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Method Development for Determination and Quantification of Ethanol in alcohol- free beer – Comparison of Size Exclusion Chromatography (SEC), enzymatic- and GC methods

The quantification of ethanol in alcohol free beer is a crucial parameter of the quality assurance in the brewery. Most laboratories use an enzymatic assay whereby ethanol is oxidized and the reduced co-factor is measured by UV spectroscopy. Here we present an alternative method which is readily automated utilizing an HPLC method with size exclusion or gel permeation chromatographic (SEC) stationary phases.
This SEC- method provides a new convenient and reliable technique to analyze ethanol in alcohol-free beer. In addition to the enzymatic and GC method the SEC- method allows the determination of ethanol in a wide concentration range with high linearity. Moreover, sugars can be analyzed in the same sample run, whereby hints for the beer production process are provided.

Descriptors: Method development, determination of alcohol analytics

1 Introduction

The quantitative determination of residual alcohol contents in non-alcoholic beers is important in the brewery. Two methods are currently used in quality laboratories: Beer distillation and the enzymatic method. The very sensitive enzymatic method gives good results down to 0.001 ww %, whereas the distillation method fails at alcohol contents below 0.05 ww %.

A reversed phase HPLC method with refractometric detection for the determination of ethanol in wine, liquor and whisky was published by J. Vogel et al. [1].

Also Kupina [2] reported another method for quantification of ethanol in grape juice by HPLC.

Here we report a further method for the very sensitive, reliable, and convenient determination of ethanol in alcohol-free beers of very low concentrations using size exclusion chromatography (gel permeation chromatography).

2 Size Exclusion Chromatography [3]

The principle of size exclusion or gel permeation chromatography (SEC) is a question of molecule volumes (Fig. 1). Various types of materials are used to form beads with various size pores. Large molecules cannot enter the matrix, intermediate size molecules can enter part of the matrix and small molecules freely enter the matrix. The matrix function is to provide a continuous decrease in accessibility for the molecules of increasing size. The largest molecules are eluted from the column first and the smallest are eluted last.

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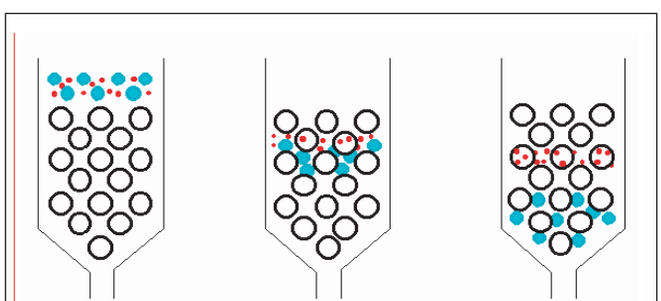

Fig. 1 Schematic principle of size exclusion or gel permeation chromatography [4]

3 Materials and Methods

3.1 SEC-Instrumentation

HPLC: RI-Detektor RI 71, Merck-Hitachi; HPLC- pump L-6000, Merck-Hitachi; Auto sampler AS 2000A-, Merck-Hitachi; ultrasonic bath; column oven.

3.2 SEC-Parameters

Column: Merck Polyspher OA HY, RT300-6.5, Nr. 1.51272; Temperature: 65°C; Eluent: 0.01 N sulfuric acid p.a.; Ethanol 99.9 %; Bi-distilled water; injection volume: 20 µl; flow rate: 0.6 ml/min; pressure ca. 75 bar; run time ca. 20 min.

3.3 SEC-Procedure

3.3.1 Low cost fast sample preparation

The beer samples were tempered to 20°C and degassed for 5 min by ultrasonic. 1 ml of the sample was transferred to teflon capped vials and put into an auto sampler. Calibration standards of ethanol in aqua bidest. were analyzed prior to each sample sequence.

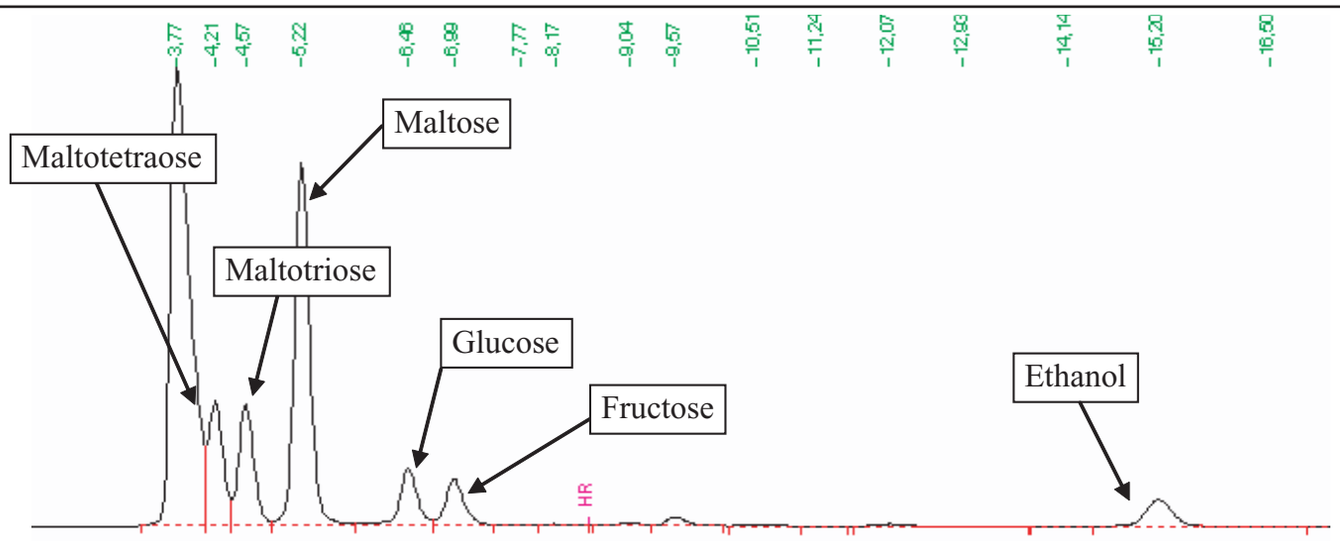

Fig. 2 SEC Chromatogram of a typical alcohol-free beer (stopped fermentation)

3.4 GC-Instrumentation

Perkin – Elmer GC AutoSystem; column: MN – FFAP 0.25 mm; 60 m * 0.25 mm, carrier gas: Hydrogen, ethanol 99.9 % (Merck 1.00983) n-butanol (p. a. Merck 1.01990).

GC Parameters: Injector temperature: 250°C; detector temperature: 250°C; detector: FID; carrier gas pressure: 150 kPa, injection volume: 1 ml; split: 1 : 40, temperature program: 50°C hold for 4 min then 40°C/min to 120°C, hold for 4 min then 40°C/min to 240°C.

3.5 GC-Procedure

The beer samples were tempered to 20°C and degassed for 5 min in an ultrasonic bath. 5 ml of the sample (beer or ethanol standard solution) was mixed with 5 ml internal standard solution (0.35 ww % n-butanol in bi-dest water). 1 ml of this mixture was transferred to Teflon capped vials and put into a GC auto sampler. Ethanol calibration standards were analyzed prior to each sample sequence.

3.6 Enzyme procedure

The enzymatic ethanol analysis method was performed utilizing an ethanol UV test kit, R-Biopharm AG (Order No. 10 176 290 035). The test was exactly performed according to the instructions of the manufacturer.

4 Results and discussion

Determination and quantification of ethanol by SEC method

When beer samples are analyzed, the SEC separates oligosaccarides by their molecular weight and tetroses elute first, followed by trioses, maltose, and glucose. Ethanol as a small molecule elutes at the end of the chromatogram (Fig. 2). Compound determination is performed by RI-Detektor and authentic references.

Fig. 3 demonstrates the SEC calibration curve of ethanol in aqua bidest. and displays the linearity of this procedure ranging ethanol concentrations from 0.001 – 1.000 ww%. According to this SEC method it is now possible to analyse different kinds of alcohol-free beers.

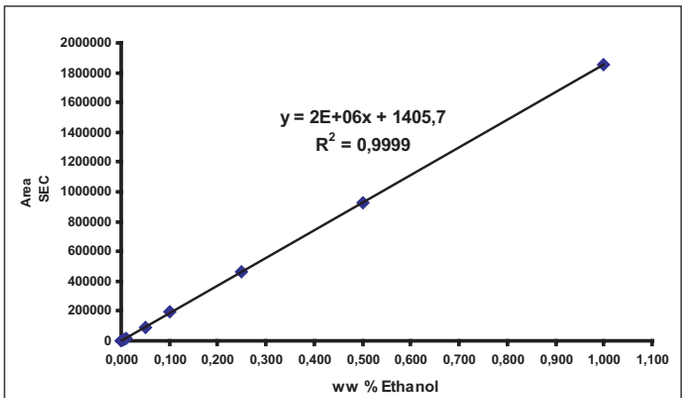

Fig. 3 SEC calibration curve of eight standard concentrations (from 0.001 to 1.000 ww %) of ethanol in aqua bidest. All calibration samples of ethanol were weighed and filled up to 100,000 g. Limit of detection: 0. 0003 ww %.

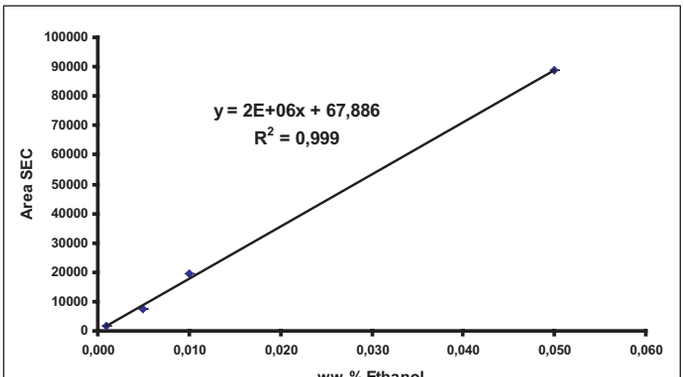

Fig. 4 SEC-calibration curve for lower concentrations of ethanol ww % (0.001 – 0.050).

For the determination of low ethanol concentrations in the range of 0.001-0.05 ww % it is recommended to monitor an extra calibration curve (Fig. 4). The determination of deviation is shown in Fig. 5 and Table 1.

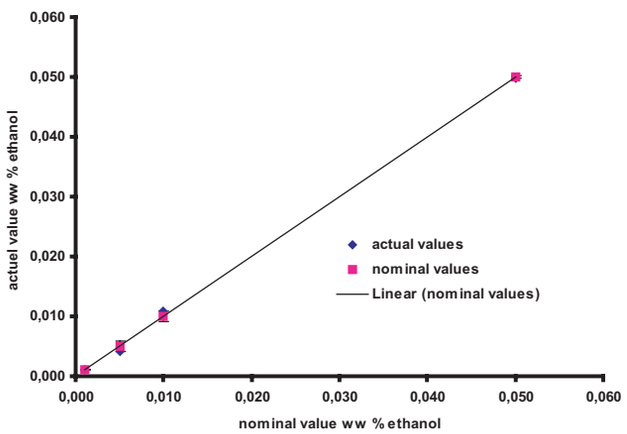

Fig. 5 Determination of deviation by comparing nominal and actual values of the SEC-calibration curve (0,001 – 0,050 ww % ethanol)

Table: 1 Nominal values, actual values and relative error for the determination of deviation

nominal value	actual value	relative error
ww %	ww %	%
0,001000	0,000996	0,39
0,005000	0,004188	16,25
0,010000	0,010959	9,59
0,050000	0,049858	0,28

4.1 Determination and quantification of ethanol by GC direct injection method

A further method to quantify ethanol in alcohol free beer is the direct injection (split injection) of the degassed sample containing the internal standard n-butanol (Fig. 6). [5]

Comparable to the newly developed SEC method, the direct GC injection analysis of ethanol from alcohol free beers is also characterized by a high linearity of 0.001–1.000 ww % (Fig.7).

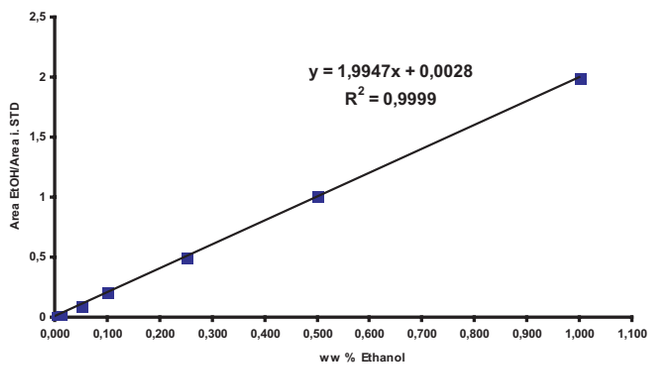

Fig. 7 Calibration curve: Direct GC injection of ethanol reference concentrations (from 0.001 to 1.000 ww %), containing n-butanol as an internal standard.

Limit of detection: 0.0004 ww %

Assuming the availability of a GC system in the laboratory, this GC method is a convenient and reproducible technique to analyze the ethanol content of a beer sample. However, the direct injection of water, sugars etc. from the complex matrix to GC capillary columns might restrict the lifespan of the GC column.

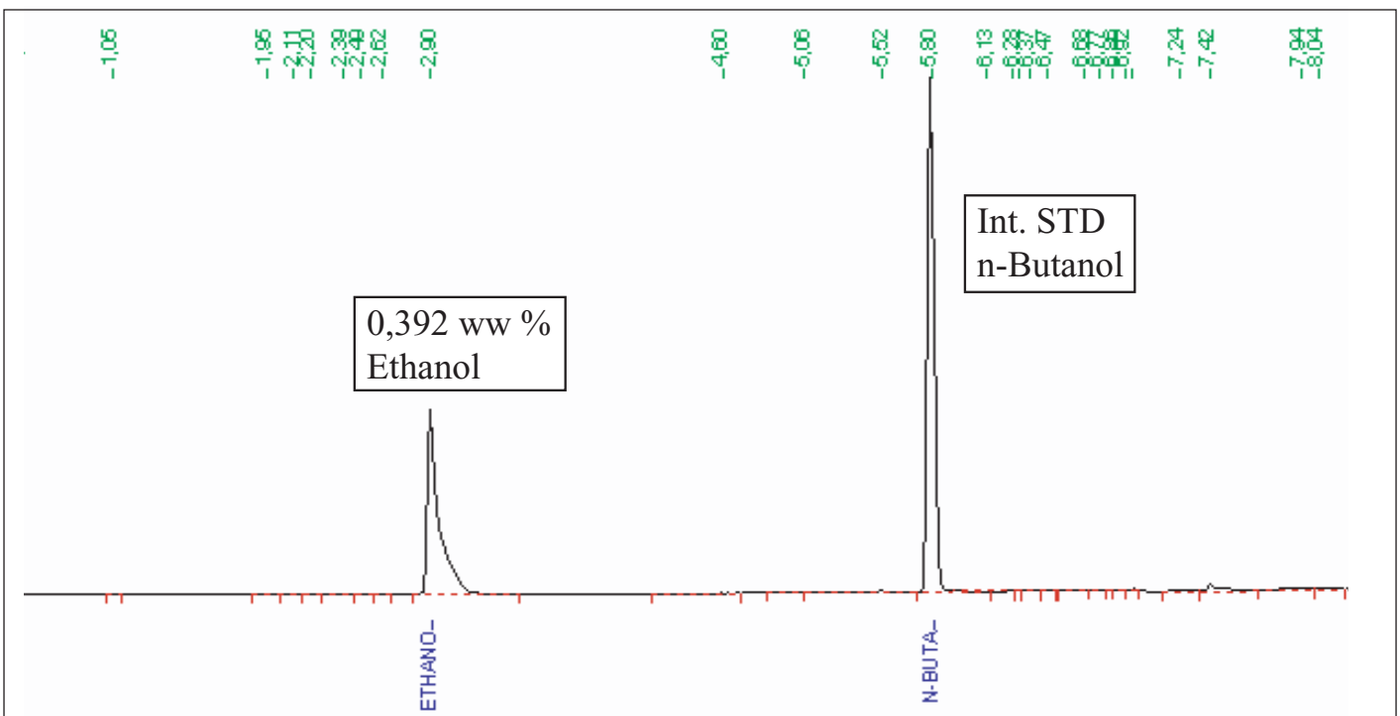

Fig. 6 Gas Chromatogram: Direct injection of alcohol free beer samples to a GC column, containing n-butanol as internal standard.

4.2 Determination and quantification of ethanol by enzymatic method

The enzymatic ethanol analysis method was performed utilizing a commercially available ethanol UV test kit (Fig. 8).

Limit of detection: 0.0001 ww %.

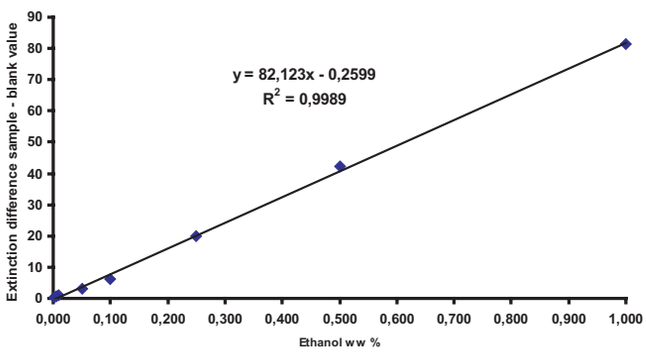

Fig. 8 Calibration curve: Enzymatic analysis of ethanol reference concentrations (from 0.001 to 1.000 ww %)

4.3 Comparison of the three methods

Ten alcohol free beer samples were analyzed using the new SEC- method, the direct GC method and the enzymatic test kit procedure. The results are shown in Fig. 9 and table 2. All three methods gave comparable results for the absolute ethanol content in the samples.

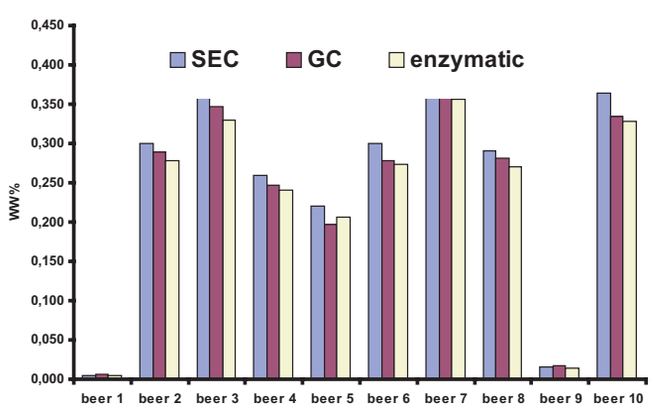

Fig. 9 Analysis of ethanol from alcohol free beers. Comparison of the ethanol concentration determined by SEC-, GC-, and enzymatic-method

4.3.1 Reproducibility

To examine and compare the reproducibility of the three described methods two non-alcoholic beers were analyzed ten times. The two samples used represent a relatively high and low amount of ethanol (Table 3).

According to table 3 the enzymatic method provides the best results in case of very low amounts of ethanol, while SEC should be used for the determination of higher amounts of alcohol.

Table 2 Content of ethanol in ten alcohol free beers from Europe in % ww. Ethanol determination by SEC -, enzymatic- and GC-method and comparison of statistical data

	SEC	GC	enzy-matic	**MW**	**SD**	**RSD%**
beer 1	0,004	0,006	0,005	**0,005**	**0,0011**	**22,38**
beer 2	0,300	0,289	0,278	**0,289**	**0,0109**	**3,77**
beer 3	0,358	0,347	0,329	**0,344**	**0,0144**	**4,19**
beer 4	0,259	0,247	0,240	**0,249**	**0,0098**	**3,94**
beer 5	0,221	0,197	0,207	**0,208**	**0,0120**	**5,77**
beer 6	0,300	0,278	0,273	**0,284**	**0,0144**	**5,07**
beer 7	0,393	0,371	0,356	**0,373**	**0,0184**	**4,92**
beer 8	0,291	0,281	0,271	**0,281**	**0,0098**	**3,48**
beer 9	0,016	0,017	0,014	**0,016**	**0,0016**	**10,27**
beer 10	0,365	0,334	0,328	**0,342**	**0,0195**	**5,69**

Table 3 Ten time analysis of sample 1 and sample 2 by SEC-, GC- and enzymatic method SD=standard deviation, RSD=relative SD

measure-ment	sample 1 ww % **SEC**	sample 2 ww % **SEC**	sample 1 ww % **GC**	sample 2 ww % **GC**	sample 1 ww % **enzyme**	sample2 ww % **enzyme**
1	0.0031	0.3821	0.0046	0.3651	0.0043	0.3320
2	0.0030	0.3868	0.0045	0.3722	0.0045	0.3290
3	0.0029	0.3888	0.0051	0.3779	0.0045	0.3280
4	0.0034	0.3800	0.0046	0.3799	0.0045	0.3280
5	0.0037	0.3803	0.0043	0.3637	0.0046	0.3330
6	0.0031	0.3867	0.0057	0.3671	0.0045	0.3320
7	0.0031	0.3822	0.0048	0.3723	0.0045	0.3350
8	0.0032	0.3810	0.0061	0.3702	0.0045	0.3200
9	0.0033	0.3875	0.0045	0.3730	0.0045	0.3270
10	0.0030	0.3821	0.0046	0.3744	0.0051	0.3310
mean value	**0.0032**	**0.3838**	**0.0049**	**0.3716**	**0.0045**	**0.3295**
SD	**0.0002**	**0.0033**	**0.0006**	**0.0052**	**0.0002**	**0.0042**
RSD%	**7.382**	**0.864**	**11.980**	**1.407**	**4.586**	**1.274**

5 Conclusion

The SEC- method provides a new convenient and reliable technique to analyze ethanol in alcohol free beer. In addition to the enzymatic and GC method the SEC-HPLC method allows the determination of ethanol in a wide concentration range with high linearity. If an HPLC system with refractive index detection and automation is available in the laboratory this method is a low cost alternative for the analysis of ethanol concentration. Moreover, sugars can be analyzed in the same sample run, whereby hints for the beer production process are provided.

6 Literature

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5. Refer to EBC-Method Section 9 Beer, Method 9.2.x

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