P. Varo and M. Rodríguez

# Determination of ethanol using the automatic enzymatic method

**The enzymatic Emit® ETS® Plus Ethyl Alcohol Assay (Emit) was used for the quantitative analysis of the ethanol content in low-alcohol beer. The assays were designed for use with the Syva ETS® Plus analyzer. The method is based on the enzymatic determination of ethanol with alcohol dehydronase. Repeatability ($r_{95}$) and reproducibility ($R_{95}$) values for aqueous controls (target value 0.05 – 0.06 v/v ethanol) were 0.006 and 0.011 respectively. In low-alcohol beer (target value alc. less than 0.5% vol.), r and R values were 0.017 and 0.038 respectively. The Emit assays were found to be rapid, precise and accurate for the determination of ethanol in low-alcohol beer. The method was tested under real conditions for the determination of ethanol in low-alcohol beer.**

BC 36 Beer

(Descriptors: Alcohol, ethanol, enzymatic determination, low-alcohol beer.

Deskriptoren: Alkohol, Ethanol, enzymatische Bestimmung, alkoholarmes Bier).

## 1 Introduction

The most important alcohols from the point of view of the analysis of food are saturates, aliphatics, methanol, ethanol and even penthanol. As alcohol in the sample is normally found mixed with other substances, separation by distillation is first necessary. Then, depending on the type of information required, the alcohols are determined as pure alcohol or, in the case of methanol as individual compounds (1). Gas chromatography is suitable for the separation and determination of the various homologs with a low ebullition point (2). Furthermore, there exist other kinds of analytical methods, among which enzymatic determination with alcohol deshydronase is particularly worthy of mention (3,4).

The enzymatic determination of the substrate satisfies all the requirements of conventional methods, reference methods and rapid methods. The enzymatic analysis of food is of special importance due to its simplicity, speed and the especificity of the model. Of special importance in the application of the enzymatic method is the analysis of carbohydrates, organic and inorganic acids, alcohols, and various nitrogen organic compounds (3). Enzymatic methods are important due to the fact that they may be developed considerably in semi-automatic or partially in completely automatic measurement processes (5). Routine enzymatic analysis stem from biochemistry and from clinical analyses. Among the methods most frequently used for the detection of alcohol in biological liquids are enzymatic analyses (6,7). These are used in areas such as biochemistry and clinical chemistry and may also be used in the analysis of food (8).

The analysis used is based on the enzymatic reaction where the ethanol is oxidized to acetaldehyde and $NAD^+$ is reduced to NADH by ADH alcohol dehydronase (ADH) (9). The conversion of $NAD^+$ to NADH results an increase in absorbance to 340 nm which is proportional to the ethanol concentration. The determination of ethanol in low-alcohol beers using the enzymatic method has been recommended by various authors (10,11,12). Commercial kits exist for the determination of ethanol in food (8). All of these are inconvenient as they are applied in a non-automatic form.

Low-alcohol beer (alcoholic content less than 1% volume) (13) presents an additional problem when determining the alcohol content. If the official method is used (14)(distillation of the beer and then measurement by picnometric method), the results can differ considerably from the real alcohol content. Therefore, the availability of rapid automatic methods with a limit of detection adequate for the alcohol content of these beverages has until now been one reason for research (15,16,17,18).

In this paper the repeatability and reproducibility of an automatic enzymatic method for the determination of ethylic alcohol in low-alcohol beer is reported.

## 2 Experimental part

### 2.1 Instrumental

A Syva ETS® Plus analyzer (Syva Co.) (Fig. 1 and Fig. 2) was used with software version 4.04. The ETS® system can carry out the Emit® ETS® Plus Ethylic Alcohol method for the quantitative analysis of ethylic alcohol present in human serum and urine samples (19).

### 2.2 Reagents

All the reagents for the Emit® ETS® Plus Ethylic Alcohol Assay, the calibrators and controls were obtained from the Syva Compa-

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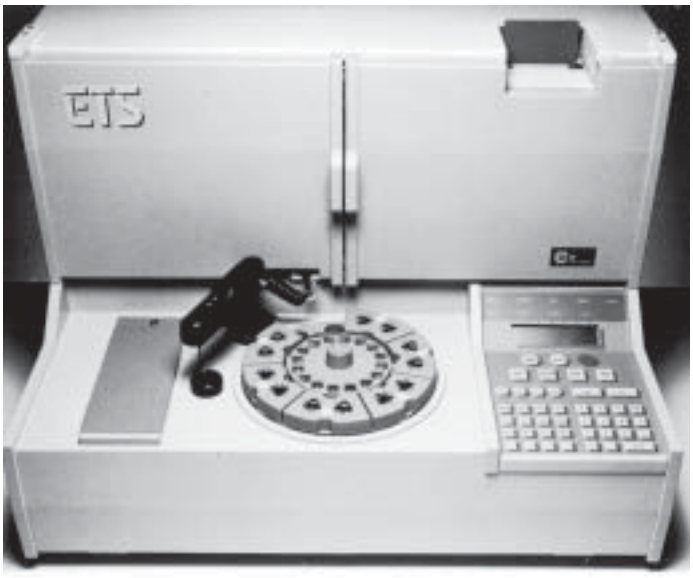


**Fig. 1 General view of the analyzer used**

**Fig. 2 Details of samples in the analyzer**

ny. The kit contains the basic components necessary to carry out the analysis, including the freeze-dried reagent and concentrated tampon. Reagent A contains the buffering system; reagent B contains alcohol dehydronase (ADH), the coenzyme nicotinamide adenine dinucleotide (NAD) and stabilizers. The concentrated tampon contains tampon and sodium azide (0.05% as a preserver). The analysis of ethylic alcohol uses a calibrator and two controls. The calibrator contains 0.13% v/v of ethanol in an aqueous solution. The low control contains ethanol in an aqueous solution in the range of 0.05-0.06 v/v and the high control in the range of 0.34-0.42% v/v. An aqueous control of ethanol of 0.09% v/v, obtained from Bio Rad Laboratories was used. All the calibrators, reagents and controls were maintained at 2 – 8 ºC.

### 2.3 Procedure

The calibration of the analyzer and the analysis of the ethanol were carried out in accordance with the recommendations of the manufacturer (20). The analysis was carried out using a calibration point of 0.13 v/v of ethanol. The tests require 50 µl of sample. However, in order to avoid evaporation losses, we used 200 µl of sample. The analyzer automatically mixed 7.5 µl of sample with 60 µl of reagent A, 60 µl of reagent B, and 187.5 µl of buffer. The mixture was incubated for 15 seconds. The photometric measurement of the reaction was produced for a period of 30 seconds. During this time the instrument took readings every 0.5 seconds of the amount of light absorbed by the reaction mixture. These readings are converted into the absorption rate of the enzymatic reaction. Once calibrated, the analyzer needs 3 minutes to determine the concentration of ethanol in the first sample. The following determinations require one minute after the analysis of the first sample.

## 3 Results and discussion

The Emit® ETS® Plus Ethylic Alcohol analysis has been reported with a detection limit of 0.01 v/v of ethanol and a linear ethanol concentration range of between 0.01% v/v and 0.76% v/v (19). The application of this reagent to the analysis of ethanol in biological fluids is linear up to 0.82% v/v ethanol (21).

Table 1 shows the results obtained for r (repeatability with speacific probability 95%), and for R (reproducibility with speacific probability 95%), following the ISO (22) criteria, for the low control (0.05-0.06 v/v), the high control (0.34 – 0.42 v/v), the control of 0.09% v/v and for a sample of low-alcohol beer. Due to the difficulty of carrying out tests in different laboratories, as laboratories where this system of analysis is available, concentrate on the analysis of clinical samples and not on food, the R has been calculated with tests within the same laboratory.

In order to compare the between-run accuracy obtained in our study with that reported in the Operators Guide for the reagent and with that reported by other authors, the values of $s_r$ (standard deviation) and $RSD_r$ (Relative standard deviation) for the aqueous controls of the same range analyzed, are presented in Table 2.

**Table 1 Accuracy obtained with the method used**

| Sample | Target value | r | R |
|---|---|---|---|
| Low control (aqueous control) | (0.05-0.06) (% v/v ethanol) | 0.006 | 0.011 |
| High control (aqueous control) | (0.34-0.42) (% v/v ethanol) | 0.029 | 0.054 |
| Medium control (aqueous control) | 0.09 (% v/v ethanol) | 0.015 | 0.032 |
| Sample of low-alcohol beer | alc. < 0.5 (% vol) | 0.017 | 0.038 |

**Table 2 Between-run accuracy (Aqueous controls)**

| Target value (% v/v ethanol) | Value found (% v/v ethanol) | $s_r$ | $RSD_r$ | Reference |
|---|---|---|---|---|
| (0.05 – 0.06) | 0.05 | 0.002 | 4.08 | (20) |
| (0.34 – 0.42) | 0.37 | 0.010 | 2.65 | (20) |
| 0.05 | 0.05 | 0.002 | 3.84 | (21) |
| 0.13 | 0.13 | 0.003 | 2.31 | (23) |
| 0.09 | 0.09 | 0.005 | 5.56 | Present paper |

$s_r$ = standard deviation, calculated under repeatability conditions

$RSD_r$ = relative standard deviation, calculated under repeatability conditions

**Table 3 Results of accuracy compared with other methods for low-alcohol beer**

| Product | Average value (% v/v ethanol) | r | R | Method | Reference |
|---|---|---|---|---|---|
| Lager beer | 0.454 | 0.026 | 0.047 | Enzymatic | (24) |
| Malt beer | 0.602 | 0.031 | 0.075 | Enzymatic | (24) |
| Low-alcohol beer | 0.55 | 0.04 | 0.07 | HPLC | (15) |
| Low-alcohol beer | 0.50 | 0.026 | 0.057 | Enzymatic | (12,18) |
| Low-alcohol beer | 0.46 | 0.017 | 0.038 | Enzymatic | Present paper |

The values found for the parameters studied are of the same order as those reported in the Operators Guide for the reagent. An aqueous control of 0.09% v/v of ethanol was analyzed and compared with the accuracy reported in other research that analyzed controls of 0.05% v/v of ethanol and 0.13% v/v of ethanol. The values found for $s_r$ and $RSD_r$ (Table 2) are comparable with those obtained by other authors (21,23).

In order to apply the method proposed to a sample of low-alcohol beer, a beer with alcohol content less than 0.05% (value shown on label) was chosen. A repeatability value of 0.017 was obtained. In the bibliography, values of 0.020 to 0.040 have been found for the same kind of beverages and with an alcohol percentage of the same order (12,18,15,24). This was to be expected, bearing in mind that our results were obtained with an analyzer. A reproducibility value of 0.038 was obtained while other authors obtained values between 0.040 and 0.080 (12,18,15,24). Table 3 shows the accuracy results for samples of low-alcohol beer.

The test method proposed is particularly useful for determining ethanol in low-alcohol beer. The detection limit is 0.01 v/v. The accuracy obtained is comparable with other methods.

On comparing the proposed method with the official one for determining the alcohol content in beer, certain advantages can be found which are; prior distillation of the sample is not necessary, incubation at a controlled temperature is not necessary for measurement of the density**.** Thus the method proposed, automatic enzyme assay, presents important advantages over the official method, as it allows an automatic analysis to be made, obtaining rapid results (one determination per minute), intermediary sample preparation is unnecessary. The addition of samples, reagents, dilutions, spectrophotometric readings and the obtaining of the results is carried out by the system itself which confers great repeatability as already reported in this paper. The limit of detection is similar to that obtained when using the tables of alcohol content expressed in % v/v for densities measured at 20/20º C with the official method. For this reason we are of the opinion that the method proposed presents sufficient advantages to be considered in the analysis of the alcohol content of low-alcohol beer.

## 4 Zusammenfassung

**Varo, P., und Rodríguez, M.: Bestimmung des Ethanolgehalts anhand des automatischen enzymatischen Verfahrens** — Monatsschrift für Brauwissenschaft 54, Nr. 11/12, 233 – 236, 2001

**BC 36 Bier**

Zur quantitativen Analyse des Ethanolgehalts von alkoholarmem Bier wurde der enzymatische Emit® ETS® Plus Ethylalkohol-Assay (Emit) verwendet. Die Assays (Tests) wurden zur Verwendung mit dem Syva ETS® Plus Analysiergerät konzipiert. Das Verfahren beruht auf der enzymatischen Bestimmung des Äthanols mittels Alkoholdehydrogenase. Die Werte für die Wiederholbarkeit ($r_{95}$) und die Reproduzierbarkeit ($R_{95}$) bei wässrigen Kontrollen (Zielwert 0,05-0,06 v/v Äthanol) betrugen 0,006 beziehungsweise 0,011. Bei alkoholarmem Bier (Zielwert für Alk. unter 0,5% Vol.), lagen die Werte r und R bei 0,017 beziehungsweise 0,038. Die Emit-Assays erwiesen sich als schnell, präzise und genau zur Bestimmung des Ethanolgehalts von alkoholarmem Bier. Das Verfahren wurde unter realistischen Bedingungen auf die Bestimmung des Ethanolgehalts von alkoholarmem Bier hin geprüft.

**Varo, P., et Rodríguez, M.: Détermination de la teneur en éthanol par un procédé enzymatique automatique** — Monatsschrift für Brauwissenschaft 54, No. 11/12, 233 – 236, 2001

**BC 36 Bière**

On a utilisé le système enzymatique Emit® ETS® Plus Ethanol Assay (Emit) pour la détermination quantitative de la teneur en éthanol dans les bières à faible teneur en alcool. Pour l'application de ce test on a conçu un appareil analytique Syva ETS ®. Le procédé repose sur la détermination de l'éthanol au moyen de l'alcooldéshydrogènase. Les valeurs de répétabilité ($r_{95}$) et de reproductibilité ($R_{95}$) sur des solutions témoins aqueuses (valeur de 0,05 – 0,06 v/v d'éthanol) s'élevaient respectivement à 0,006 et à 0,011. Pour des bières à faible teneur en alcool (valeurs en dessous de 0,5 % vol.) les valeurs de r et R étaient respectivement de 0,017 et de 0,038. Le Emit-Assays est une méthode rapide, précise et exacte pour la détermination de l'éthanol dans les bières pauvres en alcool. Le procédé a été vérifié dans des conditions réalistes pour la détermination de la teneur en éthanol dans les bières à faible teneur en alcool.

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