· Advances in Medical Sciences · Vol. 54(1) · 2009 · pp 41-46 · DOI: 10.2478/v10039-009-0020-9

Capillary zone electrophoresis method for determination of bitter (α- and β-) acids in hop (*Humulus lupulus* L.) cone extracts

Kornyšova O[1], Stanius Ž[1], Obelevičius K[2], Ragažinskienė O[2], Skrzydlewska E[3], Maruška A[1*]

1 Department of Biochemistry and Biotechnologies, Vytautas Magnus University, Kaunas, Lithuania
2 Kaunas Botanical Garden of Vytautas Magnus University, Kaunas, Lithuania
3 Deptartment of Analytical Chemistry, Medical University of Bialystok, Bialystok, Poland

* CORRESPONDING AUTHOR:
Department of Biochemistry and Biotechnologies,
Vytautas Magnus University
Vileikos st. 8,
LT-44404 Kaunas, Lithuania
Tel: +370 37 3279 07; Fax: +370 37 3279 08
e-mail: a.maruska@gmf.vdu.lt (Audrius Maruška)

Received 08.04.2009
Accepted 13.05.2009
Advances in Medical Sciences
Vol. 54(1) · 2009 · pp 41-46
DOI: 10.2478/v10039-009-0020-9

ABSTRACT

Purpose: *Humulus lupulus (H. lupulus)*, more commonly known as hop, is a member of the Cannabaceae family with male and female flowers on separate plants. It is native in Europe including Lithuania, Asia and North America. Hop has been recognized as a medicinal plant for centuries, nevertheless different medicinal activities of hop are currently investigated and discovered. An important class of hop compounds is the hop acids, which are classified as α-acids and β-acids. Different varieties of hops vary in amount and composition of hop acids.

Methods: Simple capillary zone electrophoresis method has been optimized and applied for the analysis of hop acids in hop cone extracts.

Results: With this method the analysis takes ca. 10 min. Repeatability for migration times and peak areas expressed as relative standard deviation were up to 0.21% and 5.96%, respectively.

Conclusions: Comparative results of capillary zone electrophoretic analysis of extracts of different hop varieties and conductometric titration, as a standard method for determination of α-acids, are presented. Both methods provide consistent results, however capillary zone electrophoresis is capable of separating co- form of humulones from other forms.

Key words: Capillary zone electrophoresis, conductometric titration method, hop bitter acids, humulones

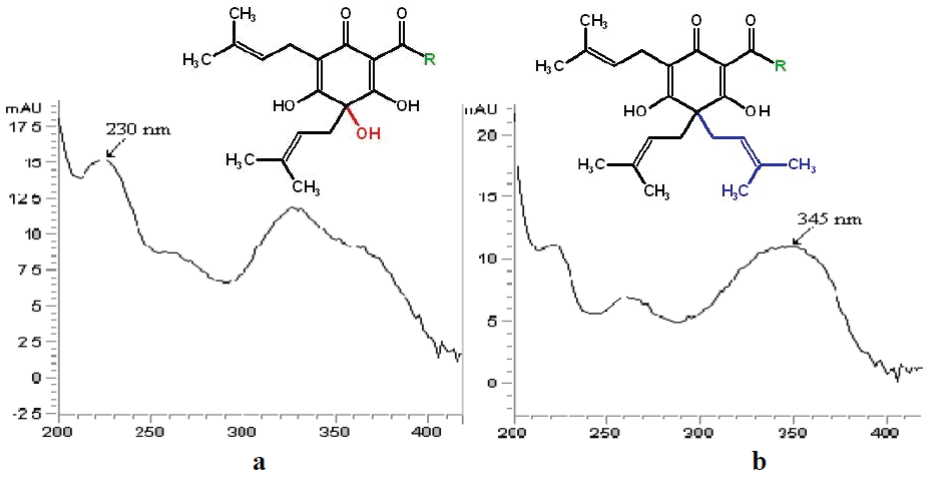
INTRODUCTION

Phytochemical research on hop plant (*Humulus lupulus* L.) was started only in 19th century. Research has been especially focused on the antibacterial properties of hop compounds and the bitter substances derived from hop [1]. Hop consists mainly of cellulose and lignin (up to 50%). Other materials of particular interest are essential oils and hop bitter acids.

Modern herbal medicine practitioners use hop as a sedative and mild hypnotic medicinal plant, as well as for its endocrine, free radical scavenging and antitumoral activities [2]. The bitter substances of hops are the so called α-acids (humulones), β-acids (lupulones) and their oxidation products. These substances consist of a mixture of homologues [3]. Hop bitter acids are shown to have different biological activities, including inhibition of angiogenesis [4], inhibition of tumor promotion by phorbol ester [5], induction of apoptosis [6], suppression of cyclooxygenase-2 gene transcription [7], antioxidant [8], antibacterial [9] and antifungal [10] actions. These substances are now recognized as potential chemotherapeutic or chemopreventive agents having antioxidative and antitumoral properties.

We focused our study on the hop bitter acids. There are two kinds of hop bitter acids humulone and lupulone, and they have several isomers as depicted in *Fig. 1*. The α-acids (humulones) are tasteless, however upon boiling they transform into the bitter tasting iso-α-acids (isohumulones). Thus they are an essential substance for beer bitterness in the beer industry. Iso-α-acids are intensely bitter, almost as bitter as quinine, which is used as a reference material for determining bitterness. β-acids (lupulones) are more interesting from the medicinal point of view.

Figure 1. **Structure of hop acids and characteristic UV absorption spectra for α-acids (humulones) (a) and β-acids (lupulones) (b). Variety of hop acids forms: N derivatives if R = $CH_2CH(CH_3)_2$; Co derivatives if R = $CH(CH_3)_2$; Ad derivatives if R = $CH(CH_3)$ CH_2CH_3.**



Several methods are employed for determination of hop bitter acids. Traditional methods of conductometric titration or spectrophotometric determination [11] are capable of estimating the sum of all bitter compound forms, such as the total amount of humulones. A more suitable method for a mixture material is the high performance liquid chromatography (HPLC), which, under isocratic conditions, can successfully separate iso-α-acids in the presence of the β-acids [12]. However, HPLC method has some drawbacks when analyzing hop acids, since the hop acids are metal chelating compounds [13] and thus require inert HPLC equipment (without mobile phase wetted metal parts) and even metal traces free stationary phase. Along with HPLC, the capillary electrophoresis emerged as a promising separation technique for the analysis of hop acids. Capillary zone electrophoresis (CZE) as the most common capillary electrophoresis mode was employed for separation of hop acids, however, it was not capable of separating different forms of humulones or lupulones [13]. Other capillary format separation techniques, such as micellar electrokinetic chromatography (MEKC) [13, 14], microemulsion electrokinetic chromatography (MEEKC) [15-17] and capillary electrochromatography (CEC) [12] were successfully used for separation of humulones and lupulones including their forms, however, these techniques are sometimes more cumbersome, particularly CEC. Therefore, to separate hop acids we optimized the most straight forward and cost effective method in capillary electrophoresis – capillary zone electrophoresis, which is capable of separating the most potent humulone - cohumulone from the two other forms. Conductometric titration with lead acetate was used as a conventional method for evaluation of developed electrophoretic method regarding its accuracy and precision.

The aim of this study was to develop and optimize the capillary electrophoresis method for bitter acids analysis in hop cone extracts, employing conductometric titration with lead acetate as a standard method for determination of α-acids, in order to evaluate precision of developed capillary electrophoresis method. Using the developed CZE method and standard conductometric titration method the composition of bitter acids in different hop varieties and wild forms were measured and compared.

MATERIALS AND METHODS

Plant material. The hop acids were studied in the 15 hop (*Humulus lupulus* L.) varieties and wild forms cultivated in Kaunas Botanical Garden of Vytautas Magnus University (Table 1).

Extraction of the hop acids. Air-dried (humidity 8%) hop cones were grinded to 0.5 – 1.0 mm particles using coffee grinder. 0.3 g of prepared material was extracted with 20 ml of methanol (p.a. grade, Merck, Germany) under continuous orbital shaking for 3 hours (Titertek shaker, Flow Laboratories, Germany). Prior to analysis extracts were filtered using paper filter and disposable membrane filter (0.2 μm).

Conductometric titration. For determination of α-acids in hop a standard conductometric titration method was used [18]. Lead acetate forms precipitating complexes particularly with α-acids, this method is specific to humulones. Therefore the method is not capable of determining β-acids. For conductometric titration a Model LF537 conductometer (WTW, Germany) was used. Lead acetate was pure for analysis grade (Lachema, Czech Republic). This method is based on the titration of methanolic hop extract with lead acetate 4 %, registering changes in conductivity. Lead salts of α-acids precipitate in methanol and therefore suppress the conductivity rise. Equivalent point was determined by extrapolation of the titration curve. The molar amount of α-acids was calculated from the used molar amount of lead acetate [18, 19].

Capillary electrophoresis. Capillary electrophoresis was performed using HP^{3D} CE capillary electrophoresis system

Table 1. **Hop varieties analyzed.**

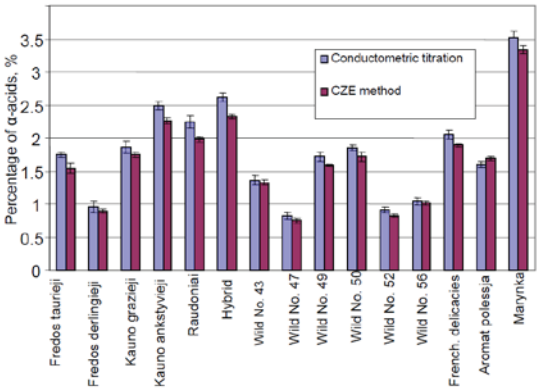
	Hop variety	Harvest year
Lithuanian varieties	Raudoniai	2005
	Kauno ankstyvieji	2005
	Kauno gražieji	2004
	Fredos derlingieji	2005
	Fredos taurieji	2004
	Hybrid A-9 (16)	2005
Wild hops from different growing regions in Lithuania	Wild No. 43 (Kaukinės v., Kaišiadorys district)	2004
	Wild No. 47 (Panemunio v., Rokiškis district)	2004
	Wild No. 49 (Gervelių v., Biržai district)	2004
	Wild No. 50 (Gervelių v., Biržai district)	2004
	Wild No. 52 (Prūdelių v., Biržai district)	2004
	Wild No. 56 (Skapiškio v., Kupiškis district)	2004
Varieties from other countries	Aromat Polessja	2004
	Marynka	2004
	French delicacies	2005

(Agilent Technologies, Waldbronn, Germany). Sodium hydroxide (chem. pure, Lachema, Czech Republic), sodium borate (g.r., Lachema, Czech Republic), sodium dihydrogene phosphate (p.a., Merck, Germany) were used to prepare buffers. Effective length of fused silica capillary (Microquarz, Germany) used for capillary electrophoresis was 60 cm, total length was 68.5 cm, i.d. was 50 µm. To prevent accumulation of the sample compounds on the wall of capillary and stabilize the electroosmotic flow, after each run the capillary was washed with 1 M NaOH for 1 min, with water for 1 min and with working buffer for 1.5 min at 5 bar pressure. Initial conditions for bitter acids separation by CZE were following: working buffer 25 mM NaH_2PO_4, 30 mM $Na_2B_4O_7$, pH 8.2; voltage 20 kV; temperature 25^0C; injection 150 mbar s. Optimizing separation process the influence of running buffer composition and pH value, amount of organic additive (methanol) in running buffer, sample dilution and analysis temperature on separation parameters were analyzed.

After optimization of the separation process the following analysis conditions were used: working buffer 25 mM NaH_2PO_4, 60 mM $Na_2B_4O_7$, pH 8.2; voltage 30 kV; temperature 20^0C; injection 150 mbar s.

Detection of α- and β-acids was performed at 230 nm and 345 nm correspondingly, since these wavelengths correspond to the absorption maxima of α- and β-acids. The peaks in electropherogram were identified according to their UV spectra acquired using the diode array detector (*Fig. 1*). Quantitative determination of α-acids was performed using certified supercritical CO_2 hop extract (amount of α-acids 51.9%) from Joh. Barth&Sohn GmbH&Co. KG (Nuernberg, Germany) as a standard material.

Figure 2. **The percentage of α-acids in different hop varieties determined by authors using conductometric titration and CZE method. Conditions of conductometric titration were as described in standard procedure [18]. Conditions for CZE analysis: 25 mM NaH_2PO_4, 60 mM $Na_2B_4O_7$, pH 8.2; voltage 30 kV; temperature 20^0C; injection 150 mbar s.**



RESULTS

The precision of conductometric method was checked analyzing certified hop extract. The results obtained (50.9±0.5%) were consistent with the certified amount of α-acids 51.9% (producer data). The same conductometric method was used for determination of α-acids in different hop varieties and wild forms. As shown in *Fig. 2* the highest amount of α-acids is in variety "Marynka". In Lithuanian hop varieties the highest amount of α-acids (2 – 2.5 %) was determined in "Kauno ankstyvieji", "Raudoniai" and "Hybrid A-9 (16)".

***Figure 3*. Electropherograms of hop extract with different sodium tetraborate concentration in working solution. Other conditions as in Fig. 2. Peaks β1 and β2 belong to lupulones. Peaks α1 and α2 belong to humulones, where α1 is the sum of n- and ad- humulones, and α2 is a co-humulone.**

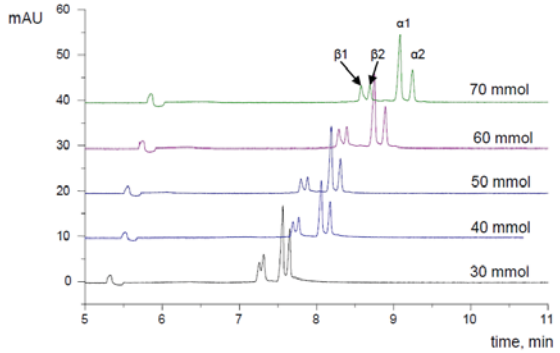
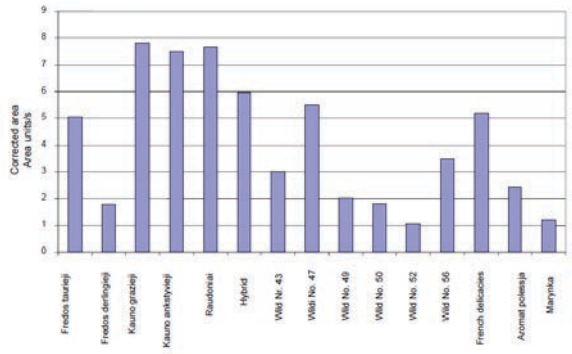


***Figure 5*. Amounts of β-acids in different hop varieties expressed by corrected peak area (ratio of peak area and migration time of the peak). Conditions of CZE as in Fig. 2.**

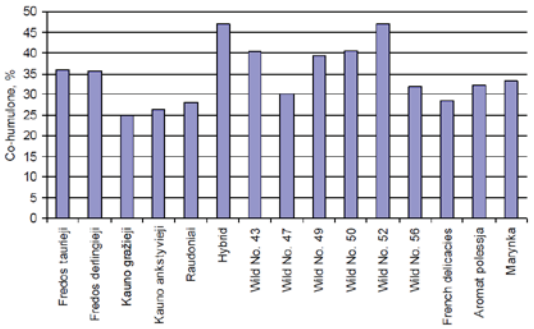


Characteristic UV absorption spectra of β-acids were obtained for the first two peaks in the electropherogram (*Fig. 3*). For the next two peaks separated the registered UV spectra were almost identical and characteristic for α-acids. Baseline separation between the resolved forms of humulone and lupulone was obtained using optimized conditions.

Using certified hop extract (51.9 % α-acids) a calibration curve was constructed in the range from 100 μg/ml to 1 mg/ml (R^2=0.9975). The limit of detection and limit of determination for α-acids were 100 μg/ml and 200 μg/ml correspondingly. The amounts (%) of α-acids determined by CZE in different varieties and wild forms of hops are shown in *Fig. 2*. Relative standard deviations of migration time and peak area for α-acids did not exceed 0.21% and 5.96% correspondingly.

The percentage of co-humulone in total amount of α-acids is presented in *Fig. 4*. The highest amount of co-humulone was determined in the wild hop form No. 52. The lowest amount of co-humulone is in Lithuanian hop variety "Kauno gražieji". Comparing Lithuanian hop varieties with selected hop varieties originating from other countries, the amount of co-humulone in Lithuanian varieties is relatively high, except that in varieties "Raudoniai", "Kauno ankstyvieji" and "Kauno gražieji".

***Figure 4*. Percentage of co-humulone in total amount of α-acids. Calculation is based on the ratio of peak areas of co-humulone and total area of humulone peaks. RSD for the measured peak areas is less than 6 %. Conditions of CZE as in Fig. 2.**



Determined relative amounts of β-acids indicate, that the highest amount of β-acids is characteristic for the varieties "Kauno ankstyvieji", "Kauno gražieji", "Raudoniai" and "Hybrid A(9)-16" (*Fig. 5*). Comparing Lithuanian varieties a low amount of β-acids was determined in "Fredos derlingieji", whereas in other Lithuanian varieties amount of β-acids was quite high.

DISCUSSION

The highest amount α-acids was found in variety "Marynka", which is supposed to be the most perspective variety for the local climatic conditions. Amount of α-acids is lower in the varieties "Aromat polessja" and "French delicacies", which are qualified as aromatic hop varieties. They are rich in essential oils and have lower amount of bitter substances. Not unexpectedly the amounts of α-acids found in wild hops were lower comparing to that in cultivated varieties, although in wild hops No. 49 and No. 50, which originate from Biržai district (North Lithuania), amount of α-acids was higher comparing to other wild hops. It should be noted that the amount of α-acids determined in all varieties is fairly low. This is probably due to the fact, that hop cones used for analysis were 1 – 2 years old, which can cause substantial reduction of the amount of α-acids in raw material. It was reported by Canbas et al. [20], that at ambient temperature (at uncontrolled storage temperature), when temperature sometimes reaches 20-30^{0}C, the amount of α-acids drops by 35% in 6 months. As reported elsewhere [21] α-acids amount in freshly dried "Marynka" hop cones ranges from 9 to 12%.

It was concluded, that CZE method can be used for separation of different forms of α- and β-acids (Fig. 3). Two peaks instead of three expected for α- and β-acids (n-, ad- and co-humulones/lupulones) indicate, that not all forms of hop acids are separated. In standard reversed-phase high performance liquid chromatography methods α- and β-acids usually are represented by the pairs of peaks. n- and ad-humulone/lupulone forms are not separated and they coelute. This can be explained by similar hydrophobicity of these

two forms, which is governed by similar side chains. The hydrophobicity of co- form is different (lower). As a result this form is separated from the other two. In capillary zone electrophoresis the result is the same, however the reason is different. Molecular weight of ad- and n- forms of α-acid (ad-humulone and n-humulone) is the same (M_r=362.4) and it is higher than M_r of co-humulone (M_r=348.4). Since separation mechanism of CZE is based on the differences of charge to mass ratio, consequently α-acid forms with different charge density (the same charge, different M_r) are separated. According to the CZE mechanism peak α1 in *Fig. 3* belongs to the n- and ad-humulones, and peak α2 belongs to co-humulone. Humulones are carried to the cathode by electroosmosis, however electrophoretically they move to anode, since they are charged negatively. Electrophoretic mobility of smaller molecules (higher charge density) will be higher. Therefore co-humulone will migrate towards the detection window more slowly. Initially the peaks of hop acids presented in electropherogram (*Fig. 3)* were not baseline separated. In order to increase the resolution, optimization of the capillary electrophoresis was carried out. The retention time and resolution depends on the concentration of the background electrolyte. The increase of the concentration of sodium tetraborate results in better resolution of α- and β-acids (*Fig. 3*). The concentration 60 mM of sodium tetraborate was determined as optimal. No remarkable affect on resolution and efficiency was observed varying sodium dihydrogene phosphate concentration in the background electrolyte from 25 to 55mM. To verify the effect of buffer pH on separation of hop acids, experiments were performed at pH ranging from 7.4 to 9.2 using the running buffer, consisted of 25 mM NaH_2PO_4 and 60 mM $Na_2B_4O_7$. Migration times increased with the increase of pH due to the greater ionization of hop acids. The increase of pH of running buffer decreased separation efficiency of α-acids however increased separation efficiency of β-acids. pH 8.2 was selected as optimum point, where efficiencies for α- and β-acids peaks were moderate. To increase separation efficiency by stacking effect the sample was diluted with water, low concentration running buffers (of various pH) and methanol. This did not remarkably affect the separation efficiency, therefore in final conditions the undiluted sample was injected. Determining the separation temperature effect, analyses were performed in the range of 15-30^{0}C. The decrease of resolution was observed increasing the separation temperature. Since efficiency at 15^{0}C and 20^{0}C did not differ remarkably and sufficient resolution 2 and 1.5 (for humulones and lupulones correspondingly) was obtained, in the final conditions separation temperature 20^{0}C was selected due to the higher electroosmosis and shorter analysis time.

Comparing conductometric titration and CZE methods, it can be concluded, that α-acids amounts obtained by both methods are consistent and variation between the methods is in the range of bias. In contrast to conductometric titration, the CZE method allows to evaluate percentage of co-humulone in total amount of α-acids. As mentioned above, higher amount of co-humulone is related to lower quality of hops for beer production, since the bitterness caused by it is more harsh. Due to the lack of β-acids reference material it was not possible to perform a quantitative analysis of β-acids in hop cone extracts. Nevertheless, CZE method allows a relative comparison of β-acids amounts in different hop varieties (*Fig. 5*).

CONCLUSIONS

Concluding it can be stated, that CZE method was optimized and home validated for analysis of hop α-acids. CZE analysis at optimized conditions takes ca. 8 min. Limit of detection 100 μg/ml and limit of quantification 200 μg/ml were calculated. Linear range of determination from 100 μg/ml up to 1 mg/ml was checked (R^2=0.9975). Repeatability for migration time (relative standard deviation up to 0.21%) and peak area (relative standard deviation up to 5.96%) were determined.

Analysis of hop cones of different hop varieties and some wild forms, collected at Kaunas Botanical Garden of Vytautas Magnus University was carried out using the optimized CZE conditions. Comparative study, using a standard conductometric titration method for determination of α-acids in hops and hop products has shown good consistence with the results obtained using the CZE method. The highest amount of α-acids (ca. 3.5 %) was determined in "Marynka" variety. Low amounts of β-acids were found in "Marynka", some wild forms (No. 52 and No. 50) and "Fredos derlingieji" (1–1.8 %), whereas in other analyzed varieties amount of β-acids was quite high.

ACKNOWLEDGEMENTS

Instrumental support from Agilent Technologies is highly acknowledged. Certified supercritical CO_2 hop extract was kindly provided by AB "Kauno alus".

REFERENCES

1. Sakamoto K, Konings WN. Beer spoilage bacteria and hop resistance. Int. J. Food Microbiol. 2003 Dec 31;89(2-3): 105–24.

2. Zanoli P, Zavatti M, Rivasi M, Brusiani F, Losi G, Puia G, Avallone R, Baraldi M. Evidence that the β-acids fraction of hops reduces central GABAergic neurotransmission. J Ethnopharmacol. 2007 Jan 3;109(1):87–92.

3. Verzele M, Potter MDE. High-performance liquid chromatography of hop bitter substances. Journal Chromatogr. 1978 May;166(1):320–6.

4. Shimamura M, Hazato T, Ashino H, Yamamoto Y, Iwasaki E, Tobe H, Yamamoto K, Yamamoto S. Inhibition of angiogenesis by humulone, a bitter acid from beer hop. Biochem Biophys Res Commun. 2001 Nov 23;289(1):220–4.

5. Yasukawa K, Takeuchi M, Takido M.

Humulone, a bitter in the hop, inhibits tumor promotion by 12-O-tetradecanoylphorbol-13-acetate in two-stage carcinogenesis in mouse skin. Oncology. 1995 Mar-Apr;52(2):156–8.

6. Chen WJ, Lin JK. Mechanisms of cancer chemoprevention by hop bitter acids (beer aroma) through induction of apoptosis mediated by Fas and caspase cascades. J Agric Food Chem 2004 Jan 14; 52(52):55–64.

7. Yamamoto K, Wang J, Yamamoto S, Tobe H. Suppression of cyclooxygenase-2 gene transcription by humulon of beer hop extract studied with reference to glucocorticoid. FEBS Lett. 2000 Jan 14;465(2-3):103–6.

8. Tagashira M, Watanabe M, Uemitsu N. Antioxidative activity of hop bitter acids and their analogues. Biosci Biotechnol Biochem. 1995 Apr;59(4):740–2.

9. Simpson WJ, Smith AR. Factors affecting antibacterial activity of hop compounds and their derivatives. J Appl Bacteriol. 1992 Apr;72(4):327–34.

10. Mizobuchi S, Sato Y. Antifungal activities of hop bitter resins and related compounds. Agric Biol Chem. 1985 Feb;49:399–403.

11. Royle L, Ames JM, Hill CA, Gardner DSJ. Analysis of hop acids by capillary electrophoresis. Food Chem. 2001 Aug;74(2):225–31.

12. Wilson RJH. HPLC analysis of hops and hop products: some problems and solutions. Ferment 1989;2:241-8.

13. Vindevogel J, Sandra P, Verhagen LC. Separation of hop bitter acids by capillary zone electrophoresis and micellar electrokinetic chromatography with UV-diode array detection. J High Resolut Chromatogr. 1990 Apr;13:295-8.

14. Vindevogel J, Sandra P. Micellar electrokinetic chromatography of ionic compounds: the analysis of hop bitter acids. J High Resolut Chromatogr. 1991 Dec;14:795-801.

15. Szücs R, Van Hove E, Sandra P. Micellar and microemulsion electrokinetic chromatography of hop bitter acids. J High Resol Chromatogr. 1996 Apr;19:189-92.

16. McLaughlin GM, Weston A, Hauffe KD. Capillary electrophoresis methods development and sensitivity enhancement strategies for the separation of industrial and environmental chemicals. J Chromatogr. A. 1996 Sept;744:123-34.

17. Vanhoenacker G, Dermaux A, De Keukeleire D, Sandra P. Single-run capillary electrochromatographic analysis of hop acids and prenylated hop flavonoids. J Sep Sci. 2001 Jan, 24, 55–8.

18. Хмель-сырец и хмель прессованный. Методы испытаний, ГОСТ 21948–21976.

19. Conductometric methods for hops and hops products. Forster A. Descriptors: acids, hop analysis. Lead conductometric value. EBC Symposium on “hops”, 17. - 19. September 1987 [cited 2008 Dec 12] Available from: http://www.barthhaasgroup.com/cmsdk/content/bhg/research/scientific1/27.html#anchor1537470

20. Canbas, A, Erten, H, Ozsahin F. The effects of storage temperature on the chemical composition of hop pellets. Process Biochemistry 2001 May;36(11):1053–8.

21. Australian craft Brewing [Internet]. Hop variety specifications; c2009 [cited 2009 May 05]. Available from: http://oz.craftbrewer.org/Library/Methods/HopCo/Origin.shtml