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Characterisation of Hop Varieties Grown in Romania Based on their Contents of Bitter Acids by HPLC in Combination with Chemometrics Approach

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Abstract

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Reverse-phase high performance liquid chromatography technique was used for studying the evolution of bitter acids from three varieties of hop growing in Romania during the development of hop cones and the pelletisation process in order to provide the information on the bitter acids profile of each cultivar. Chemometrics methods were applied for highlighting the statistical correlations existing between the genotype (variety), chemotype (composition), and phenotype (phenophase of cone development) with respect to the classes of biologically active compounds investigated (bitter acids). The bitter acid content of each hop cultivar was not only significantly dependent on the phenophases of the cones, but was also influenced by the harvest year. The variations in the α/β ratio as well as cohumulone and colupulone contents were low in both experimental years and the cohumulone/∑α did fraction not exceed 30% in any of the three varieties.

Keywords: α-acids; β-acids; Romanian hop cultivars; *Humulus lupulus;* principal component analysis

Hop cones, the female inflorescence of *Humulus lupulus* L., are used in the beer production in various forms (whole cones, pellets, and extracts) to give the beer the hop flavour and bitterness (Jelínek *et al.* 2010). The lupulin inside the cones is in particular the substance which provides these characteristics (Lorenzana *et al*. 2010), containing hop bitter resins, essential oils, tannins, and polyphenols.

The hop components that contribute to the bitterness are known generically as α-acids and consist of a mixture of three compounds: cohumulone, humulone, and adhumulone (Khatib *et al*. 2007), and of β-acids: colupulone, lupulone, and adlupulone (Negri *et al*. 2010). Considering the significant impact of α- and β-acids on the beer flavour, it is important for brewers to be able to measure accurately their concentrations in order to maintain the standards for the known brands or to create a new brew with the desired characteristics. Bitter acids can be used as potential cancer chemopreventive agents (Gerhäuser 2005) and in recent years, hops have gained considerable interest due to the biological and potential cancer chemopreventive activities of some of their constituents (Negri *et al*. 2010). Humulone possesses antioxidative, anti-inflammatory, and other biological activities, such as antitumorpromoting effects on mouse skin carcinogenesis (Van Cleemput *et al*. 2009; Negri *et al*. 2010).

The average α- and β-acid weight percentage varies among the varieties of hops but is typically between 3 and 15% with β-acid concentration between 2 and 8% (Sahlstrom & Rostad 2011). One of the primary distinguishing method divides the hop cultivars according to the average content of alpha bitter acids: (*i*) high α-acid, (*ii*) intermediate α-acid, and (*iii*) aroma (noble) varieties (Shellie *et al*. 2009). In the

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brewing process it is very important to determine the bitterness profile, if it is fine or hard, in order to obtain beer of high quality. Several chromatographic techniques, such as high performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE), nuclear magnetic resonance spectroscopy (NMR), and thin layer chromatography (TLC), used for the determination of bitter acids and described in the literature claim advantages and disadvantages between the procedures including different extraction techniques. The concentrations of α- and β-acids can be determined by a HPLC separation followed by UV-Vis detection (De Cooman *et al*. 1998; Zhang *et al*. 2003; Jaskula *et al*. 2007; Ceslová *et al*. 2009; Sahlstrom & Rostad 2011).

The price of hops is given per kilogram of the dry product and depends on the content of α-acids (Hoek *et al*. 2001; Lorenzana *et al*. 2010). Year by year fluctuations in the α-acid content of hops is a common occurrence (Mitter *et al*. 2009). In the last two decades, brewers have become more specific in their requirements for the amounts and types of α- and β-acids, as well as for the qualitative and quantitative differences of essential oil profiles of hop cultivars (Beatson *et al*. 2003).

The objective of the present investigation was to characterise three varieties of hop growing in Romania by bitter acids content using liquid chromatography and statistical analysis. Furthermore, we investigated the bitter acids content during the main phenophases of the of hop cones vegetation, in order to provide information on the characteristic composition of each cultivar, and also to assess the losses in bitter acids during the pelleting process.

MATERIAL AND METHODS

The dry cones and pellets samples were used for chemical analysis of bitter substances over a period of two consecutive years.

Plant material. The samples from three different varieties of the *Humulus lupulus*, cultivars Magnum (MG), classified as high α-acid, Hüller Bitterer (HB), and Aroma (AR), classified as aroma (low α-acid), were collected in 2011 and 2012 year crops. The hop cultivars were cultivated in the climatic areas of Transylvania, in the Seleuş farms from Mureş County, an area with a tradition in hops culture. The female hop inflorescence (cones) were picked during three phenophases of development (end of August/beginning of September). In phenophase I (PHP I), the cones were of small-size, measuring between 2–2.5 cm. This phenophase coincides with the period of the cone and lupulin gland shaping. In the phenophase II (PHP II), the cones had a medium size measuring approximately 3 cm and the lupulin glands were fully formed being in full ripeness. In the last phenophase (PHP III), the cones were the technological maturity for the harvest, measuring 4 cm. The cones were dried for 48 h in a cool dark place for the conservation of the active principles. The pellets (P) samples were obtained from the pelletisation station. All hop samples were labelled and stored at –20°C until the analysis.

Chemicals. All chemicals used for the separation were of analytical grade: methanol (Chem-Lab NV, Zedelgem, Belgium), lead acetate solution (20 g/l), diethyl ether (peroxides free) (Lab-Scan Analytical Sciences, Gliwice, Poland), hydrochloric acid (Merck) (0.1 M). Methanol (HPLC super gradient grade) and *ortho*-phosphoric acid were purchased from Merck (Darmstadt, Germany). The international calibration extract, namely the standard ICE 2 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) was purchased from Labor Veritas AG (Zurich, Switzerland).

Extraction of the hop acids. The quantification of α-acids from hop cones and pellets was carried out according to the European Brewing Convention (Analytica-EBC).

The conductometric and HPLC methods used were: moisture content of hops and hop products (Analytica-EBC v. 7.2, 2006), Lead conductance method for hops, hop powder, and pellets (Analytica-EBC v. 7.4, 2006), and α- and β-acids in hops and hop products by HPLC (Analytica-EBC v. 7.7, 2006).

Hop bitter acids were extracted from ground cones (10 g) with 120 ml of methanol : diethyl ether (1 : 5) solution for 30 min under continuous agitation. Next, 40 ml of hydrochloric acid (0.1 M) were added into the mixture and the extraction ran for another 10 minutes. Five ml of the supernatant were transferred into a volumetric flask (50 ml) which was filled up with methanol. The content of the flask was filtered through a 0.45-μm PTFE Millipore filter, prior to HPLC analysis. The extraction of bitter acids resembled the above mentioned methods (HPLC and CV).

Conductometric analysis (CV). The conductometric analysis of α-bitter acids was performed using an automatic titrator and conductometer system (Schott, type TA 20 PLUS; Schott, Mainz, Germany).

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High performance liquid chromatography (HPLC). The separation of hop bitter acids was performed using a HPLC equipment from Shimadzu (Kyoto, Japan), with a UV detector. The column was a Grom Bier Bitter, 7 µm, 125 × 4 mm, from Alltech (Grace Davinson, USA). The mobile phase consisted of the mixture of solvents: A (methanol) and B (methanol: water: *ortho*-phosphoric acid 775 : 10 : 9, v/v/v). The time of analysis was 20 min, the flow rate was 1 ml/minute. The injected volume was 10 µl and the column temperature was set at 40°C. The wavelength used for the detection of all samples was 314 nm.

Statistical analysis. Matlab (v. 7.2.0232, 2006) was used to evaluate the data from the bitter acids analyses using the basic statistic functions, multi-factorial analysis of variance (ANOVA) and Spearman correlations. The same computer program was used for the cluster analysis (CA) with the Euclidean distances.

For the characterisation of the hop varieties studied, the obtained results were subjected to principal component analysis (PCA) with cross-validation (full model size and centre data). The statistical analyses were performed using Unscrambler X software v. 10.1 (CAMO Software AS, Oslo, Norway).

RESULTS AND DISCUSSION

Analysis of the hop acids. An example of the shape and size of the cones from the cv. Aroma during the phenophases is presented in Figure 1.

The dynamics of the bitter substances accumulation (Table 1) are a matter of interest, because they provide information on the bitter acids development during the phenophase, also including its consecutive steps. According to these data optimum cropping period can be estimated, as well as the beginning of the post maturation phenomen. The cv. Aroma presents a different accumulation of bitter acids in the years of the study. In the first year, it is really interesting to notice that the maximum content of α-acids is achieved in phenophase II of vegetation (9.6%), after which it decreases in phenophase III (9.36%) due to the processes of post-maturation, which lead to losses in bitter acids. In the second experimental year, a low debut of bitter acids is reported due to the climatic conditions such as inclement weather, hail damage (Dumbrăveni meteorological stations, TM 11, Synoptic code: 15 189 m, 46°13'40''N, 24°35'30''E, Alt: 318, Hb: 319). All these led to difficulties in managing the plantations of this variety which, according to the experimental results, was the most affected of all. However, the cv. Aroma showed a high ecomalleability and managed to recover in the course of the year 2012 and reach high values in PHP III (8.94%). For the Hüller Bitterer hop variety, in the experimental year 2011, it was noted that PHP III (10.63%) was richer than all the other phenophases from the point of view of α-acids accumulation. In the second experimental year, this variety showed in PHP II (10.71%) a higher build-up of α- and β-acids than in all other phenophases. The cv. Magnum presented ascending accumulation of bitter substances in each experimental year, maximum accumulation having been recorded in phenophase three of the development.

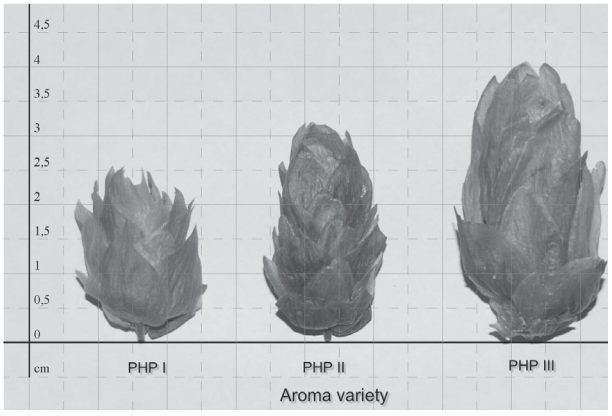

Figure 1. Shape and size of cones in the three phenophases of development for cv. Aroma

The total content of alpha bitter acids was established by conductometry, the method being useful especially for the evaluation of the hop chemical quality in view of commercialisation. Alpha acids content is expressed in terms of the conductometer value (EBC v. 7.5), as this describes more accurately the brewing value of hop pellets or whole hop and is generally used for everyday calculations of hop additions (Mitter *et al*. 2009).

Alpha-acids (cohumulone, humulone, adhumulone) and β-acids (colupulone, lupulone, and adlupulone) were identified in the hop samples and quantified using HPLC technique. The results of CV and HPLC analyses are listed in Table 1 (2011 and 2012) and are reported in percentages of dry matter. The internal validation of the methods was performed in order to confirm the quality results (Salanță *et al*. 2013).

Aroma variety. The aroma hop cultivar *Humulus lupulus* L. ssp. *europeus* Ryb. was created at UASVM, Cluj-Napoca (Romania) through clonal selection from the cv. Hüller Bitterer (Tofană 2006). The content of

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Table 1. The bitter acids content during development of hop cones varieties and in pellets analysed by CV and HPLC methods (2011 and 2012)

Hop cultivar	Phenophases	Harvest data	α-Acids conductometric (% ± SD)	Bitter acids (HPLC)						α/β Acid ratio
				α-acids			β-acids			
				(% ± SD)	% of total α-acids		(% ± SD)	% of total β-acids		
					CoH ± SD	AdH ± SD		CoL ± SD	AdL ± SD	
2011										
Aroma (AR)	I	12.08.	8.69 ± 0.00	6.55 ± 0.07	30.915 ± 0.01	69.09 ± 0.01	4.43 ± 0.08	56.70 ± 0.12	43.29 ± 0.12	1.5
	II	29.08.	13.58 ± 0.00	9.60 ± 0.00	21.945 ± 0.01	78.05 ± 0.01	5.10 ± 0.00	43.89 ± 0.08	56.11 ± 0.08	1.8
	III	09.09.	12.85 ± 0.48	9.36 ± 0.15	23.76 ± 0.00	76.24 ± 0.00	4.71 ± 0.11	46.07 ± 0.21	53.92 ± 0.21	1.9
Hűller Bitterer (HB)	I	12.08.	8.74 ± 0.21	6.26 ± 0.25	23.72 ± 0.06	76.28 ± 0.06	3.37 ± 0.13	44.51 ± 0.00	55.49 ± 0.00	1.9
	II	29.08.	11.68 ± 0.00	8.18 ± 0.00	21.76 ± 0.00	78.24 ± 0.00	5.43 ± 0.01	38.82 ± 0.05	61.17 ± 0.05	1.5
	III	09.09.	13.77 ± 0.20	10.63 ± 0.04	29.48 ± 0.03	70.52 ± 0.03	5.49 ± 0.04	60.29 ± 0.05	39.70 ± 0.05	1.9
	P	10.12.	11.05 ± 0.16	8.93 ± 0.06	34.325 ± 0.02	65.675 ± 0.02	5.42 ± 0.01	57.95 ± 0.00	42.03 ± 0.06	1.7
Magnum (MG)	I	12.08.	13.93 ± 0.00	11.52 ± 0.32	18.67 ± 0.31	81.13 ± 0.03	9.66 ± 0.22	35.44 ± 0.08	64.56 ± 0.00	1.2
	II	29.08.	15.11 ± 0.21	13.58 ± 0.08	20.175 ± 0.08	79.82 ± 0.08	11.00 ± 0.08	37.48 ± 0.06	62.52 ± 0.06	1.2
	III	09.09.	15.17 ± 0.00	17.16 ± 0.18	21.74 ± 0.01	78.26 ± 0.10	8.06 ± 0.13	40.30 ± 0.14	59.70 ± 0.14	2.1
	P	10.12.	13.54 ± 0.00	13.09 ± 0.67	21.00 ± 0.11	79.00 ± 0.11	7.26 ± 0.35	40.64 ± 0.62	59.35 ± 0.62	1.8
2012										
Aroma (AR)	I	12.08.	6.45 ± 0.00	4.47 ± 0.08	19.91 ± 0.06	79.76 ± 0.41	2.73 ± 0.04	41.02 ± 0.12	58.97 ± 0.12	1.6
	II	29.08.	7.82 ± 18.00	5.96 ± 0.06	23.30 ± 0.25	76.69 ± 0.25	3.70 ± 0.08	46.02 ± 0.01	53.98 ± 0.01	1.6
	III	09.09.	9.70 ± 0.16	8.94 ± 0.06	21.7 ± 0.14	78.30 ± 0.14	5.10 ± 0.04	45.00 ± 0.04	54.99 ± 0.04	1.8
Hűller Bitterer (HB)	I	12.08.	9.16 ± 0.20	8.37 ± 0.27	21.26 ± 0.01	78.48 ± 0.36	5.91 ± 0.23	42.98 ± 0.21	57.02 ± 0.21	
	II	29.08.	11.77 ± 0.00	10.71 ± 0.06	28.46 ± 0.09	71.58 ± 0.10	6.71 ± 0.04	51.45 ± 0.04	48.55 ± 0.04	
	III	09.09.	11.69 ± 0.40	10.15 ± 0.20	22.74 ± 0.05	77.25 ± 0.05	6.40 ± 0.04	47.74 ± 1.08	54.25 ± 0.25	
	P	10.12.	8.23 ± 0.00	7.40 ± 0.01	29.10 ± 0.13	77.25 ± 0.13	4.48 ± 0.01	52.73 ± 0.07	47.27 ± 0.07	
Magnum (MG)	I	12.08.	10.56 ± 0.13	9.12 ± 0.01	13.59 ± 0.02	70.90 ± 0.02	7.67 ± 0.04	29.70 ± 0.05	70.29 ± 0.05	
	II	29.08.	13.45 ± 0.35	10.54 ± 0.03	16.89 ± 0.08	86.40 ± 0.08	8.03 ± 0.01	34.24 ± 0.06	65.75 ± 0.06	
	III	09.09.	18.71 ± 0.37	16.27 ± 0.04	17.66 ± 0.01	83.11 ± 0.00	8.09 ± 0.03	38.19 ± 0.13	61.86 ± 0.05	
	P	10.12.	12.44 ± 0.07	2.20 ± 0.05	18.48 ± 0.10	82.34 ± 0.10	6.32 ± 0.03	37.81 ± 0.05	62.18 ± 0.05	

CoH – cohumulona; AdH – adhumulone; CoL – colupulone; AdL – adlupulone

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α-acids varied from 4.47% to 9.6% during the cones phenophases. Although it is considered an aromatic variety, high levels of α-acids in the two experimental years were determined, highlighting a dual character. On average, the α-acids content was lower than that in the cv. Hüller Bitterer. The situation was similar with the β-acids content, which varied from 2.73% to 5.1%. Alpha/beta acid ratio was lower than 2.0 and the cohumulone content did not exceed 30%, which is characteristic of aromatic varieties. Our data are in a good agreement with other ones reported, thus Krofta (2003) found the ratio of α/β acids too be between 0.67–2.19 and Jelínek *et al.* (2010) reported the ratio of α/β acids to be between 0.95–1.02 for the aroma varieties.

Hüller Bitterer cultivar. Considering an aroma variety with good quality bitterness, the content of α-acids during the stage of the cones development varied from 6.26% to 10.71%. The content of α-acids were higher than that reported by German Hop Growers Association (5–7%) for these cultivar. Tofană *et al.* (2012) reported the content of α-acids in cones being between 7.7–10.3% in 2008–2011 years crops. The cv. Hüller Bitterer had a good suitability, with no major differences between the registered maximum α-acids values. Regarding the content of β-acids, it varied from 3.37% to 6.71%. Cohumulone content did not exceed 30%, except for 2011 pellets (P) samples, when their proportion increased from 29.48% as recorded in PHP III to 34.32% in P samples, due to the decreased levels of humulone, more sensitive to oxidation. This variety presented the highest concentration of colupulone and a higher colupulone/β acids ratio than the other two varieties. The ratio of α/β bitter acids for the cones at technological maturity varied between 1.6 to 1.9, which is typical of aromatic varieties (Krofta 2003).

Magnum cultivar. The cv. Magnum presents elongated cone shape and had significantly higher α-acids content (9.12–17.16%) than those having oval or round cones (AR and HB). Our findings corroborate those of Srečec *et al.* (2010) for wild hops from the Croatia region. Cohumulone proportions were in relatively wide ranges of 17–21%, lower in comparison with the results of Krofta (2003) for these varieties (24.1–28.5%). The content of β-acids varied from 7.67% to 11% in the cones phenophases. The bitter hop cv. Magnum can be readily distinguished from the other two varieties by its relatively low cohumulone and colupulone proportions. The α/β acid proportion in the cones at technological maturity (2–2.1) resembles that presented by Krofta (2003) for this variety grown in the Czech Republic (1.69–2.34).

The loss of the brewing value of hop pellets was registered with both varieties HB and MG, due to the processing of hops and aggressive drying in specialised installations. As the composition of hop cones bitter acids is influenced by the environmental factors (harvest year) and stage development (phenophases) of cones, we used multi-factorial ANOVA to evaluate the interdependence. We found that the bitter acid contents were significantly dependent on the phenophases of cones and were also influenced by the harvest year. The harvest year is dependent on the environmental factors (weather conditions, disease infections, pest damage, and plant ontogenesis) (Patzak *et al.* 2010). We also found some significant differences in the contents between varieties.

As it can be observed in Table 1, the (α/β) ratio was higher in the high alpha and bitter cultivar (MG). On the other hand, this ratio was lower in the aroma cultivars (AR and HB), similar results having been reported by Jelínek *et al.* (2010) for the hop cultivars from the Czech Republic. In all three varieties

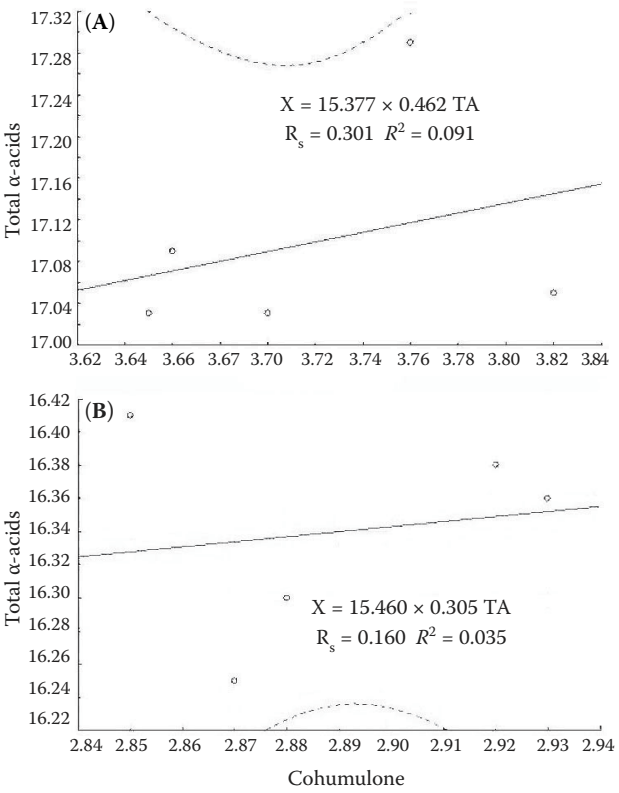

Figure 2. The correlation between the amount of total α-acids and cohumulone for hop cv. Magnum: (**A**) 2011 year and (**B**) 2012 year

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of hops, the cohumulone/∑α fraction was higher in the year 2011 than in 2012, but the values reported did not exceed 30%, thus recommending these varieties for the proportions of quality beers. In the same variety variations may appear in bitter acids content, due to factors such as: the weather conditions, type and pH of the soil, geographic region, postharvest conditions, time of harvesting, and genetic variations. Nevertheless, the variations in the α/β ratio and cohumulone and colupulone contents were low in both experimental years.

As a result of applying the Spearman correlations, it was found that the variation of cohumulone did not have a strong impact on the total α-acids in the cones, which led to the conclusion that cohumulone is accumulated from the first phenophase in a lower concentration than the other alpha compounds, and that it remains constant. It can be observed that for cv. Magnum (Figure 2) a poor influence exists (R = 0.301/2011 and R = 0.160/2012) of the content of cohumulone on the total quantity of alpha acids (the same goes for the other two varieties), suggesting that this variety has a good suitability.

Principal component analysis. Chemometric methods for the characterisation or classification of herbs according to the origin, quality, or variety are powerful tools and have been already widely used (Gad *et al*. 2013). In the present study, Principal Component Analysis (PCA) and Cluster Analysis (CA) were applied on the data obtained from the HPLC analyses in order to differentiate the three studied cultivars (Aroma, Hüller Bitterer, and Magnum) based on their bitter acids composition. The amounts of the individual and total α- and β-acids, ratios between total amounts of α- and β-acids, ratios of cohumulone and colupulone in α- and β-acids, and the Lead conductance value of the hop samples were used as the input parameters in the chemometric methods.

The cluster analysis affords a dendrogram which demonstrates similarities between the hop cultivars based on bitter acids content. Cultivar Magnum markedly differed from the others, which allowed an easy discrimination of this cultivar. The dendrogram highlights similarities between cvs Aroma and Hüller Bitterer, due to the fact that they are genetically related and have the same character (aroma), both having very similar proportions of α-acids analogues.

According to PCA, the first two principal components explained 96% (2011; Figure 3A) and 98% (2012; Figure 3B) of the variance of the data, showing a good discrimination between the samples phenophases and types (cones and pellets). In the case of the cv. Magum, a clear distinction can be noticed (Figure 3A) between the cone samples from phenophases I and the other two phenophases. Also, the cones sample from PHP II had a profile close to those from phenophase III, while the PHP II cones samples profile resembled those of the pellets samples (Figure 3B).

For the cv. Hüller Bitterer, a clear distinction between the three phenopases and the pellet samples can be observed. Although this may imply that the sample of cones in PHP III and the sample of pellets may have a closely similar patterns (due to the fact that the pellets are obtained from the cones harvested in PHP III), a difference still exists. This can be attributed to a decrease in the concentration of the compounds selected after processing the cones into pellets. PCA and CA both gave comparable results for the separation of the hop samples according to the variety.

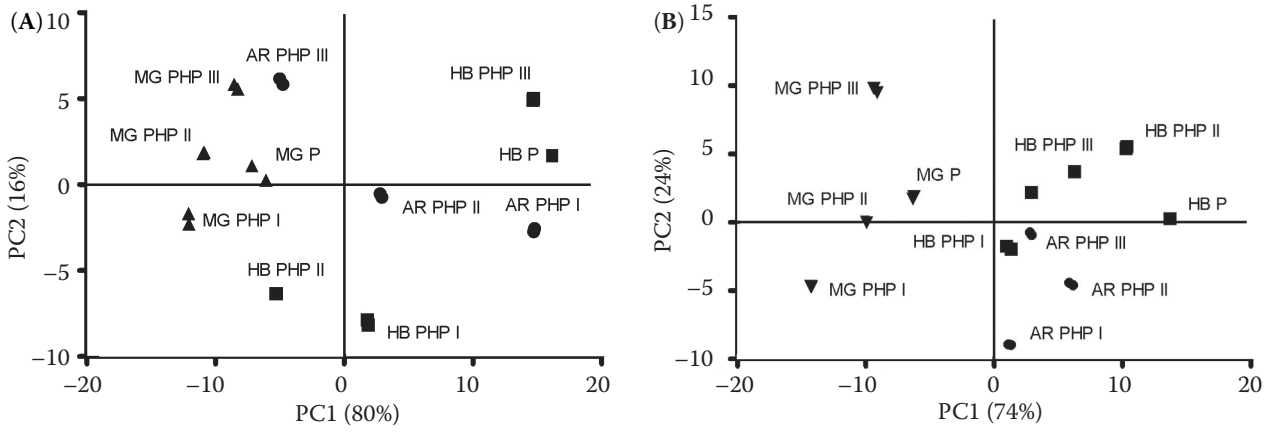

Figure 3. Principal components analysis bi-plots of bitter compounds from three hop cultivars; The first two components together explained 96% (**A**) and 98% (**B**) of the data variation

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CONCLUSIONS

Three Romanian hop cultivars were analysed for the contents of α- and β-bitter acids analogues during the development of cones. The content of α-bitter acids in the cv. Aroma ranging from 8.94% to 9.6% (PHP III). In the cv. Hüller Bitterer, the content of α-acids (PHP III) was in the range of 10–11%, and in the high-alpha hop (cv. Magnum) it was 17%. Cohumulone proportion was below 30%, which recommends these varieties for the production of quality beers.

Therefore, it can be concluded that HPLC combined with chemometric methods (CA and PCA) is a useful tool in the discrimination of the hop varieties based on their composition of bitter substances. Furthermore, statistical approach (PCA) provided the possibility of monitoring the accumulation of bitter substances during the agricultural year, and at the same time, the differentiation of the phenophases of certain varieties. Also, statistical processing of the data provided essential information for the hops growers on the influence of the pelletisation process on the content of bitter substances. The performed analyses represent a first step in the characterisation of Romanian hop varieties. Considering the annual changes of the bitter acids markers it is quite difficult to use only these compounds for the identification purposes. The next step in our future researches will be a comparative study on the composition of the volatile oils isolated from hop varieties enabling the identification of each Romanian hop cultivar.

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