BIOLOGIJA. 2005. Nr. 3. P. 81–84

Capillary electrophoresis method for determination of bitter (α- and β-) acids in *Humulus lupulus* L. Lithuanian varieties

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The research was performed in 2000–2005 at Vytautas Magnus University (VMU) Department of Chemistry and Kaunas Botanical Garden of Vytautas Magnus University (KBG). The object of the research was varieties of *Humulus Lupulus*: moderately early 'Kauno Graþieji', 'Kauno Ankstyvieji', moderately late 'Fredos Derlingieji', 'Fredos Taurieji' and 'Raudoniai' nurtured in 1952–1975 by hybridization. These hop varieties have inherited the characteristic properties from local wild hops acquired by pas the whole vegetation period, including the complete ripening of cones, in the climatic conditions of Lithuania.

The method of capillary zone electrophoresis (CZE) was optimized and validated for analysis of hop α- and β-acids. By the CZE method, cones of five hop Lithuanian varieties cultivated and preserved in the collection of KBG were analyzed.

Key words: *Humulus lupulus* L., Lithuanian varieties, bitter acids, capillary electrophoresis

INTRODUCTION

Hop (*Humulus lupulus* L.) is a perennial, climbing, gramineous, dioecious herb of the *Cannabaceae* family, *Dilleniidae* subclass, *Magnoliopsida* class, *Magnoliophyta* section with male and female flowers on separate plants, native in Europe (including Lithuania), Asia and North America [1]. According to the Global Strategy for Plant Biological Diversity Conservation [2] and National Programme of Plant Genetic Resources [3], *Humulus lupulus* is cultivated, preserved and studied in open air conditions in the *ex situ* collection of KBG. The hop collection belonging to the KBG is part of the National Genetic Resources. The collection has been created and is continuously enlarged since 1925. Presently the collection is consisted of 31 varieties, one hybrid and 25 wild forms [4, 5]. Selection of cultural hops was carried out since 1952 till 1975 by Dr. S. Gudanivièius by means of hybridization. He interbred varieties from West Europe with male individuals of hops growing in Lithuania, nurturing five novel varieties: the moderately early 'Kauno Graþieji' and 'Kauno Ankstyvieji', moderately late 'Fredos Derlingieji', 'Fredos Taurieji' and 'Raudoniai', which are exclusive due to good biological properties, favorable agroeconomical indicators and a short vegetation period, which is consistent with local climatic conditions [6, 7]. The chemical investigation of hops was started only in the 19th century. The research was particularly focused on antibacterial properties of hop compounds and the bitter substances derived from hop [8]. We focused our studies on the hop bitter acids. There are two kind hop bitter acids, humulone and lupulone, and they have several isomers. **The aim** of this study was to develop, optimize and validate the CZE method for bitter acids analysis in hop cone extracts and evaluate the composition of bitter acids in Lithuanian hop varieties.

MATERIALS AND METHODS

Materials. Research object: five varieties of *Humulus lupulus* L.: moderately early 'Kauno Graþieji' and 'Kauno Ankstyvieji', moderately late 'Fredos Derlingieji', 'Fredos Taurieji' and 'Raudoniai', cultivated in the hop collection *ex situ*, and cones as a medicinal raw material. The research was performed in 2000–2005 in Central Lithuania (Kaunas), in a hop collection *ex situ* (800 m^2) at KBG of VMU. Five hop varieties from the collection were studied by the methods modified for collections and outdoor tests [9, 10]. Mass weight for each individual (g) was determined and evaluated from three replicates investigating dry hop cones from different varieties and recalculating into kg/ha [11]. Investigation results were

statistically evaluated by the correlation and regression statistical analysis methods [9, 12]. Statistical analysis of the results was performed using the STATENG and ANOVA programmes from the Selection and IRRISTAT software packages.

Extraction of hop acids. Air-dried (humidity 8%) and chopped up 0.3 g of hop cones were exposed to 20 ml of methanol under continuous shaking for 3 hours. Prior to analysis the extract was filtered using paper filter and 0.2 μm pore size disposable membrane filter.

Conductometric titration. For determination of α-acids in hop, a standard conductometric titration method was used [13]. This method is based on the titration of methanolic hop extract with lead acetate, registering changes in conductivity. α-acids form lead salts, which are not soluble in methanol and therefore suppress the conductivity. The equivalent point was determined by extrapolation of the titration curve, and the molar amount of α-acids was calculated from the molar amount of lead acetate used [13, 14]. The method's suitability was evaluated.

Capillary electrophoresis. Capillary electrophoresis was performed using an HP^{3D} CE capillary electrophoresis system (AgilentTechnologies, Waldbronn, Germany). The effective length of the capillary column was 60 cm, total length 68.5 cm, internal diameter 50 μm. Initial conditions for bitter acids separation by CZE were as follows: working buffer 25 mM NaH_2PO_4, 30 mM $Na_2B_4O_7$, pH 8.2; voltage 20 kV; temperature 25 °C; injection 150 mbar·s. Optimizing the separation process, the influence of the composition and the pH value of running buffer, the amount of organic solvent in the running buffer, sample dilution and analysis temperature on the separation parameters were analyzed. Detection of α- and β-acids was performed at the 230 nm and 345 nm wavelengths simultaneously, since these wavelengths correspond to the absorption maxima of α- and β-acids. The peaks in electropherogrammes were identified according to their characteristic spectra acquired with the aid of a diode array detector. Quantitative determination of α-acids was performed using certified (α-acids 51.9%) hop extract from Joh. Barth & Sohn GmbH & Co. KG company (Nuernberg, Germany) as the standard material.

RESULTS AND DISCUSSION

According to vegetation duration, the hop varieties studied are classified to early, moderately early and late [6, 7]. The optimal varieties for cultivation in Lithuania are moderately early and moderately late [16]. In 2000–2005, studying Lithuanian hop varieties, namely the moderately early 'Kauno Graþieji' and 'Kauno Ankstyvieji' and moderately late 'Fredos Derlingieji', 'Fredos Taurieji' and 'Raudoniai', it was determined that the total vegetation period for two early hop varieties is *ca*. 144 days. The period since the vegetation beginning till blooming for these varieties is 1.2 times shorter (101 days) and since blooming till complete ripening of cones 2 times shorter (43 days) than that for the moderately late hop varieties. Evaluation of the productivity of five hop varieties showed that the amount of cones for two moderately early hop varieties was 1.7 times lower than that for three moderately late hop varieties. During the investigation period, the maximum amount of cones for the moderately early varieties was 964 kg/ha in 2000 and the minimum amount 356.9 kg/ha in 2002. The maximum amount of cones for moderately late hop varieties was 1534.5 and 1589.3 in 2000 and 2001, respectively, and the minimum amount being 601.1 kg/ha in 2002. The moderately early hop varieties 'Kauno Graþieji' and 'Kauno Ankstyvieji' may be classified as hops of fluctuating productivity and irregular yield. Their productivity and yield are highly dependent on meteorological conditions. A regular amount of raw material is characteristic of the moderately late hop variety 'Raudoniai', and the best productivity is a characteristic feature of the moderately late varieties 'Fredos Derlingieji' and 'Fredos Taurieji'.

The accuracy of the conductometric titration method was checked by analyzing a certified supercritical CO_2 extract of hop. The obtained results were 50.9 ± 0.5% consistent with the certified amount of α-acids 51.9% (producer's data). The same conductometric method was used for α-acids determination in different hop varieties. As is shown in Fig. 1, in Lithuanian hop varieties the highest amount of α-acids (2–2.5% in dried cones) was determined in 'Kauno Ankstyvieji' and 'Raudoniai'. It should be noted that the amount of α-acids determined in all varieties was fairly low. Analysis of bitter acids in five hop varieties was also performed using the CZE method. UV absorption spectra almost identical characteristic of β-acids were registered for the first two peaks. For the next two peaks, UV spectra typical of α-acids were registered. They were almost identical, however, differed substantially from the spectra of the first two migrating compounds. Therefore, the first two migrating peaks were attributed to the different forms of bitter β-acids and the next two to the forms of α-acids. It has been concluded that this method can separate two of the existing α- and β-acid forms (n-, ad- and co-humulones/lupulones). This means that not all forms are separated. In the capillary zone, electrophoresis separation is governed by the charge density of the analytes. The molecular weights of ad- and n- forms of α-acids (ad-humulone and n-humulone) are the same (M_r = 362.47) and are higher than M_r of co-humulone (M_r = 348.44). According to the CZE mechanism, the peak α1 belongs to the n- and ad-humulones and the peak α2 belongs to co-humulone.

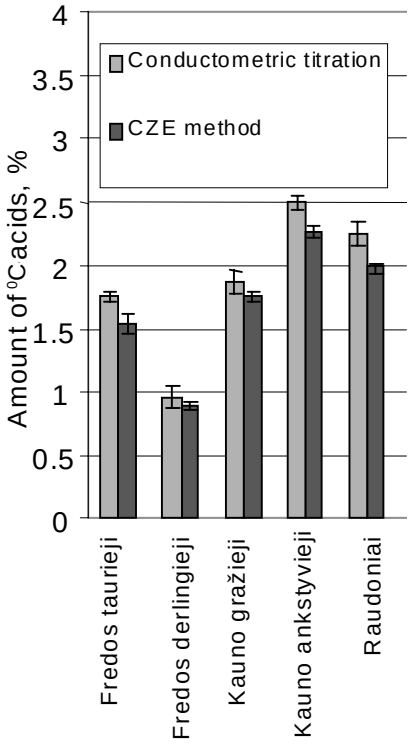

Fig. 1. Content of α-acids (%) determined in dried hop cones by conductometric titration and CZE method in different hop varieties

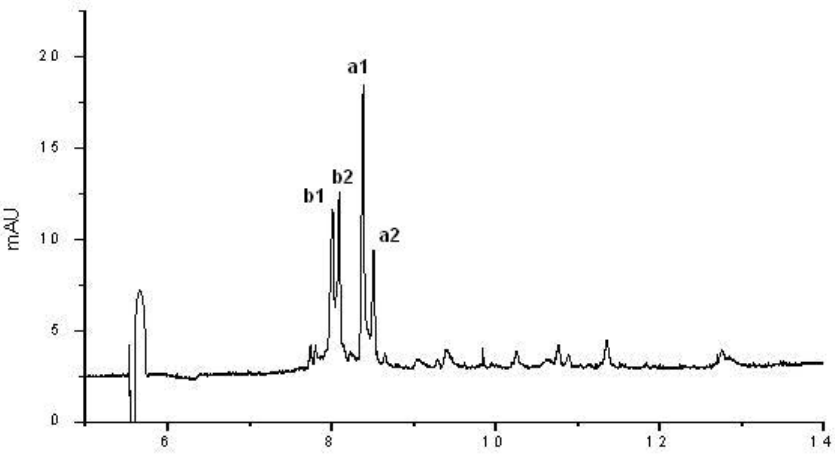

Fig. 2. Electropherogramme of hop methanolic extract. Analysis conditions: 25 mM NaH_2PO_4, 30 mM $Na_2B_4O_7$, pH 8.2; voltage 20 kV; temperature 25 °C; injection 150 mbar·s. The first two peaks were identified as β-acids, and the next two peaks identified as α-acids

As is obvious from Fig. 2, the resolution is not sufficient, as the separation of peaks of different forms of humulones and lupulones is incomplete. Therefore, the CZE method was further optimized. After the separation process optimization, the following analysis conditions were selected: working buffer 25 mM NaH_2PO_4, 60 mM $Na_2B_4O_7$, pH 8.2; voltage 30 kV; temperature 20 °C; injection 150 mbar*s. Baseline separation between humulones and lupulones was obtained using the optimized conditions.

Using the certified hop extract (51.9 % α-acids), a calibration curve was obtained in the range from 100 μg/ml to 1 mg/ml (R^2 = 0.9975). The detection limit calculated for α-acids was 100 μg/ml and the limit of determination 200 μg/ml. The amount (%) of α-acids determined by CZE in different hop cone extracts is shown in Fig. 1. Comparing the conductometric titration and CZE methods, we can state that the results for α-acids obtained by the CZE and conductometry as a reference method are consistent.

Differently from conductometric titration, CZE method allows to evaluate the percentage of co-humulone in the total amount of α-acids. The higher amount of co-humulone is related to the lower quality of hops in beer production, whereas its provided bitterness is more harsh. In Table, the percentage of co-humulone in the total amount of α-acids is presented.

Because of the lack of β-acids reference material we could not make a quantitative analysis of β-acids in hop cone extracts. Nevertheless, the CZE method allows a relative comparison of β-acid amounts in different hop varieties (Fig. 3). Relative standard deviation (RSD%) for β-acid amounts did not exceed 4.8%. Our results show that the content of β-acids is highest in the varieties 'Kauno Ankstyvieji', 'Kauno Grąbieji', and 'Raudoniai'. It is in 'Fredos Derlingieji', whereas in other Lithuanian varieties the content of these acids is quite high.

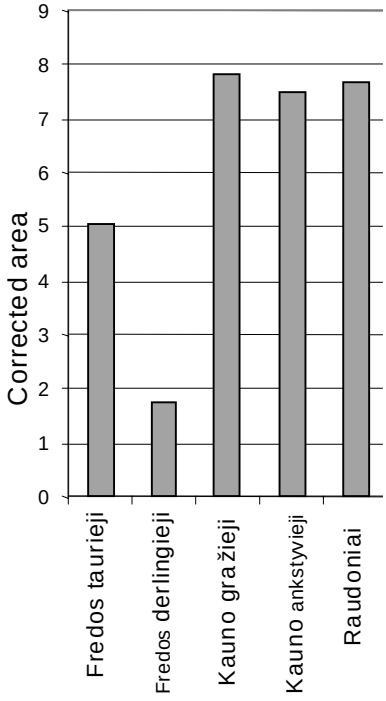

Fig. 3. Relative comparison of β-acids content in different Lithuanian hop varieties (expressed by corrected areas of the peaks)

Table. **Percentage of co-humulone in total content of α-acids**

Hop variety	Co-humulone, %
'Fredos Taurieji'	35.87
'Fredos Derlingieji'	35.64
'Kauno Graþieji'	24.98
'Kauno Ankstyvieji'	26.36
'Raudoniai'	27.95

CONCLUSIONS

Lithuanian hop varieties cultivated in the collection in the collection of the Kaunas Botanical Garden pass the whole vegetation period till complete ripening of seeds (cones) in the climatic conditions of Lithuania. The amount of the hop cones was the highest in the two moderately late hop varieties 'Fredos Derlingieji' (1327.1 kg/ha) and 'Fredos Taurieji' (1529.7 kg/ha) and the lowest in the moderately early variety 'Kauno Ankstyvieji' (653.7 kg/ha), regular yield is characteristic of the moderately late hop variety 'Raudoniai' (868.2 kg/ha). The CZE method was optimized and validated for analysis of hop α-acids. A comparative study using the standard conductometric titration method for determination of α-acids has shown a good consistency with the results obtained by the established CZE method. The highest content of α-acids (2–2.5%) was determined in 'Kauno Ankstyvieji' and 'Raudoniai'. A low content of β-acids was found in 'Fredos Derlingieji', whereas in other Lithuanian varieties the content of these acids was quite high.

ACKNOWLEDGEMENTS

The work was carried out in the frame of the National Programme on Plant Genetic Resources, 2004–2008, Research project No. 7, Assesement of genetic stability and variability of genetic resources in medical and aromatic plants and development of their preservation strategies, Contract No. 12. Instrumental support from Agilent Technologies is highly acknowledged. Certified supercritical CO_2 hop extract was kindly provided by AB 'Kauno alus'.

Received 10 May 2005
Accepted 9 August 2005

References

1. Jankevièienë R (red.). Botanikos vardø þodynas. Vilnius, Botanikos institutas, 1998.
2. Global Strategy For Plant Conservation. Decision VI (9), 2002. Adopted by the Convention on Biological Diversity (CBD).
3. Augalø nacionaliniø genetiniø iðtekliø ástatymas ir poástatyminiai aktai. Vilnius, 2004.
4. Grybauskas K. Kultûriniai apyniai ir jø auginimas. Kaunas, 1936.
5. Ragaþinskienë O, Rimkienë S, Ðeinauskienë E, Obelevièius K. Biologija 2002; 4 (priedas): 62–5.
6. Gudanavièius S. Apyniø auginimas. Vilnius, 1979.
7. Gudanavièius S. Biologijos instituto darbai 1958; 3 t.: 325–30.
8. Sakamoto K, Konings WN. Int. J Food Microbiol 2003; 89: 105–24.
9. Arvasas J, Stancevièius A. Lauko bandymø duomenø ávertinimo metodika. Kaunas, Akademija, 1981.
10. Ì åòîäèêà èññëåäîâàíèé ïðè èíòðîäóêöèè ëåêàðſòâåííûõ ðàſòåíèé (îáçîðíàÿ èíôîðìàöèÿ). Ñåð. Ëåêàðſòâåííûå ðàſòåíèåâîſſòâî. 3. Ì îſêâà, 1984.
11. Êîëîſſ ÃÅ. Õìåëåâîſſòâî 1988; 8: 27–33.
12. Tarakanovas P, Raudonius S. Agronominiø tyrimø duomenø statistinë analizë taikant kompiuterines programas ANOVA, STAT, SPLIT-PLOT iš paketo SELEKCIJA ir IRRISTAT. Kaunas, Akademija, 2003.
13. Õìåëü-ñûðöè è õìåëü ïðåſſîâàííûé. Ì åòîäû èſſëäîâàíèé, ÃÎÑÒ 21948–76.
14. Conductometric methods for hops and hop products. http://www.johbarth.com/scientific/27.html.
15. Cortacero-Ramirez S, Hernainz-Bermudez deCastro M, Segura-Carretero A, Cruces-Blanco C, Fernandez-Gutierrez A. Trends in Analytical Chemistry 2003; 22: 440–55.
16. Obelevièius K. Botanica Lithuanica 2003; Suppl. 5: 87–95.

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KAPILIARINËS ELEKTROFOREZËS METODAS KARÈIOSIOMS RÛGÐTIMS (α- IR β-) NUSTATYTI *Humulus lupulus* L. LIETUVOS VEISLËSE

Santrauka

Tyrimai atlikti 2000–2005 m. Vytauto Didþiojo universiteto Chemijos katedroje ir Vytauto Didþiojo universiteto Kauno botanikos sode. Tyrimo objektas – paprastojo apynio veislës – vidutinio ankstyvumo 'Kauno Graþieji', 'Kauno Ankstyvieji', vidutinio vëlyvumo 'Fredos Derlingieji', 'Fredos Taurieji' ir 'Raudoniai', sukurtos 1952–1975 m. hibridizacijos metodu Kaune, Vidurio Lietuvoje. Introdukuojamos ir tiriamos apynio veislës paveldëjo vietinio laukinio apynio adaptacines savybes, praëjo visà vegetacijà iki spurgø brandos respublikos klimatinëmis sàlygomis. Optimizuotas kapiliarinës elektroforezës metodas α- ir β-rûgðtims nustatyti ir atlikti jo áteisinimo þingsniai. Apskaièiuotos α-rûgðèiø aptikimo (100 mg/ml) ir nustatymo (200 mg/ml) ribos. Naudojant optimizuotà kapiliarinës elektroforezës metodà analizuotos penkios Lietuvoje sukurtos paprastojo apynio veislës.