

Article

## Simple and Rapid Determination of Ethanol Content in Beer Using an Amperometric Biosensor

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**Abstract:** An alcohol dehydrogenase-based biosensor was prepared and tested for its use to determine ethanol in beer. The biosensor is based on a screen-printed carbon electrode (SPCE) modified by rhodium dioxide and immobilized with a biocatalytic layer containing the enzyme. Function of the enzyme biosensor was tested in model ethanol samples, in which it showed a linear range of 15–120 g·L<sup>−1</sup> with a detection limit of 3.3 g·L<sup>−1</sup> (established as 3σ) and response time of 19 s. In a potential window from −0.2 to +0.45 V, interferences of both ascorbic and uric acids were negligible. Several types of marketed beers of Czech provenance were selected and subjected to measurements under optimized conditions but without any pretreatment of real samples. When compared with the reference method (gas chromatography), the results were in quite good agreement for beers of the pale lager type but higher contents of ethanol were indicated in the samples of dark lager beers.

**Keywords:** ethanol; amperometry; screen-printed carbon electrode; alcohol dehydrogenase; biosensor; beer

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## 1. Introduction

Together with coffee, beer is one of the most used stimulants in the world. In the territory of the Czech Republic, beer is the most frequently consumed alcoholic beverage. Beer is also considered to be one of the symbols of the country and since 2008, the mark “Czech beer” (“České pivo”) has been protected as a geographical indication [1]. It is known that beer is quite a complicated disperse system composed of various compounds [2]. In addition, it represents a colloidal solution of numerous macromolecules, such as albuminous matters, nucleic acids, sugars and lipids. Chemical composition of beers varies in wide bounds; in dependence on the brewer’s grains, it can contain 2%–6% of extractable compounds. The main constituents of extracts are saccharides (dextrins, mono- and oligosaccharides, maltose, maltotriose, pentose). Nitrogenous substances form *ca.* 6%–9% of extract. Among others, beer also contains polyphenolic compounds ( $100\text{--}180\text{ mg}\cdot\text{L}^{-1}$ ), bitter substances from hops ( $15\text{--}40\text{ mg}\cdot\text{L}^{-1}$ ), dyes (melanoids), glycerol, lipids, heterocyclic compounds and vitamins (especially those of the B group). Beer can be used as a beverage suitable for relieving thirst, but also for its nutritional value, namely an especially suitable balance of ions and minerals, vitamins and polyphenols. The beneficial effects of beer on the human body that can occur when consumed moderately do not outweigh the negative effects of alcohol. Conversely, alcohol has a positive impact on the reduction of cardiovascular disease. In this context, the death rate among temperate drinkers is reduced by 20%–50% compared to the total abstainers or heavy drinkers. Thus, especially in the case of elderly persons, beer is not unhealthy; one 0.5-L glass daily is better than none.

To control the fermentation process and quality of beer production, simple and fast analytical procedures are highly desirable. Determination of all matrix compounds (individual phenolic acids, polyphenols, flavonoids and related antioxidants) is usually carried out by high-performance liquid chromatography [3]. To determine the ethanol content, gas chromatographic and spectrometric methods are often used [4,5]. However, such methods are time-consuming and require relatively expensive equipment. In contrast, electroanalysis using corresponding biosensors can represent an acceptable alternative to the methods mentioned above as they are not expensive and the sample preparation is simple as well (it should be mentioned that sensors based on the fluorescent probe may also be constructed [6]). Moreover, two advantages are combined in electrochemical sensors, first enzyme specificity and secondly, transfer of the signal of biocatalytic reaction to an electrochemical one (see, e.g., [7–10]). To prepare simple biosensors, the screen-printing technology may advantageously be applied [11].

In biosensors for ethanol determination, alcohol oxidase and alcohol dehydrogenase enzymes are widely used. Alcohol oxidase is catalytically active for alcohols containing short alkyl chains, alcohol dehydrogenase is more selective for alcohols other than methanol as the corresponding reaction rate decreases rapidly with the size of the molecule [12]. As discussed in a recent review [13], the use of alcohol oxidase enzyme on the analysis of ethanol in complex samples allows a considerable enhancement in specificity. Examples of such a sensor developed for the analysis of beer have also been presented [14,15]. However, alcohol oxidase is less stable and oxygen-dependent; interferences of other oxidable compounds like both ascorbic and uric acids or paracetamol are also undesirable. Evidently, the influence of the last two substances cannot be taken into account when beer samples are analyzed. However, interference of ascorbic acid must be considered as the compound is added to beer

not only as an essential ingredient for the human body and an antioxidant (allowed within EU under E300) but because its addition prevents the clouding of beer.

The second substance—alcohol dehydrogenase—is a nicotinamideadenine-dinucleotide (NADH) depending enzyme and the biosensors for ethanol based on it require the co-immobilization of both enzyme and co-enzyme [16]. A presence of the  $\text{NAD}^+$  cofactor (coming from NADH) is needed in higher levels and thus, the whole sensor seems to be quite complicated in terms of its fabrication [17–22]. However, this can be solved using appropriate immobilization techniques.

A few years ago, we studied the oxides of platinum metals as potential catalysts in amperometric biosensors [23–25]. Among others, we recommended rhodium dioxide, which has been found to be applicable for measurements not affected by the presence of interfering acids mentioned above. In addition, its stability in flow injection analytical procedures was absolutely satisfactory. Subsequently, we reported on screen-printed biosensors modified by  $\text{RhO}_2$  and dehydrogenases [26,27], and determination of ethanol in alcoholic drinks like wine, vodka and whisky was also tested [27]. This biosensor worked even at low input potentials, where other easily oxidable or reducible molecules often present in real samples did not contribute to resulting amperometric signals. The aim of this paper was to check how far the biosensor could be used for the determination of ethanol content in much more complicated matrices (like beer is) without prior treatment of the samples; the results are reported here.

## 2. Experimental Section

### 2.1. Chemicals, Reagents and Solutions

Alcohol dehydrogenase (EC 1.1.1.1 from *Saccharomyces cerevisiae*, with specific activity  $300 \text{ U} \cdot \text{mg}^{-1}$ ; ADH), rhodium dioxide, *m*-phenylenediamine, nicotinamideadenine-dinucleotide ( $\text{NAD}^+$ ), ascorbic acid and uric acid were purchased from Aldrich (St. Louis, MO, USA). All other chemicals used for the preparation of buffer, stock and standard solutions were of analytical reagent grade and purchased from Lachema (Brno, Czech Republic). Phosphate buffers were prepared by mixing aqueous solutions of sodium dihydrogen phosphate and disodium hydrogen phosphate (both  $0.1 \text{ mol L}^{-1}$ ) to achieve the required pH. Doubly distilled water was used throughout the work.

### 2.2. Instrumentation

A modular electrochemical system AUTOLAB connected with modules PGSTAT 30 and ECD (Ecochemie, Utrecht, Holand) was used in combination with a corresponding software (GPES, Ecochemie) under Windows XP<sup>®</sup>. The flow injection system consisted of a peristaltic pump (Minipuls 3, Gilson SA., France), a sample injection valve (ECOM, Ventil C, Czech Republic), and a self-constructed thin layer electrochemical flow-through cell. The working electrode was fixed via rubber gaskets (thickness 0.6 mm) directly to the back plate of the thin layer cell. The reference electrode was  $\text{Ag}/\text{AgCl}/3\text{M KCl}$  (RE-6, BAS, West Lafayette, IN, USA), the stainless steel back plate represented the counter electrode of the cell. The responses were evaluated using the peak heights (differences between background and response current of the analyte). Corresponding pH values were controlled potentiometrically using a portable pH-meter (CPH 52 model, Elteca, Turnov, Czech Republic)

equipped with a measuring cell (an OP-0808P combined glass pH-sensor, Radelkis, Budapest, Hungary) calibrated with buffer solutions of the conventional activity scale.

### 2.3. Biosensor Preparation, Measuring Procedure and Sample Processing

A screen-printed carbon electrode (SPCE) was used as a support for a biosensor, which was prepared similarly as described previously: carbon ink (0.095 g, Gwent C50905D1, Pontypool, UK) and rhodium dioxide (0.05 g) were thoroughly mixed and subsequently sonicated; the mixture was used for screen-printing to obtain a SPCE/RhO<sub>2</sub> support [26] on which the enzyme was immobilized. Measurements were performed by direct current amperometry in both flow injection and batch arrangements. All operational variables (*i.e.*, applied potential, pH and flow rate) were optimized [26,27]. It should be noted that all of the prepared and used biosensors were stored in the fridge to keep their long term stability; their activities retained constant even after 60 injections, and that no loss of the original responses occurred after three weeks of measurements.

Beer samples were treated using a shaking machine (for 30 min) and ultrasound (10 min). No other treatment was applied. For analyses, sample volumes of 200 µL were always taken and injected into the flow injection system.

### 2.4. Reference Gas Chromatographic Method

A gas chromatographic procedure using a GC 2010 (Shimadzu, Tokyo, Japan) chromatograph equipped with a flame ionization detector (GC/FID) was selected as a reference, the separation was carried out using a capillary column (length, 30 m; internal diameter, 0.25 mm; film thickness, 0.25 µm; DB/% ms J&W Scientific, Agilent Technologies, Palo Alto, CA, USA). As a carrier gas, helium of ultrahigh purity was used at a linear flow speed of 30 cm s<sup>-1</sup> and at corresponding temperatures as follow: injector, 180 °C; column starting at 40 °C (3 min) with a gradient of 20 °C min<sup>-1</sup> to 260 °C (5 min); detector, 265 °C.

### 2.5. Statistical Analysis

The arithmetic means ( $\bar{x}$ ) and ranges ( $R$ , difference between the highest and the lowest value in set of data) were calculated for three replications ( $n = 3$ ). Both precision and mutual agreement of results obtained with the two procedures could be evaluated applying a simple Lord's  $u$ -test, which has been known for some time [28], but is nonetheless still very useful; it is suitable for the quick comparison of low number of results obtained by two different methods A and B. In the test,  $u$ -values are calculated according Equation (1)

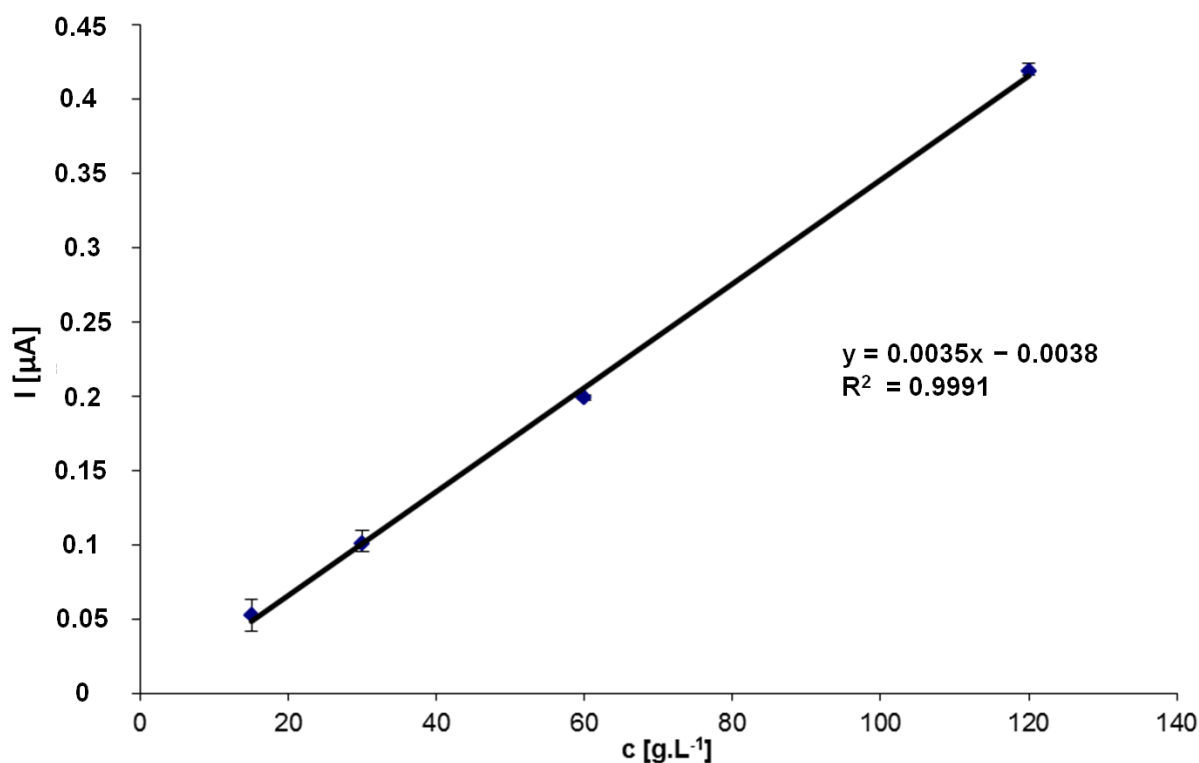
$$[u = |\bar{x}_A - \bar{x}_B| / (R_A + R_B)] \quad (1)$$

and compared with the critical value  $u_{\text{crit}} = 0.636$  for a given number of replications, usually for the significance level  $\alpha = 0.05$  (95% probability). If the calculated  $u$  value is higher than  $u_{\text{crit}}$ , the difference in arithmetic means is statistically significant.

### 3. Results and Discussion

As reported previously [26,27], attention needs to be paid to execute the immobilization correctly when dehydrogenases are to be used. Not only enzymes, but also their cofactors ( $\text{NAD}^+$  or  $\text{NADP}^+$ ) are water-soluble. Thus, they may rapidly be washed out of the electrode surface, especially during measurements in flowing liquids (like in the flow injection analysis). From that point of view, immobilization through electropolymerization with *m*-phenylenediamine was considered the most appropriate.

In addition, a choice of input potential is very important as it significantly affects the biosensor selectivity. Plotting the current response against the input potential, it was observed [27] that oxidation starts at around +0.2 V, and with increasing potential the response increases too [27]. No catalytic activity was observed in the potential range of  $-0.05$  to  $+0.15$  V, which implies that, to determine ethanol, an appropriate potential should be chosen in the range of  $+0.2$  to  $+0.6$  V. As shown [26], possible interfering species (both ascorbic and uric acids) are electroactive if the potential of  $+0.5$  V is applied but their responses are negligible at potentials from  $-0.2$  to  $+0.45$  V. Formerly, effects of flow rate and pH were also studied [26]. At optimum conditions (batch volume,  $200\text{ }\mu\text{L}$ ; input potential,  $+0.25$  V; flow rate,  $0.6\text{ mL}\cdot\text{min}^{-1}$ ), a calibration dependence of the biosensor response to ethanol was studied. The calibration (see Figure 1) was linear in a range of  $15\text{--}120\text{ g}\cdot\text{L}^{-1}$  and could be expressed by equation  $I\text{ }[\mu\text{A}] = 3.5 \times 10^{-3}c\text{ }[\text{g}\cdot\text{L}^{-1}] + 0.0038$ ;  $R^2 = 0.9991$ . The corresponding detection limit, established as  $3\sigma$ , was  $3.3\text{ g}\cdot\text{L}^{-1}$ .



**Figure 1.** Biosensor (screen-printed carbon electrode (SPCE)/ $\text{RhO}_2$ /ADH) response current to ethanol concentration.

As real samples, several types of marketed beers were finally selected and subjected to analyses. Measurements were performed under optimized conditions, the values obtained are listed in Table 1. When compared with results of the reference method (gas chromatographic determinations), the found contents of ethanol are in quite good agreement except for samples of both dark and half-dark beers, probably due to other technologies leading to other interfering species in these samples. Concerning sample #3, the alcohol content found by the proposed method agreed tolerably with that given by a producer but the content indicated by GC was lower. In the sample of non-alcoholic beer (sample #1), results of the reference method agreed well with the value declared by the producer but no response was obtained using the biosensor procedure; it seems that the level of ethanol was under its detection limit.

**Table 1.** Determination of ethanol content in real beer samples.

Beer Type		Proposed	Reference	$n$	$u$	$u_{\text{crit}}$	Declared
		Method	Method				
		$\bar{x} \pm R[\%]$			[%]		
1	non-alcoholic	<DL	0.41 ± 0.01	3	–	0.636	≤0.49
2	light	3.8 ± 0.3	3.46 ± 0.09		0.872		4.1
3	lager	4.7 ± 0.4	3.31 ± 0.08		2.896		4.9
4	lager	5.1 ± 0.2	5.28 ± 0.09		0.621		5.0
5	lager	4.1 ± 0.2	4.07 ± 0.03		0.130		4.4
6	diabetic	3.8 ± 0.1	3.75 ± 0.10		0.250		4.0
7	strong lager	5.7 ± 0.4	5.39 ± 0.05		0.689		6.5
8	porter	9.1 ± 0.4	7.66 ± 0.05		3.200		8.0
9	half dark lager	5.9 ± 0.2	4.35 ± 0.06		5.962		4.0
10	lager	4.7 ± 0.3	4.87 ± 0.05		0.486		4.7
11	tap lager beer	4.2 ± 0.1	4.25 ± 0.06		0.313		3.5

*n*—number of measurements;  $\bar{x}$ —arithmetic mean; *R*—range; *u*<sub>crit</sub> and *u*—critical and calculated values of the Lord's test (for 95% probability); DL—detection limit.

#### 4. Conclusions

Based on the observations described above, it can be concluded that the screen-printed biosensor containing RhO<sub>2</sub> as an electron transfer and alcohol dehydrogenase entrapped by *m*-phenyldiamine as a biocatalytic layer can successfully be applied in rapid control analyses of lager type beers using a flow injection analytical procedure under the optimized conditions described in the preceding paragraph. With regard to the complex nature of the samples, the results can mostly be classified as satisfactory. At the potential selected for measurements, interference of ascorbic acid is negligible as well as interferences of other compounds (polyphenols and other electroactive substances) which is clear from analyses of non-alcoholic beer where no responses were indicated. Concerning analysis of beers of the dark lager and porter types, the proposed biosensor procedure is less suitable because it leads to higher results. This could probably be solved using standard addition experiments.

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## Author Contributions

V.P. prepared and tested biosensors and performed all electrochemical measurements; A.E. contributed with gas chromatographic analysis of real beer samples; K.V. suggested and supervised the experiments and writing the final text as well.

## Conflicts of Interest

The authors declare no conflict of interest.

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