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The validation of the HPLC Hop Bitter acids method

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Abstract

Two methods for the determination of alpha and iso-alpha bitter acids composition by HPLC, are validated in the Food Safety and Quality Testing Laboratory (LICSA) of USAMV Cluj-Napoca in order to asses the capability of the laboratory to assure quality results. In the validation study, the following quality parameters were determined: specificity, linearity, precision and accuracy of the method. The method is selective for: co-isohumulone, isohumulone, Ad-isohumulone, Cohumulone and Colupulone and isn't selective for the separation of normal-humulone from ad-humulone, which appear together as a single peak. The same thing goes for the normal-lupulone and ad-lupulone peak.

Keywords: hop, bitter acids determination, HPLC, method validation.

1. Introduction

The analysis of bitter acids from hop is a very complex process due to bitter acids composition but also due to their isomerization during brewing. Thus, if in hop and hop products we have to dose the alpha and beta acids, in beer we have to dose the iso-alpha acids.

The determination of bitter acids from hop cones, hop pellets and hop extracts, represents an important step in brewing process because depending on the bitter acids content of hop the dosing of beer in bitter compounds is made. The bitter acids are, as presented in previous studies, complex mixtures of homologues and analogues compounds, which, during brewing process, offers certain bitterness.

For the dosing of beer bitterness, the analyses for determination of total content of alpha bitter acids (which are representative for beer bitterness) are: conductometric method (1-4) and spectrophotometric method (5). In the brewing process it is very important to determine the bitterness profile, if it is fine or hard, in order to obtain a high quality beer. Thus, the determination of alpha and beta bitter acids composition, by HPLC (6,7) method is necessary. The methods are described in Analytica EBC (European Brewery Convention), which represents the European authority in the field of analysis of bitter compounds from hop. Also, during the brewing process, the alpha bitter acids are transformed in iso-alpha acids which have to be quantitatively dosed. The methods presented in Analytica EBC are validated methods through interlaboratory studies and are recommended to be used in the analysis of hop, hop products and beer. The compounds that can be determined by HPLC method are: alpha acids (total) and the homologues compounds: cohumulone, normal-humulone and adhumulone; beta acids (total) and homologues: colupulone, normal-lupulone and adlupulone; iso-alpha acids (total) and homologues: co-isohumulone, isohumulone and ad-isohumulone.

Taking in consideration the fact that the two methods for the determination of alpha and iso-alpha bitter acids composition by HPLC, are validated through interlaboratory studies, still the capability of the Food Safety and Quality Testing Laboratory (LICSA) of USAMV Cluj-Napoca to assure quality results has to be proved.

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2. Materials and Method

In order to realize the internal validation of the HPLC method of the determination of bitter acids, hop pellets (from S.C HOPTRADE SRL-Romania), standard hop extract (alpha and beta bitter acids in known concentration) and the complex of iso-alpha acids with diciclohexylamine (from Veritas Labor - Swisse) were used. The determination of alpha acids (1-7), iso-alpha and their homologues was achieved using HPLC equipment from Schimadzu, with a UV detector. The column was a 250x4mm, filled with ODS RP18, NUCLEOSIL 5xC18 type; pre-column for HPLC analysis with the role of protecting the analysis column.

In the validation study, the following quality parameters were determined: specificity, linearity, precision and accuracy of the method.

Specificity

The specificity of the method was determined through the analysis of one blank sample, prepared in the solvent used for the dilution of standard samples. The investigation of specificity is made toward a calibration curve. –Requirement – the chromatograms of the specificity analyses doesn't have to present peaks at the retention time of the analyt. Although, if these are present, their peak area has to be with 20% higher then the analyt area at the quantification limit.

Linearity

In order to determine the linearity of the method, a number of 6-8 concentrations of standard samples in the matrix, at least in three different series, will be analyzed. The accuracy (the percentual difference between the analyt quantity calculated from the calibration curve and the entered analyt quantity) will be calculated for each point from the calibration curve.

Requirements: the calibration curve is acceptable if :

- The regression coefficient is higher than 0.98;
- The accuracy is between the limits ±20% at CC1 and ±15% at the other concentrations; 4 from the 6 calibration points have to fulfil this condition, including the inferior and superior quantification limit.

Precision and accuracy

In one lot of validation, three replicates of control samples will be analyzed for each concentration level (inferior – medium – superior), towards the calibration curve (precision and accuracy for analyses made in the same day).

In three different series, one sample for each control concentration level (inferior – medium – superior), will be analyzed towards the calibration curve (precision and accuracy for analyses made in different day/series).

The mean concentration, the standard deviation (SD) and the variation coefficient (CV%) of measured concentrations will be calculated and reported.

Requirements: the precision and accuracy will be acceptable if the variation coefficient of measured control concentration is not higher then ±15% for the determinations made in the same day or in the different days or series.

3. Results and Discussion

Specificity

No interferences with the retention times of iso-humulone, iso-cohumulone, iso-adhumulone, cohumulone, N/adhumulone, colupulone and N/adlupulone were observed.

In figure 1 is presented the chromatogram of a blank sample overlaid with a chromatogram of bitter acids at the lowest concentration.

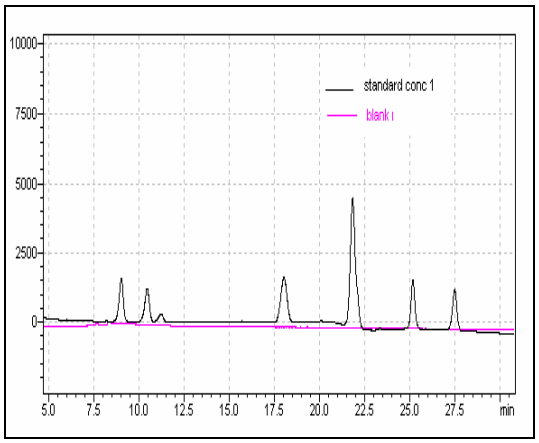

Figure 1. The chromatogram of a blank sample overlaid with a chromatogram of standard sample in the lowest concentration

Regarding the specificity / interferences of the bitter acids determination method by HPLC, the interlaboratory studies showed that, on one hand the methods are specific for the bitter acids from hop, and on the other hand, no interferences were noticed in the analyses, this being mentioned also in the EBC methods. The preparation system of the samples, including the reagents and extraction solvents, do not absorb in the UV at the absorption wavelengths of iso-alpha, alpha and beta bitter acids. The method is selective for: co-isohumulone, isohumulone, Ad-isohumulone, Cohumulone and Colupulone, but isn't selective for the separation of normal-humulone from ad-humulone, which appear together as a single peak. The same thing goes for the normal-lupulone and ad-lupulone peak.

Linearity

The calibration curves were linear – with adequate precision and accuracy – in the concentration interval presented in table 1.

The correlation coefficients were higher then 0.99 and the correlation is better for the level 2 of concentration. The recalculated concentrations based on the calibration curve, showed that the residuals don't have a certain tendency neither for low or high concentrations. From the calibration curve were excluded all the points for which the accuracy has values higher than 16%.

Table 1. The accuracy of cohumulone concentrations for the calibration curves on two levels of concentration and from different series.

1st level of concentration				**2d level of concentration**			
Nominal conc. %	**Accuracy %**			**Nominal conc. %**	**Accuracy %**		
	Curve 1	**Curve 2**	**Curve 3**		**Curve 1**	**Curve 2**	**Curve 3**
0,0289	11,21	11,61	4,43	0,1445	6,91	12,77	13,00
0,0578	-	- 3,37	4,34	0,289	6,32	6,66	6,20
0,0722	8,22	9,46	9,91	1,445	4,16	1,24	- 2,27
0,1445	- 0,7	4,76	4,97	2,89	1,77	- 4,22	-8,98
0,289	- 1,24	- 0,93	- 1,35	3,6125	2,07	-	-
r	**0,9977**	**0,9987**	**0,9994**	**r**	**0,9992**	**0,9997**	**0,9993**

Precision

Precision – The method precision is highly correlated with the results of the independent tests obtained from the homogenized material and in the actual working conditions. The precision is express through the repeatability and reproducibility and can be estimated when the validation is made by interlaboratory studies. In the absence of such studies estimations of the repeatability (same analyst – duplicate samples) and reproducibility (two analysts – duplicate samples) will be made.

Requirements regarding the method performance: The variation coefficient (CV) offers an image regarding the method performance for the same concentration level. Thus, the following situation can be distinguished:

For CV lower than 5% , we have a good method performance;

For CV higher than 10 %, we have a weak method performance;

In order to estimate the method performance we need to observe first the mean value of the concentration. Thus, at low concentrations, the value of CV can be high, while at high concentrations the CV values are generally smaller.

Table 2 . The quality parameters of the HPLC method of the bitter acids from hop pellets determination – repeatability conditions.

Analysis	**Concentration%**						
	Co-iso-humulone	**Iso-humulone**	**Ad-iso-humulone**	**Co-humulone**	**N/Ad-humulone**	**Co-lupulone**	**N/Ad-lupulone**
Repeatability conditions							
1	0.026072	0,111365	0.056265	2.351994	4.419439	2.506062	1.820904
2	0.032265	0.128725	0.072509	2.654757	5.020645	2.857632	2.075937
3	0.032664	0.127604	0.069482	2.577474	4.878462	2.772900	2.014743
4	0.034527	0.131594	0.075128	2.618219	4.962165	2.815666	2.047794
5	0.034454	0.128554	0.073366	2.551904	4.830287	2.732808	1.988268
6	0.034100	0.127881	0.072907	2.548447	4.828068	2.726350	1.988562
7	0.033669	0.128902	0.072907	2.548447	4.828068	2.689645	1.972426
8	0.033209	0.129843	0.069197	2.557977	4.838175	2.705062	1.993692
9	0.033896	0.130554	0.068486	2.576553	4.869598	2.716918	1.999543
The quality parameters of the method							
Min. Conc. %	0.026072	0.111365	0.056265	2.351994	4.419439	2.506062	1.820904
Max. Conc. %	0.034527	0.131594	0.075128	2.654757	5.020645	2.857632	2.075937
Mean Conc. %	0.032762	0.127225	0.069611	2.553332	4.827761	2.724783	1.989097
SD %	0.002624	0.006082	0.005518	0.083903	0.168465	0.098625	0.071012
CV %	**8.009721**	**4.780839**	**7.926601**	**3.286023**	**3.489509**	**3.619558**	**3.570085**
MDL %	0.008805	0.020409	0.018514	0.281527	0.565266	0.330925	0.238274
DL %	0.008660	0.020072	0.018209	0.276880	0.555935	0.325463	0.234341
QL %	0.026241	0.060824	0.055178	0.839031	1.684651	0.986251	0.710124

4. Conclusions

- Regarding the specificity / interferences of the bitter acids HPLC determination method, the interlaboratory studies showed that, on one hand, the methods are specific for the bitter acids from hop, and on the other hand, no interferences were noticed in the analyses, this being mentioned also in the EBC methods;
- The preparation system of the samples, including the reagents and extraction solvents, do not absorb in the UV at the absorption wavelengths of iso-alpha, alpha and beta bitter acids, as it can be seen from the overlaid chromatograms of a blank and a sample;
- The method is selective for: co-isohumulone, isohumulone, Ad-isohumulone, Cohumulone and Colupulone;
- The method isn't selective for the separation of normal-humulone from ad-humulone, which appear together as a single peak. The same thing goes for the normal-lupulone and ad-lupulone peak;
- Regarding the specificity, the calibration curve is acceptable because:
  - The regression coefficient for all the investigated compounds is higher than 0.98;
  - The accuracy is under the limit of ±20% at the lowest concentration (the inferior quantification limit) and under ±15% at the other concentrations; 4 of the calibration points fulfil this condition, including the inferior and superior quantification limit.
- The requirements regarding the method performance are fulfilled:
  - For all the performed analyses in the repeatability and reproducibility conditions, for the majority of the investigated compounds, the Cv value was under 5%, resulting a good performance of the method;
  - For co-Iso-Humulone and Ad-Iso-Humulone, determined in the repeatability and reproducibility conditions the CV value was between 5% and 10%; for these compounds the method has a average performance;
  - This average performance may be due to low concentrations of these compounds in the analyzed samples; thus, at lower concentrations, the value of CV can be high, while at higher concentrations the CV values are generally smaller.

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