13 samples), Celeia (15 samples), Magnum (nine samples) and Savinjski golding (18 samples). The procedure was tested on 62 samples of these varieties: Aurora (12 samples), Bobek (three samples), Celeia (nine samples), Magnum (six samples) and Savinjski golding (32 samples). They originated from different Slovenian hop growing areas, were representative of the 1998, 1999 and 2000 crops and were collected with the help of the Slovenian Agricultural Advisory Service (Žalec, Slovenia).

2.2. Sample preparation and analysis

Samples of hop essential oil were isolated by steam distillation using a distillation apparatus of Clavenger type (Howard, 1970): 50 g of pulverized, air dried hop cones (ground in a coffee mill) were placed into a 2000-ml distillation flask, 1000 ml of deionized water were added and the mixture was distilled for 4 h. Oil was collected from the condenser and 0.2 ml were diluted with 5 ml of *n*-hexane. The components were separated by GC analysis on a Hewlett Packard 5890 gas chromatograph (Palo Alto, CA, USA) equipped with a flame ionization detector: 1.4 μl of sample were injected using flow splitting of 1:50, at 0.5 ml/min carrier gas flow (N_2, 5.0) onto a HP-1 capillary column (25 m×0.2 mm, 0.11 μm; Hewlett Packard, Palo Alto, CA, USA). The temperatures of injector and detector were 180 °C and 280 °C, respectively. The temperature programme was 3 min at 60 °C, from 60 °C to 190 °C at a rate of 2.5 °C/min, 1 min at 190 ° from 190 °C to 240 °C at a rate of 70 °C/min and 11 min at 240 °C. The chromatograms were recorded and integrated using a Hewlett Packard 3396A integrator. The results of chromatography were recorded as relative areas of all essential oil components.

2.3. Chemometric processing

From all the chromatographic peaks, a minimal number was selected for chemometric processing. The following criteria were considered: the repeatability of each peak, its separation from other peaks and its changes due to ageing of the sample as well as correlation with other oil constituents (as determined by cluster analysis). The procedure included several successive steps. After 187 different hop essential oil constituents were determined in each chromatogram recorded under conditions specified in Section 2.2, every sample was consequently described by 187 parameters in the form of relative chromatographic peak areas. Since such a number of parameters was too high for statistical software and as not all of them were considered significant, a reduction in their number was performed. The criteria mentioned earlier were used in successive steps. Firstly, all peaks with poor reproducibility were omitted, since they contribute only "noise" to the collected data. Secondly, all only partially separated peaks were eliminated, since their relative areas are, at best, uncertain and they may even disappear because of coelution with larger peaks. The composition of essential oil depends also on ageing processes, which can significantly influence the ability of a chemometric system to differentiate between different

varieties. Therefore, all peaks sensitive to ageing were left out. Further on the presentation of chromatographic data with relative peak areas were abandoned and the so-called indexes were introduced. Each index was calculated as the quotient of the relative peak area of the component and the sum of the relative peak areas of all the chosen essential oil components. In this way, data on each sample depended on the contents of the chosen components. The last step for reducing the number of components was to determine correlations between the contents of different parameters/compounds arising from similar biosynthetic pathways by cluster analysis of the variables.

This procedure left us with each sample described by the minimal number of parameters (16) in the form of indexes, as described earlier. Samples described by such indexes were processed or grouped using two methods: cluster analysis (nearest neighbour method, squared euclidean) and principal component analysis. The statistical software used was Statgraphics Plus for Windows 4.0 (Manugistic, Rockville, USA).

2.4. Identification of the selected essential oil components

The compounds selected for chemometric procedures were identified by means of retention times, Kovats retention indexes, separation into essential oil fractions and MS spectra of the pure compounds (Table 1). The electron impact spectra and chemical ionisation spectra were recorded on a Varian GC/MS system (STAR 3400 CX gas chromatograph coupled with a Saturn 2000 ion trap mass spectrometer; Walnut Creek, CA, USA). A sample of essential oil was prepared and diluted in the same way as described in Section 2.2. GC/MS chromatograms were obtained by split injection (1:30); 1 μl of solution being injected at an injector temperature of 180 °C on a RTX-5MS capillary column (Restek, Bellefonte, PA, USA; 30 m×0.25 mm, 0.25 μm), the carrier gas being helium 5.0 at a flow rate of 1.5 ml/min and the temperature programme being 3 min at 60 °C and from 60 °C to 150 °C at a rate of 1 °C/min. Electron impact mass spectra were acquired under the following conditions: ion trap temperature: 180 °C, ionization energy: 70 eV and scan range: 50–400 m/z. Molecular masses were determined by chemical ionization using methanol as a reagent gas at an ion trap temperature of 180 °C and scan range of 60–249 m/z. More details on component identification are presented elsewhere (Kovačevič & Kač, 2001).

Table 1
Chromatographic peaks used in chemometric analysis

Peak no.	Component name	RI[a]	RI[b]
47	2-Nonanone	1070	1093
51	Linalool	1084	1100
56	Methyl octanoate	1109	1125
71	Methyl nonanoate	1209	1226
77	N.i.[c] oxygenated compound (170 amu)	1238	1258
80	N.i.[c] oxygenated compound (168 amu)	1254	1277
83	2-Undecanone	1274	1295
85	Methyl 4-decenoate (trans)	1291	1311
86	Methyl 4,8-decadienoate	1293	1316
87	Methyl geranate	1302	1324
98	α-Copaene	1363	1366
105	N.i.[c] sesquiterpene	1412	1418
107	α-Bergamotene	1428	1430
114	γ-Muurolene	1459	1468
118	α-Selinene	1476	1483
124	γ-Cadinene	1493	1503

[a] Retention Index on HP 1 column.
[b] Retention Index on RTX-5MS column.
[c] Not identified.

3. Results and discussion

3.1. Selection of the representative essential oil components

Fig. 1 gives a typical chromatogram. All recorded chromatograms of hop essential oils were checked for presence of the 187 constituents. Peaks with relative areas less than 0.1% were eliminated, as their relative standard deviations determined by repeatability studies were to high (between ±7 and ±100%). Additionally, all poorly separated peaks were eliminated and these two steps left us with 50 peaks describing each chromatogram/sample. In order to eliminate components sensitive to ageing, 24 already analyzed hop samples were stored for 9 months and then re-analyzed. Evaporation of essential oil took place and an enrichment of less volatile constituents as well as reduction of concentrations of more volatile components were observed. Oxidation was observed as a build up of low volatile oxides and epoxides with retention times more than 36 min. To avoid the influences of evaporation and oxidation, components having retention times less then 12 min and more than 36 min, were eliminated. Each sample was thus described by only 23 chromatographic peaks presented as relative areas.

The processing of data by cluster analysis, gave unsatisfactory results because significant differences between fresh and aged samples were observed. As a consequence an aged sample could be misidentified, which is a major drawback since typical laboratory samples are up to 1 year old. The role of ageing as a major factor preventing successful distinguishing between varieties, was explained in terms of the indirect influence of ageing on relative areas of 23 selected chromatographic peaks. It appears that the problem lies in presenting chromatography data as relative peak areas calculated as a quotient between the peak area in question and the

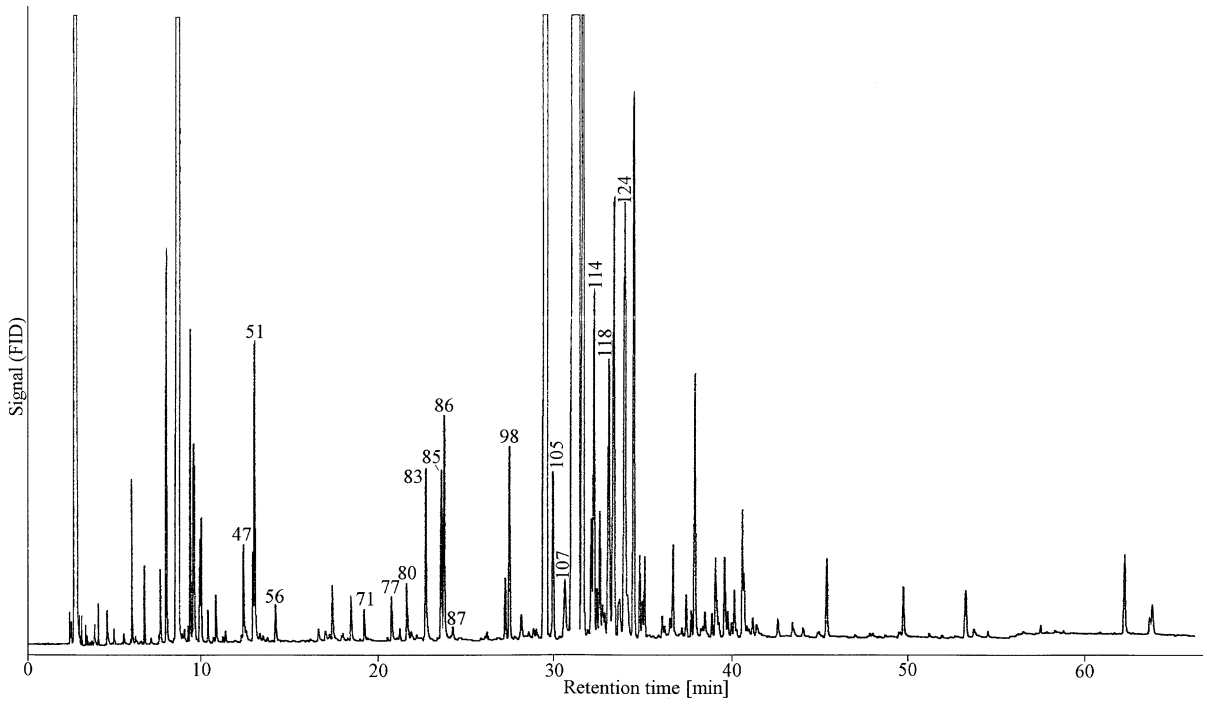

Fig. 1. A typical gas chromatogram of hop essential oil, variety Savinjski golding. For peak identities see Table 1, for experimental conditions see Section 2.2.

sum of peak areas of all essential oil components in the chromatogram. If, for example, a highly volatile major essential oil component evaporated due to long storage, the relative areas of other components increased because the sum of all peaks in the chromatogram decreased. This is in spite of the fact that the major compound in question may not be among the 23 selected components/peaks, which are supposedly insensitive to ageing. To avoid such indirect influence of ageing, the so-called indexes were introduced. In this way data on each sample depended only on those (23) components selected for chemometry (see Section 2.3). The samples were next processed by cluster analysis, giving results that clearly showed five groups, each representing samples of one variety. By determining the correlation between essential oil components arising from similar biosynthetic pathways, the number of parameters was additionally reduced, so that finally only 16 essential oil components remained for purposes of determination and verification of hop varieties. Using analogy with indirect influence of major components on relative peak areas, major essential oil components (more than 5%) were avoided if they have correlated with a non-major component. So, in practice all components that were used for chemometric processing, were in the concentration range between 0.1 and 5%, which is considered as a new approach, since all previous work on identification of hop varieties was done using data on major components. For identities of chosen components see Table 1.

3.2. Determination and verification of hop varieties

In order to determine and verify hop varieties, a reference database containing 78 samples was established. The data were processed by two methods. Cluster analysis gave the dendrogram presented in Fig. 2. It is branched on five well separated groups, each containing samples belonging to one variety. The separation of the groups belonging to varieties Aurora and Bobek is least obvious, which is not surprising due to their high genetic similarity. The three-dimensional graphical representation of principal component analysis is shown in Fig. 3 and contains 79% of the information of

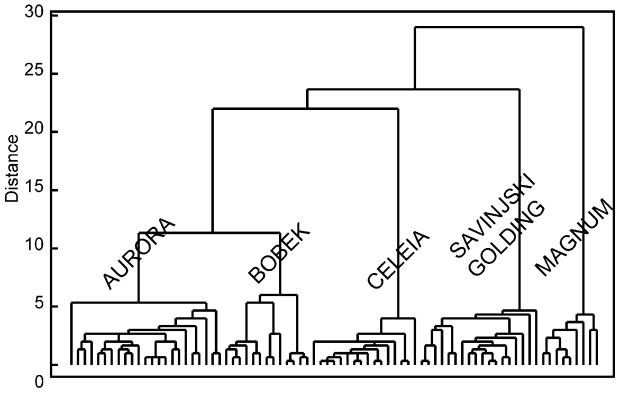

Fig. 2. Dendrogram obtained by cluster analysis (nearest neighbour, squared Euclidean) of 78 hop samples.

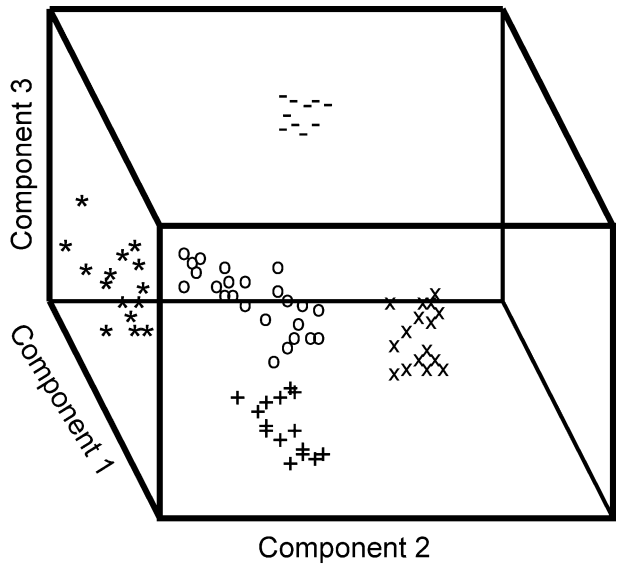

Fig. 3. Principal component analysis of 78 hop samples presented in three dimensional space: Aurora (o), Bobek (+), Celeia (x), Magnum (−) and Savinjski golding (*).

the original data. It is obvious that the 78 samples form five well separated groups defining the five varieties.

In practice, the hop sample was analyzed by gas chromatography, indexes for the 16 selected components were calculated and the results were added to the reference database. After cluster analysis and principal component analysis, the position of the sample among reference samples on graphical presentations was noted. If the sample fell in the group of a certain variety using both chemometric methods it was concluded that it belongs to that variety. The procedure was tested on 62 commercial (test) samples belonging to hop varieties used in the method development. In 59 cases correct answers were obtained and in three cases the answers were incorrect, giving a success rate of approximately 95%. In laboratory practice this can be considered sufficient for verifying and determining hop varieties. However, it would be desirable to analyse other hop constituents also, such as hop resins, in order to make the decision even more reliable. In the case of testing other hop varieties (not belonging to one of the five varieties included in the study), the only possible answer is that such a sample does not belong to any of the five varieties discussed here. To determine such samples, new reference samples need to be included. Despite usefulness of the described procedure, it has to be emphasized that on the scale of world hop production, where numerous varieties with minor differences are grown, such a system could lack the necessary resolution. But in the case of our laboratory, where only hops from Slovenia are analyzed (included varieties are all commercially important varieties in Slovenia) the system works very well. The same is true for most everyday problems dealing with practical identification and verification of hop varieties (only a limited number of varieties is involved).

3.3. Determination of mixtures

The same procedures were also used to perform some preliminary studies on mixtures of hops. Since a hop sample which is declared as a pure variety could possibly be a mixture of two varieties, the possibility of detecting mixtures was also studied. The composition of the essential oil of such a potential binary mixture was simulated, assuming that the composition of essential oil from a mixture is a linear combination of the compositions of both varieties in question. Indexes for the 16 selected components were calculated and the results grouped together with reference samples by cluster analysis and principal component analysis. The procedure was repeated using data on different hop varieties in different ratios and the following results were obtained:

If the mixture contains more than 20% of a second variety, identification of the bulk variety (80% of the sample) becomes impossible. "Judgement" is therefore negative and a sample of such a mixture does not pass the test as a pure variety. Addition of less than 20% has no decisive effect on the identification and the sample appears to be a pure variety. It is important to note however that the added variety, and obviously the bulk variety, were selected from the five varieties included in the study and have rather similar essential oils.

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