Comparison of Czech Hop Cultivars Based on their Contents of Secondary Metabolites

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Abstract

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Seven Czech hop varieties (dry hop cones) coming from the harvest of 2008 (Agnus, Bor, Harmonie, Premiant, Rubín, Sládek, and Saaz) were compared for their composition depending on their varietal differentiation. These cultivars were analysed for the contents of α- and β-bitter acids analogues, essential oils, and polyphenols. Hop essential oil constituents significantly contribute to the individual hop varieties. The dichotomous key for the authentication of Czech hop varieties was established based on some characteristic varietal markers.

Keywords: hop; Czech hop varieties; polyphenols; essential oils; α-bitter acids; β-bitter acids

Dry hop cones and hop products (pellets, extracts etc.) are the major raw materials employed in brewing which provide bitterness and characteristic aroma to beer. Due to this fact, the identification of the hop cultivar is a very actual question as well as the development of the methods for this purpose. Hop producers need methods for distinguishing the cultivars to claim their rights and expect high precision; on the other hand, the brewers need to keep standard quality of beer by using standard quality raw materials, and they expect the identification methods to be simple and well reproducible.

Nowadays, there are two major methods for the identification of the hop cultivars. The first one is based on using DNA markers (Araki *et al.* 1998; Čerenak *et al.* 2004). This method provides highly accurate results, but it is applicable for native hop samples (leaves, cones) only. In addition, specific equipment is required, thus it is clear that this method is not acceptable for a common brewing laboratory.

The second method is based on the unique compound composition of each hop cultivar. The contents of secondary hop metabolites (bitter acids, essential oils, and polyphenols) may provide information on the characteristic composition of each cultivar. For example, the Czech hop variety Saaz is unique by its very high content of β-farnesen (Perpéte *et al.* 1998), while Magnum variety contains a high amount of *p*-coumaric acid (Goiris *et al.* 2005). Kenny described a unique content of cohumulone for Nugget variety (Kenny 1990). Indeed, all of those factors are highly influenced by various effects, like the breeding area (Green 1997), climatic conditions (de Keukeleire *et al.* 2007),

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manufacturing technique (Srečec *et al.* 2009), or storage temperature (Canbas *et al.* 2001).

Some authors propose identification methods based only on the specific contents of the selected essential oils (Peacock & McCarty 1992; Eri *et al.* 2000), other authors suggest to use the contents of hop essential oils and bitter acids (Kenny 1990). De Cooman *et al. (*1998) described some characteristic markers (characteristic contents of bitter acids, oils, and flavonoids) in the Saaz, Wye Target, and Nugget cultivars.

The main purpose of this work was to determine the composition of bitter acids, essential oils, and polyphenols in seven Czech hop cultivars, to find some characteristic varietal markers and establish a dichotomous key for Czech hop varieties authentication.

MATERIALS AND METHODS

Samples. Seven samples of Czech hop cultivars (dry hop cones) from the harvest of 2008 (Agnus, Bor, Harmonie, Premiant, Rubín, Sládek, and Saaz) were obtained from V. F. Humulus, Ltd., Žatec, Czech Republic.

Chemicals. Acetonitrile (HPLC super gradient grade) and methanol (HPLC super gradient grade) were purchased from Lab-Scan (Dublin, Ireland). The international calibration extract of α- and β-bitter acids (cohumulone – 14.45% w/w, humulone + adhumulone – 34.94% w/w, colupulone – 12.92% w/w, lupulone + adlupulone – 12.02% w/w) ICE 2 was purchased from Labor Veritas AG (Zurich, Switzerland).

The standards of gallic acid (97% w/w), gentisic acid (98% w/w), vanilic acid (97% w/w), sinapic acid (98% w/w), ferulic acid (99% w/w), protocatechuic acid (97% w/w), caffeic acid (99% w/w), syringic acid (95% w/w), *o*-coumaric acid (98% w/w), ellagic acid (99% w/w), *trans*-cinnamic acid (99% w/w), (+)-catechin hydrate (96% w/w), (–)-epicatechin (90% w/w), rutin hydrate (95% w/w), naringenin (95% w/w), quercetin dihydrat (98% w/w), and morin (98% w/w) were purchased from Sigma-Aldrich (Steinheim, Germany).

Coumarin (97% w/w) and 4-hydroxybenzoic acid (99% w/w) were purchased from Fluka (Buchs, Switzerland) and borneol was purchased from Merck (Geneva, Switzerland).

Sample preparation. Hop bitter acids were extracted from crushed cones (10 g) with 120 ml of methanol-diethylether (1:5) solution for 30 min on a vortex mixer. Next, 40 ml of hydrochlorid acid (0.1M) was added into the mixture and the extraction ran for another 10 minutes. 5 ml of the supernatant was transferred into a volumetric flask (50 ml) which was filled up with methanol. 1 ml sample was passed through a 0.2 µm PTFE filter and subjected to HPLC analysis *(*Anonymus 2009*)*.

In the case of hop polyphenols, 5 g of crushed hop material was extracted with dichloromethan (2 × 100 ml for 1 h) for removing the hop bitter acids. The residual solid sample was extracted with 70% v/v acetone (3 × 50 ml, 30 min). The organic solvent (acetone) was evaporated and the residue was filled up to the volume of 50 ml with distilled water. 1 ml sample was passed through a 0.2 µm cellulose acetate filter and subjected to HPLC analysis.

Essential oils were steam-distilled using 100 g of crushed hop material (3 h). The raw distillate was purified in the separating funnel with diethylether (50 ml 2 min). The organic phase was dried out overnight with waterfree Na_2SO_4. The organic solvent was removed by evaporation under vacuum to the constant weight. The sample for GC analysis was prepared by mixing 0.25 ml of the distilled essential oils with 0.25 ml of borneol solution (7.2 g borneol/100 ml *n*-hexane).

HPLC. The separations of hop bitter acids and polyphenols were performed using the column Agilent Eclipse XDB-C18 (5 µm, 4.6 × 150 mm; Agilent Technologies, Santa Clara, USA) in the HPLC system Agilent 1100 equipped with a photodiode array detector working in the range of 190–810 nm, a quarternary pump, and an autosampler (Agilent Technologies, Santa Clara, USA).

The mobile phase consisted in the case of bitter acids of solvents A (acetonitrile – 0.05% w/w *o*-phosphoric acid) and B (water – 0.05% w/w *o*-phosphoric acid). The separation was performed using the gradient conditions: from 40% to 20% of solvent B in the first 40 min, from 20% to 0% in further 45 min, and from 0% to 40% in the next 50 minutes. The analysis duration was 55 min, the flow rate was 0.8 ml/minutes. The injected volume was 10 µl and column temperature was 30°C. The wavelength used for the detection of all samples was 314 nm.

In the case of polyphenols, the mobile phase consisted of solvent A (methanol – 0.1% v/v glacial acetic acid) and solvent B (water – 0.1% v/v glacial acetic acid). The separation was performed using the gradient conditions: from 90% to 50% of solvent B in the first 35 min, from 50% to 10% in

further 43 min, and from 10% to 90% in the next 54 minutes. Each run was followed by an equilibration period of 6 minutes. The analysis duration was 60 min, the flow rate was kept at 1 ml/minute. The injected volume was 20 µl. The wavelenghts used for the detection were 253 nm (protocatechic acid, 4-hydroxybenzoic acid, vanilic acid, rutin, ellagic acid, morin, and quercetin), 280 nm (gallic acid, (+)-catechin, syringic acid, (–)-epicatechin, cumarin, *o*-cumaric acid), *trans*-cinnamic acid and naringenin), and 320 nm (gentisic acid, coffeic acid, ferulic acid, and sinapic acid).

GC-MS. The separation of hop esential oils was performed on Agilent GC 6890 using the column HP-5MS (30 m × 0.25 mm, film layer – 0.25 µm). The detector was Agilent GC – MSD – 5975. The carrier gas was helium, thermal program 60°C (5 min), 150°C (2 min), 225°C (20 min), analysis duration 27 minutes. All essential oils were determined using borneol as the internal standard.

Statistical methods. Statistical evaluation of the results achieved was done employing the computer program Statistica 8.0 for Windows (StatSoft, Inc., Seattle, USA). The same software was used also for cluster analysis of the data.

RESULTS AND DISCUSSION

Compositions of Czech hop cultivars

The purpose of the method for varietal identification was to find out some markers for each hop cultivar. In this case, the term "marker" represents characteristic contents or the contents ratios of the selected secondary metabolites (bitter acids, essential oils or polyphenols) for a cultivar or a group of cultivars.

One of the most basic distinguishing method divides the hop cultivars according to the average content of alpha bitter acids (α-BA) into four groups: fine aroma group (α-BA 3.5 – 4% w/w), aroma group (α-BA 3.5 – 6.5% w/w), bitter group (α-BA < 8% w/w), and high alpha group (α-BA < 15% w/w). Due to a low accuracy of this subdivision (high variability of α-BA content), this system can be used only in the last step of identification for the verification of the results. Table 1 demonstrates bitter acid compositions and group types (Krofta 2002, 2003; Kroupa 2007) of seven Czech hop cultivars. The Saaz cultivar is the only single representative of the fine aroma group. This cultivar has very low contents of both α- and β-bitter acids (α-BA and β-BA) and almost the same relative abundance of lupulone and colupulon. The cvs Rubín and Agnus belong to the high alpha group, but only the cv. Rubín has the content of α-BA higher than 11% w/w. The cv. Harmonie (aroma) is the only cultivar with (β-BA) content higher than 7% w/w and its lupulone–colupulone ratio is very similar to that of the cv. Saaz.

α-BA/β-BA ratio (α/β) can also provide some information which can be useful for the identification purposes. As can be seen in Table 1, the (α/β) ratio is higher in high alpha and bitter cultivars. On the other hand, this ratio is lower in the fine aroma and aroma cultivars.

Table 2 shows the essential oils composition of Czech hop cultivars. β-Farnesen (Figure 1) is one of the most important key compound for the hop identification. β-Farnesen is a therpenic compound which occurs in the hop cones in a relatively high and stable concentration. Peacock and McCarty (1992) divided the hop varieties into two groups according to the relative abundance of β-farnesen. The first one has the characteristic content of β-farnesen above 5% rel., the second one lower than that.

Table 1. Bitter acid (BA) composition of Czech hop cultivars

Variety	Type	α-BA (w/w %)	Cohumulone (rel. %)	Humulone (rel. %)	β-BA (w/w %)	Colupulone (rel. %)	Lupulone (rel. %)	α/β
Agnus	high alpha	10.16	31.59	52.02	4.83	55.78	32.48	2.10
Bor	bitter	7.93	20.81	68.62	3.71	46.61	41.24	2.14
Harmonie	aroma	6.89	20.43	67.36	7.28	41.49	42.82	0.95
Premiant	bitter	8.89	18.42	71.28	4.61	41.96	46.00	1.93
Rubín	high alpha	11.33	26.25	60.23	3.57	49.08	40.40	3.17
Sládek	aroma	7.07	23.11	65.72	6.92	50.31	38.39	1.02
Saaz	fine aroma	2.32	23.38	63.24	3.40	41.11	45.53	0.68

Table 2. Essential oils composition of Czech hop cultivars (rel. %)

Essentials oils	Agnus	Bor	Harmonie	Premiant	Rubín	Sládek	Saaz
β-Pinene	0.69	0.29	0.36	0.25	0.16	0.12	0.31
β-Myrcene	44.31	18.73	21.42	18.87	11.45	10.97	21.71
2-MBI	0.70	0.53	0.20	0.67	0.57	0.21	N.D.
Limonene	0.60	0.46	0.41	0.36	0.31	0.25	0.38
Ocimene	0.14	0.74	0.11	0.40	0.19	0.25	0.04
2-Nonanone	0.05	0.12	0.22	0.24	0.09	0.20	0.21
Linalool	0.57	0.46	1.01	1.11	0.55	0.36	0.56
Nonanal	N.D.	0.16	0.12	0.13	N.D.	0.09	0.35
Methyl octanoate	0.17	0.75	0.27	0.36	0.21	0.71	0.17
2-Decanone	0.05	0.10	0.26	0.31	0.05	0.18	0.36
2-Decanol	0.01	N.D.	0.02	N.D.	N.D.	N.D.	0.07
Methyl nonenoate	0.13	0.29	0.24	0.36	0.16	0.42	0.31
Geraniol	1.17	0.31	0.18	0.35	0.83	0.43	0.30
2-Undecanone	0.43	0.97	1.12	0.99	0.53	1.59	0.98
2-Tridecanol	N.D.	N.D.	0.02	N.D.	N.D.	N.D.	0.03
Methyl decenoate	1.19	2.79	1.69	2.44	2.46	2.10	1.42
Methyl geranate	1.88	1.43	0.68	0.77	1.12	1.00	0.23
Methyl decanoate	0.12	0.23	0.24	0.32	0.11	0.47	0.09
α-Cubebene	0.08	0.08	0.05	0.06	0.06	0.10	0.04
α-Ylangene	0.10	0.18	0.10	0.15	0.16	0.19	0.07
Copaene	0.34	0.48	0.35	0.47	0.51	0.64	0.23
2-Dodecanone	0.06	0.14	0.18	0.18	0.07	0.22	0.23
Caryophyllene	14.23	13.64	9.62	13.37	12.29	17.88	8.00
β-Cubebene	0.44	0.74	0.50	0.67	0.50	0.85	0.33
α-Bergamotene	N.D.	N.D.	N.D.	0.09	N.D.	N.D.	1.34
Humulene	23.27	43.83	26.63	42.56	30.01	43.00	23.36
β-Farnesene	N.D.	0.16	0.05	2.26	0.11	0.03	24.87
ν-Selinene	N.D.	N.D.	1.98	N.D.	1.98	N.D.	N.D.
Muurolene	1.06	1.22	0.90	1.23	1.37	1.96	0.67
β-Selinene	0.77	0.57	9.48	0.49	11.61	0.48	0.51
α-Selinene	1.14	0.56	10.81	0.57	11.84	0.54	0.49
2-Tridecanone	0.60	0.60	1.48	N.D.	0.48	1.02	0.97
ν-Cadinene	1.18	1.41	1.28	1.49	1.76	2.06	1.15
Geranyl buthyrate	0.21	0.96	N.D.	0.59	0.33	0.62	N.D.
σ-Cadinene	1.95	2.62	2.02	2.61	2.65	3.68	1.46
α-Calacorene	0.06	0.09	0.09	0.10	0.11	0.17	0.07
Caryofylen epoxid	0.33	0.26	0.46	0.43	0.33	1.24	0.58
Humulen epoxid	0.57	0.89	1.28	1.52	0.83	2.71	2.02
Cubenol	0.11	0.20	0.20	0.24	0.23	0.46	0.18
σ-Ccadinol	0.26	0.44	0.34	0.42	0.40	0.53	0.24
Eudesmol	N.D.	N.D.	0.14	N.D.	0.13	N.D.	N.D.
11-Selinen-4-α-ol	0.04	0.08	1.47	0.07	1.18	0.08	0.01
α-Limonene epoxid	0.57	0.82	0.75	0.62	1.30	1.11	1.09
1,9-Dodecadiene	0.32	0.26	0.28	0.25	0.55	0.69	0.29
Pentadecanon	0.07	0.10	0.14	0.08	0.08	0.19	0.13
Farnesol	0.01	1.29	0.85	1.56	0.35	0.21	0.17
Total oils (w/w %)	2.11	0.75	1.19	1.16	1.15	1.10	0.38

N.D. – not detected

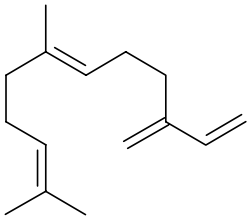

Figure 1. Structure of β-farnesene

The Saaz and Premiant are two cultivars which can be distinguished by the relative abundance of β-farnesen above 1% rel. The Saaz is also the only cultivar which does not contain 2-MBI (2-methyl butyl iso-butyrate). The Harmonie and Rubín are both cultivars with very high amounts of selinenes, and only these two cultivars contain measurable amounts of eudesmol. The cv. Agnus has nearly twice as high content of total oils than the other cultivars. β-Farnesene as well as α-bergamotene was not detected in the cv. Agnus.

Polyphenols are the most problematic compounds for the hop cultivar identification, mainly due to their ability for oxidation. Table 3 shows the contents of the selected polyphenolic compounds in Czech hop cultivars. The cv. Saaz hop has the highest content of total polyphenols. Quercetine was not detected in this cultivar and it also had the highest relative abundance of catechin. The cv. Agnus is rich in individual polyphenolic markers. It can be distinguished by the highest relative abundance of rutin, protocatechuic acid, epicatechin, and the lowest relative abundance of catechin. The cvs Sládek and Bor have very similar compositions of polyphenols and their distinguishing based on the characteristic composition of these compounds is complicated. The cv. Harmonie can be distinguished by the lowest amount of morin while the cv. Premiant had the highest amount of quercetin (1.2 % rel.). The Rubín is a cultivar with the lowest content of total polyphenols and a very high relative abundance of vanilic acid.

Table 3. Polyphenols composition of Czech hop cultivars (rel. %)

Polyphenols	Agnus	Bor	Harmonie	Premiant	Rubín	Sládek	Saaz
Protocatechuic acid	4.2	3.1	3.6	3.7	2.7	2.5	1.4
p-Hydroxybenzoic acid	1.6	1.4	1.7	1.6	0.6	0.9	1.4
Vanilic acid	0.9	0.8	1.2	1.2	2.4	0.5	0.6
Rutin	13.2	11.1	12.9	8.8	12.2	9.3	9.1
Ellagic acid	2.7	1.3	2.2	1.4	2.7	2.0	1.9
Morin	3.0	1.0	0.5	1.5	2.7	1.9	1.0
Quercetin	0.4	0.4	0.8	1.2	0.8	0.5	N.D.
Gallic acid	3.8	6.0	6.5	3.7	5.9	4.5	3.2
Catechin	15.6	26.9	26.6	38.1	25.0	28.9	42.3
Syringic acid	1.3	0.8	0.9	1.0	1.0	0.7	0.8
Epicatechin	9.9	4.0	5.3	6.2	4.7	4.3	6.4
Coumarin	13.0	11.1	11.3	7.9	9.8	12.1	9.4
o-Coumaric acid	0.2	0.2	0.4	0.2	0.1	0.2	0.2
trans-Cinnamic acid	0.2	0.3	0.1	0.3	0.2	0.2	0.3
Naringenin	0.4	0.3	0.4	0.4	0.5	0.2	0.2
Gentisic acid	22.7	23.5	18.7	17.2	22.2	23.6	17.4
Caffeic acid	2.6	3.5	3.1	2.3	3.1	3.5	1.7
Ferulic acid	1.7	1.4	1.7	1.3	1.5	1.4	1.1
Sinapic acid	2.7	2.7	2.1	1.8	2.1	2.5	1.6
Total polyphenols (mg/100 g)	247.1	330.3	263.5	260.1	220.6	374.2	504.8

N.D. – not detected

Chemical composition of hop cones is not only varietal dependent. There are many factors influencing the contents of secondary metabolites (growing season, growing area, climatic conditions, postharvest conditions, etc.), which must be taken into account.

Identification of Czech hop cultivars

The compositions of secondary metabolites of some hop cultivars may be very similar, thus their dividing is quite problematic. That is why all the results pass through the statistic analysis (cluster analysis), which affords three dendrogrames, one for each group of compounds. Figure 2 demonstrates similarities of the hop cultivars based on bitter acids, essential oils, and polyphenols composition. In all three cases, the cv. Agnus markedly differed from the others, which allowed an easy identification of this cultivar.

The dendrogram based on the bitter acids composition (Figure 2A) demonstrates a high similarity of the cvs Bor (bitter), Premiant (bitter), and Harmonie (aroma). All of these have very similar ratios of α-acids analogues, the cvs Premiant and Harmonie have also almost identical distribution of lupulone and colupulone. The cv. Agnus is unique, especially due to the highest relative abundance of cohumulone and colupulone.

The comparison of similarity of the hop varieties in view of the essential oils composition is demonstrated in Figure 2B. There are two cultivars markedly distinguished from others – Agnus and Saaz. The connection distances for most of the remaining cultivars are also relatively long. This fact confirms the correctness of using essential oils as the most useful markers for the identification purposes. The highest rate of similarity was noted for the cvs Premiant and Bor. The cultivars with a high relative abundance of selinenes (Harmonie and Rubín) also form a pair, but their connection distance is long enough for precise distinguishing.

The comparison of similarities in polyphenols composition is illustrated in Figure 2C. Also in this case the cv. Agnus is the most distinguishable cultivar, mainly due to the highest ratio of several polyphenols and the lowest amount of catechin. The cvs Saaz and Premiant can be also well separated from the others. These two cultivares have similar amounts of most polyphenols (the amounts of several polyphenols are very different), but there are many significant differences in comparison with

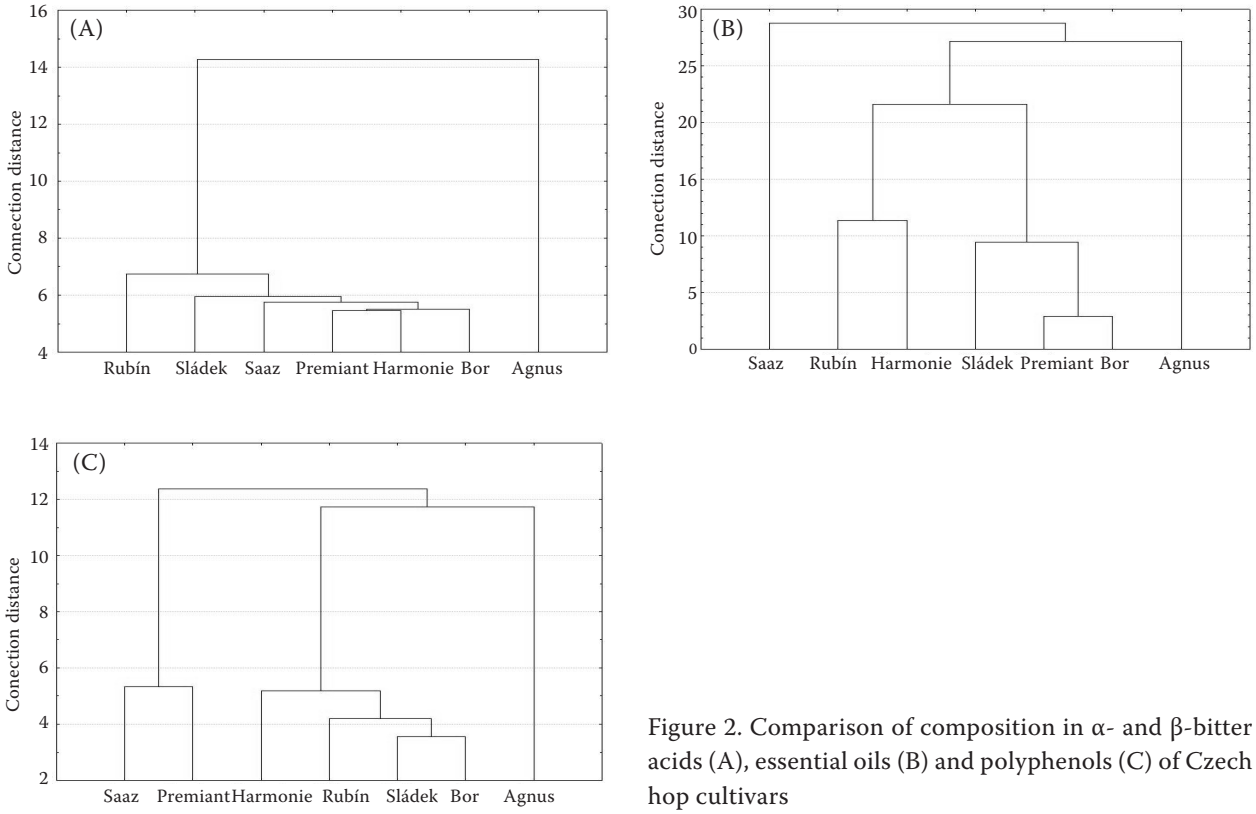

Figure 2. Comparison of composition in α- and β-bitter acids (A), essential oils (B) and polyphenols (C) of Czech hop cultivars

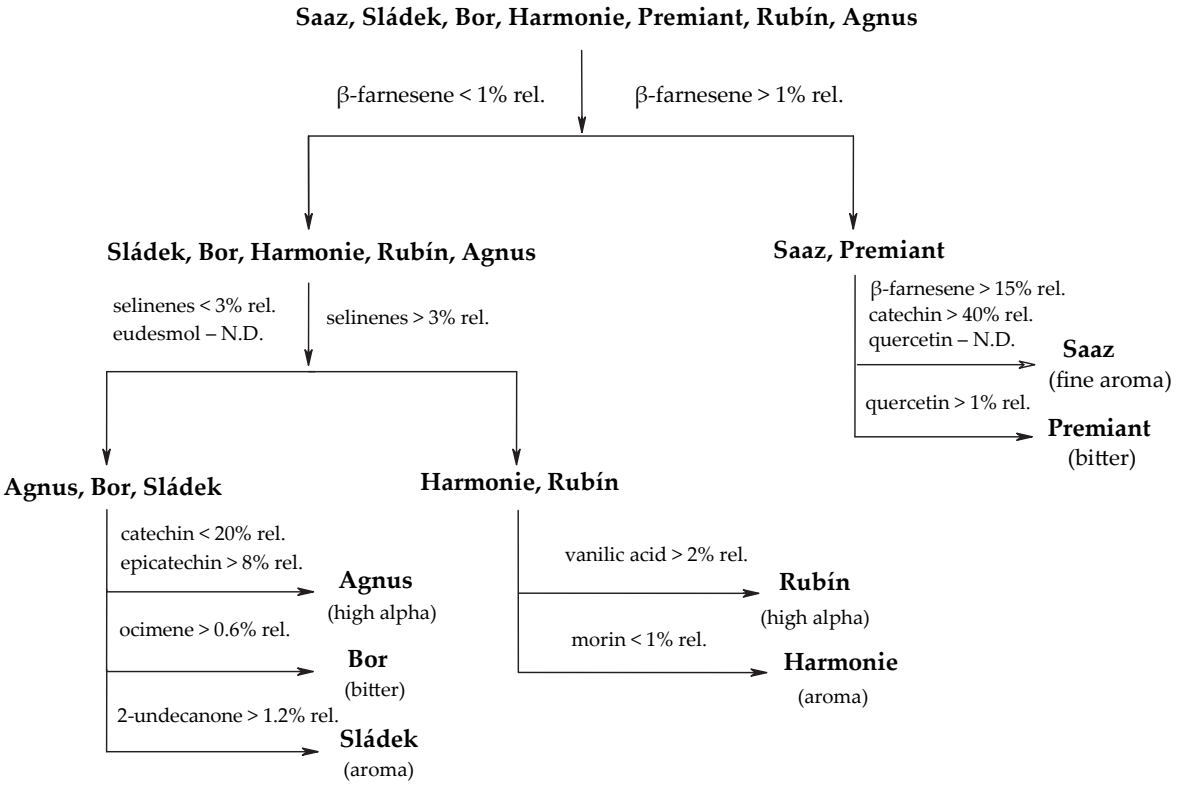

Figure 3. Dichotomous key diagram for identification of Czech hop cultivars

other Czech cultivars. That is the reason why these varieties are well distinguishable from the others and keep their high connection distance.

Figure 3 shows the dichotomous key diagram for the identification of Czech hop varieties. The cvs Saaz and Premiant are a pair of cultivars which can be separated from the others due to β-farnesene content (> 1% rel.). The Saaz is a cultivar with many others markers, thus it may be easily distinguished from the cv. Premiant, for example by β-farnesene (> 15% rel.), catechine (> 40% rel.) or quercetine (not detected) contents. The quercetine ratio is a marker also for the Premiant (> 1% rel.).

The Sládek, Bor, Harmonie, Rubín, and Agnus are the hop cultivars with the β-farnesene relative abundance below 1% rel. We can divide these cultivars into two groups by using the total content of selinenes (3% rel.). The cultivars in the first group (Agnus, Bor, Sládek) have the total selinenes amount below 3% rel. and, in addition, they do not contain eudesmol. The cv. Agnus can be distinguished thanks to catechin (< 20% rel.) and epikatechin contents (> 8% rel.). The cv. Agnus is also the only one variety in this group the belonging to the high alpha category. The other two cultivars are distinguished with the help of the characteristic relative abundance of one essential oil. The second group has the proportion of selinenes higher than 3% rel. It contains the cultivars Rubín and Harmonie. Both of these cultivars have a characteristic relative abundance of some polyphenols.

CONCLUSIONS

Seven Czech hop cultivars were analysed for the contents of α- and β-bitter acids analogues, essential oils, and single polyphenol. Out of these contents, some markers were selected characteristic for each cultivar. All the results of the hop analyses were statistically evaluated for the identification of varieties with similar compositions, which confirmed the uniqueness of the Agnus and Saaz cultivars. We made up the dichotomous key diagram dividing primarily the varieties according to their characteristic contents of β-farnesene. In this diagram, we used only the markers of essential oils or polyphenols. Bitter acids markers were not used for the identification purposes, mainly due to their annual changes. With the help of this diagram, we are able to identify each Czech hop cultivar.

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