

## Influence of Growing Area, Plant Age, and Virus Infection on the Contents of Hop Secondary Metabolites

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### Abstract

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Hops and hop products (pellets and extracts) belong to the major raw materials employed in brewing industry. Many effects such as the growing area, hop plant age, and virus infection influence the contents of the brewing-important hop secondary metabolites ( $\alpha$ - and  $\beta$ -bitter acids, essential oils, and polyphenols). The clones of the Czech cultivars Saaz and German cv. Taurus were used in this work and compared with the aim to investigate the influence of the effects mentioned on the contents of hop secondary metabolites.

**Keywords:** *Humulus lupulus*; hop plant age; polyphenols; essential oils;  $\alpha$ - bitter acids;  $\beta$ -bitter acids

The external conditions such as the growing area, virus infection, and cultivar age have very strong impacts on the yield of hop cone secondary metabolites commonly employed in brewing (bitter acids, essential oils, and polyphenols).

GREEN (1997) compared the amounts of essential oils, and bitter acids (BA) in the hop cultivar Tettmanger (crops 1990–1993) grown in North America, Australia and Europe. He pointed out the considerable differences between German and all other Tettmanger samples. KISHIMOTO *et al.* (2008) monitored the impact of the growing region on the amount of 4-mercapto-4-methylpentan-2-one (4MMP) in the hop cultivars Perle and Nugget (crops 2005 and 2006). According to their study, the hop samples from the plants grown in Germany contained no 4MMP in contrast to those grown in USA.

In recent years, the effort of many scientists has been to investigate the influence of the virus infection on the contents of hop secondary metabolites, mainly BA. They found out that many viruses may affect both the resin yield and composition. According to PATZAK *et al.* (2001), *Hop latent viroid* (HLVd) may significantly reduce the content of  $\alpha$ -BA, whereas  $\beta$ -BA content stays stable or slightly increases.

Pethybridge and colleagues investigated the effects of *Hop latent virus* (HpLV), *Hop mosaic virus* (HpMV), *Prunus necrotic ringspot virus* (PNRSV-A and PNRSV-I) and their combinations on the cultivars Nugget, Opal, Pride of Ringwood, and Victoria (PETHYBRIDGE *et al.* 2002). The result of this work was not only a detailed characterisation of the influence of the virus infection on the cone yield and  $\alpha$ - and  $\beta$ -BA levels, but also the finding that

some undesirable virus effects can be ameliorated by co-infection with another virus.

The main aim of this work was to find out the extent of the influence of the virus infection, growing area, and plant age on the contents of brewing-important hop secondary metabolites ( $\alpha$ - and  $\beta$ - BA, essential oils, and polyphenols).

## MATERIAL AND METHODS

**Samples.** Hop samples (dried cones) originating from the harvest of 2009 were grown on the locality Stekník and Velká Bystřice.

**Sample preparation.**  $\alpha$ - and  $\beta$ -BA were extracted from 10 g of crushed hop cones with 120 ml of the methanol-diethylether (1:5) mixture for 30 min on a vortex mixer. Next, 40 ml of 0.1M hydrochloric acid was added into the mixture and the extraction proceeded for another 10 minutes. 5 ml of the supernatant was transferred into a volumetric flask (50 ml) which was filled up to the mark with methanol. The sample was filtered by 0.2  $\mu$ m PTFE filter and subjected to HPLC analysis (ANONYMUS 2009).

In the case of hop polyphenols, 5 g of the crushed hop material was extracted with dichloromethan (2  $\times$  100 ml for 1 h) for removing the hop bitter acids. The residual solid sample was extracted with 70% v/v acetone (3  $\times$  50 ml, 30 min). The organic solvent (acetone) was evaporated and the residue was made up to the volume of 50 ml with distilled water. 1 ml sample was passed through a 0.2  $\mu$ m cellulose acetate filter and subjected to HPLC analysis. (JELÍNEK *et al.* 2010).

The essential oils were 3 h steam-distilled from 100 g of the crushed hop cones. The raw distillate was extracted with diethylether (50 ml 2 min) in a separating funnel. The extract was dried with anhydrous Na<sub>2</sub>SO<sub>4</sub> overnight. Diethylether was evaporated 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 containing 7.2 g borneol/100 ml *n*-hexane (JELÍNEK *et al.* 2010).

**HPLC analysis.** The separations of hop BA and polyphenols were performed using the column Agilent Eclipse XDB-C18 (5  $\mu$ m, 4.6  $\times$  150 mm) in the HPLC system Agilent 1100 equipped with a photodiode array detector (both Agilent Technologies, Santa Clara, USA). The mobile phase consisted in the case of BA 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 5 min, and from 0% to 40% in the next 10 minutes. The analysis duration was 55 min, the flow rate was 0.8 ml/minute. The injected volume was 10  $\mu$ l and the column temperature was 30°C. The wavelength used for the detection in all samples was 314 nm (JELÍNEK *et al.* 2010).

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 following gradient conditions: from 90% to 50% of solvent B in the first 35 min, from 50% to 10% in further 8 min, and from 10% to 90% in the next 12 minutes. Each run was followed by an equilibration period of 5 minutes. The analysis duration was 60 min, the flow rate was kept at 1 ml/minute. The injected volume was 20  $\mu$ l. The wavelengths used for the detection were 253 nm (rutin), 280 nm ((+)-catechin, (–)-epicatechin, and coumarin), and 330 nm (gentisic acid).

**GC-MS.** The separation of the hop essential oils was performed on Agilent GC 6890 using the column HP-5MS (30 m  $\times$  0.25 mm, film layer – 0.25  $\mu$ m) and detector 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 (JELÍNEK *et al.* 2010).

Table 1. Characteristic of hop fields in Stekník and Velká Bystřice

Growing area	GPS location	Altitude (m)	Average temperature (°C)	Average rainfall (mm)	Soil character
Stekník	50°19'19.449"N 13°37'23.674"E	201	9.7	450	muck soil, slightly acid or neutral reaction, humus content < 2%
Velká Bystřice	49°34'58.227"N 17°21'38.908"E	253	9.2	606	dry soil, neutral reaction, humus content – 2.4%

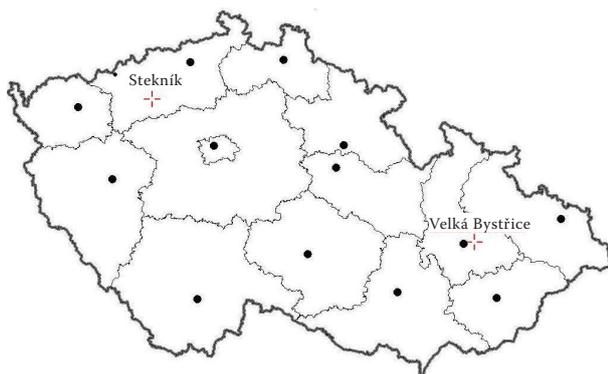


Figure 1. Location of Stekník and Velká Bystrice

## RESULTS AND DISCUSSION

### Effect of the growing area

The growing area is an important factor for the contents of secondary metabolites in the hop cones. This influence includes effects such as altitude, average temperature, rainfall, soil characteristics, and many others. The hop plant prefers primarily areas with average annual temperatures from 8°C to 10°C (warm summers and cold winters), deep

and fertile soil (slightly acid or neutral reaction), and adequate supplies of water (500 mm/year of rainfall) (PRIEST & STEWART 2006; BASAŘOVÁ *et al.* 2010; KROFTA *et al.* 2010).

The effect of the growing area was investigated for two cultivars Sládek and Saaz (Osvald clone 72) from the Žatec (Stekník) and Tršice (Velká Bystrice) regions. The distance between these two localities is about 280 km (Figure 1). The characteristics of these regions are shown in Table 1. These areas differ significantly only by the rainfall amount. It is obvious that Žatec area is rather arid, which can be the main cause of the changes in the hop secondary metabolites concentrations and compositions (assuming the same growing conditions and the occurrence of the same number of pests).

The composition of bitter acids and their four analogues is given in Table 2. A slight decrease was found in the case of  $\alpha$ -BA and a significant decrease in the case of  $\beta$ -BA contents in the hops grown in Velká Bystrice. A similar trend was observed with all analogues of  $\alpha$ - and  $\beta$ -BA. On the other hand, the reduction of total oils was found in the hops grown in Stekník. The composition of essential oils was relatively stable in both cultivars

Table 2. Secondary metabolites composition of hops originated from Stekník and Velká Bystrice

	Saaz		Sládek		
	Stekník	Velká Bystrice	Stekník	Velká Bystrice	
Bitter acids	$\alpha$ -BA (% w/w)	3.83	2.60	7.93	6.38
	cohumulone	0.87	0.58	1.84	1.60
	humulone	2.69	1.64	5.11	3.83
	$\beta$ -BA	5.12	3.79	7.47	2.47
	colupulone	2.04	1.58	3.52	1.21
	lupulone	2.46	1.72	3.07	0.95
Essential oils	total oil (% w/w)	0,37	0,46	0,96	1,16
	$\alpha$ -humulene (% rel.)	34.77	35.28	46.82	41.63
	humulene epoxid (% rel.)	2.83	5.11	1.69	4.02
	$\beta$ -caryophyllene (% rel.)	10.87	8.05	18.83	14.93
	caryophyllene epoxid (% rel.)	2.83	5.11	0.71	1.12
	$\beta$ -farnesene (% rel.)	20.64	19.01	N.D.	N.D.
	selinenes (% rel.)	0.80	0.71	1.10	0.76
$\beta$ -myrcene (% rel.)	12.10	11.60	14.73	13.83	
Polyphenols	$\Sigma$ polyphenols (% w/w)	0.73	0.63	0.35	0.30
	rutin (% rel.)	18.69	13.58	17.18	14.64
	catechin (% rel.)	44.80	43.19	29.89	27.97
	epicatechin (% rel.)	4.45	5.39	5.74	7.66
	coumarin (% rel.)	2.53	4.36	11.69	8.09
gentisic acid (% rel.)	16.56	22.66	13.50	25.63	

originated from the different growing areas. The only exception was observed in the case of epoxy-compounds which occurred in significantly higher concentrations in the samples from Velká Bystrice.

Higher amounts of polyphenols were found in the hop samples from Stekník. No direct effect was found of the place of growing on the composition of the individual phenolic compounds except for gentisic acid, which occurred in significantly higher amounts in the hops from Velká Bystrice.

Generally, the hops grown in areas with more rainfalls contain higher amounts of  $\alpha$ - and  $\beta$ -BA. On the other hand, the hops from arid areas contain a lower amount of essential oils.

### Effect of hop plant age

Different contents of some secondary metabolites can be observed during different phases of the hop plant life. The cvs Saaz – O. clone72 (age 8 and 13 years), Saaz – O. clone 114 (age 8 and 13 years), and Taurus (age 1 and 8 years) were used for the purposes of this work. These plants

originated from the same region and were grown under the same conditions.

The contents of the selected secondary metabolites are shown in Table 3. The samples of all older varieties showed slightly higher contents of both  $\alpha$ - and  $\beta$ -BA. The differences were most evident with the cv. Taurus, which probably did not yet reach full fertility in its first year of life. The same behaviour was observed with all analysed analogues of  $\alpha$ - and  $\beta$ -BA.

A similar trend was also observed in the contents of total essential oils. However, this finding does not coincide with the conclusions of PLUHÁČKOVÁ *et al.* (2011), who found a higher amount of total oils in younger plants. The compositions of most essential oils were very similar for each pair of samples. The only exception was the group of selenenes, which was found to be contained in greater amounts in the older hops.

A completely different behaviour was observed with the hop polyphenols. Their total amount was higher in younger cultivars, which may correspond to the assumption that these compounds were present in plants as a protection against

Table 3. Secondary metabolites composition of hops originated from fields with different age

	Saaz – O. C. 114		Saaz – O. C. 72		Taurus		
	8 years	13 years	8 years	13 years	1 year	8 years	
Bitter Acids	$\alpha$ -BA (% w/w)	3.36	3.74	3.08	3.62	13.69	16.24
	cohumulone (% w/w)	0.77	0.82	0.70	0.82	2.68	3.23
	humulone (% w/w)	2.15	2.34	1.97	2.34	9.18	10.88
	$\beta$ -BA (% w/w)	3.91	4.32	3.65	4.42	3.64	4.80
	colupulone (% w/w)	1.62	1.77	1.50	1.82	1.44	1.90
	lupulone (% w/w)	1.86	2.06	1.73	2.11	1.71	2.27
Essential oils	total oil (% w/w)	0.42	0.45	0.45	0.46	1.32	1.47
	$\alpha$ -humulene (% rel.)	26.88	26.72	26.45	26.86	29.63	25.75
	humulene epoxid (% rel.)	5.35	5.96	6.87	5.61	0.67	1.19
	$\beta$ -caryophyllene (% rel.)	7.44	8.00	7.71	7.71	9.81	8.54
	caryophyllene epoxid (% rel.)	2.07	1.46	1.57	1.39	0.18	0.32
	$\beta$ -farnesene (% rel.)	23.02	22.38	22.26	22.85	0.10	0.05
	selenenes (% rel.)	0.60	1.13	0.65	1.04	17.64	21.09
	$\beta$ -myrcene (% rel.)	10.97	11.54	11.09	12.60	26.92	23.51
Polyphenols	$\Sigma$ polyphenols (% w/w)	0.69	0.59	0.83	0.57	0.17	0.14
	rutin (% rel.)	12.51	12.97	12.94	13.58	24.99	34.20
	catechin (% rel.)	48.77	46.50	49.14	43.88	10.32	9.78
	epicatechin (% rel.)	6.63	5.97	7.11	6.51	8.46	7.82
	coumarin (% rel.)	3.09	3.44	2.95	4.63	8.31	7.54
	gentisic acid (% rel.)	19.63	21.42	19.74	21.27	16.36	14.09

external influences (BEART *et al.* 1985). This was also confirmed by a high percentage of flavan-3-ols (catechin and epicatechin) in younger hops, as these are substances with proven antioxidant effects (ARON & KENNEDY 2008).

### Effect of virus infection

The infections caused by *Hop latent viroid* (HLVd) and *Hop stunt viroid* (HSVd) are the main causes of the resins decrease in the Czech hops. For this reason, the cv. Saaz was subjected to the process of recovery, through which virus free cultivars were developed. It was proven, that the virus infection significantly reduces the content of  $\alpha$ -BA, but there is a small amount of information on the behaviour of essential oils and polyphenols contents.

The concentrations of the selected secondary metabolites in virus-infected cultivars and virus-free cultivars (Saaz – O. C. 72 and 114) are shown in Table 4. A significant decrease of  $\alpha$ - and  $\beta$ -BA (and their analogues) was detected in both infected hops, which is in contradiction with the work by PATZAK *et al.* (2001). The contents of total essential

oils in all samples did not significantly differ. The relative percentages of the individual compounds were also very similar. Slightly increased values of  $\beta$ -myrcene were found in the infected varieties. Virus-free Saaz O. C. 72 contained approximately half the amount of humulene epoxid than the infected cultivar. This trend was confirmed by many authors who consider that the essential oil content is sufficiently stable for the purposes of hop varieties identification (ERI *et al.* 2000; KOVAČEVIČ & KAČ 2002; JORGE & TRUGO 2003).

It is well known that plant phenolic compounds such as simple phenols, phenolic acid, flavanols, and dihydrochalcones evince allelopathic and anti-viral properties (DROEBNER *et al.* 2007; BEN SASSI *et al.* 2008). It has been also shown that higher plants use some polyphenols as protectants against some stress factors (IMPERATO *et al.* 2006). It can be assumed that the cultivars infected by the *Hop latent viroids* (HLVd) will contain increased amounts of polyphenols.

This assumption was confirmed in the case of our samples, where higher amounts of polyphenols were found in the infected cultivars (Table 4). In both cases were detected reduced concentrations

Table 4. Secondary metabolites composition of virus-infected and virus-free hops

	Saaz – O. C. 114		Saaz – O. C. 72		
	virus	virus free	virus	virus free	
Bitter acids	$\alpha$ -BA (% w/w)	3.36	5.76	3.08	4.56
	cohumulone (% w/w)	0.77	1.23	0.70	1.00
	humulone (% w/w)	2.15	3.86	1.97	2.98
	$\beta$ -BA (% w/w)	3.91	5.91	3.56	5.53
	colupulone (% w/w)	1.62	2.36	1.50	2.23
	lupulone (% w/w)	1.86	2.90	1.73	2.70
Essential oils	total oil (% w/w)	0.47	0.53	0.46	0.45
	$\alpha$ -humulene (% rel.)	26.88	24.09	26.45	26.71
	humulene epoxid (% rel.)	9.35	8.15	6.87	3.87
	$\beta$ -caryophyllene (% rel.)	7.44	7.17	7.71	8.09
	caryophyllene epoxid (% rel.)	2.07	1.93	1.57	0.93
	$\beta$ -farnesene (% rel.)	17.00	18.97	22.26	25.06
	selinenes (% rel.)	0.60	0.44	0.43	0.45
	$\beta$ -myrcene (% rel.)	10.97	13.69	11.09	13.60
Polyphenols	$\Sigma$ polyphenols (% w/w)	0.69	0.62	0.83	0.56
	rutin (% rel.)	13.51	13.10	12.94	12.14
	catechin (% rel.)	48.77	42.88	49.14	41.74
	epicatechin (% rel.)	7.37	6.63	7.11	6.29
	coumarin (% rel.)	3.09	4.08	2.95	6.13
	gentisic acid (% rel.)	19.63	20.83	19.74	17.73

of flavan-3-ol (catechin and epicatechin) in the virus-free hops. On the other hand, coumarin was detected in a higher concentrations in the infected varieties. It is well known that the group of coumarins exhibits a wide range of biological activities including the antioxidant (JUN *et al.* 2005) and antiviral activities (KOSTOVA *et al.* 2006), depending on the substituent on the benzopyrone structure (TRAYKOVA & KOSTOVA 2005). Coumarin itself shows a very low activity compared with substituted benzopyrones, and therefore its higher content in the virus-free hops is not surprising. The content of rutin was stable in both cultivars investigated. It is implied that the virus infection has no significant effect on the content of this polyphenol.

### CONCLUSION

The investigation of the impact of the growing area on the contents of secondary hop metabolites was carried out using hop cultivars Saaz and Sládek. It has been shown that the hops grown in the drier and warmer areas provide higher concentrations of bitter acids and polyphenols. On the other hand, the hops grown in colder areas with a higher amount of rainfall contain higher amounts of essential oils.

The hop cultivars Saaz (Osvald clones 72 and 114, 8 and 14 years old) and Taurus (1 and 8 years old) were used for studying the effect of the hop plant age on the contents of secondary metabolites. It was proven that the older plants contain higher contents of bitter acids and essential oils. A completely different behaviour was found out in the case of polyphenols, which were detected in higher amounts in younger plants.

The effect of the virus infection was carried out with the hop cv. Saaz (Osvald clones 72 and 114) either infected by HLVD or virus free. In the infected plants, a significant reduction of bitter acids and their analogues was detected while no differences were found in the concentrations of total essential oils in the infected and virus free hops. The bracts originated from infected plants had increased contents of phenolic compounds, especially flavan-3 ols.

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