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Capacitive Field-Effect Biosensor Applied for the Detection of Acetoin in Alcoholic Beverages and Fermentation Broths

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An acetoin biosensor based on a capacitive electrolyte–insulator–semiconductor (EIS) structure modified with the enzyme acetoin reductase, also known as butane-2,3-diol dehydrogenase (*Bacillus clausii* DSM 8716^T), is applied for acetoin detection in beer, red wine, and fermentation broth samples for the first time. The EIS sensor consists of an Al/p-Si/SiO_2/Ta_2O_5 layer structure with immobilized acetoin reductase on top of the Ta_2O_5 transducer layer by means of crosslinking via glutaraldehyde. The unmodified and enzyme-modified sensors are electrochemically characterized by means of leakage current, capacitance–voltage, and constant capacitance methods, respectively.

1. Introduction

Some of today's common groceries, such as wine, beer, and cheese, are assumed to have been first produced by accident.[1] For instance, the genesis of wine may have its roots more than 7000 years ago, when damaged grapes spontaneously fermented in harvesting vessels. Once fermentation was discovered randomly, it was directly performed on a regular basis and became common.[2] From that point on, for thousands of years, fermentation processes were carried out with no proficiency about the mechanisms behind it or the knowledge of microbes that drive the fermentation.[3] Nevertheless, as time progressed, quality management became more important. In the case of German beer production, political regulation of quality management started already in 1516, when Duke Wilhelm IV released the Bavarian purity law, which determines permissible ingredients in beer and still has a crucial impact on methods used in German beer breweries today.[4] During the last century, fermentation processes started to be used in a wide range of industry branches, for example, for the production of organic acids, antibiotics, and enzymes.[3] Fermentation processes were further improved and more specified by the different industrial producers, who want to sell a unique product with maintaining quality. With the knowledge build-up, production control happens nowadays on a molecular level.[5–9]

One important aspect of quality control in the food industry is taste. For maintaining a specific taste, flavoring agents are frequently used. Acetoin and diacetyl are flavorings, which can be found, for example, in cheese and yoghurt, or in alcoholic beverages such as beer and wine.[10] Acetoin is a product of fermentative metabolisms of different microorganisms, which exposes a butter-like aroma.[11] During the production process of wine and beer, the acetoin concentration depends on different stages of fermentation and maturation. Due to this, the acetoin level can indicate how far the process has progressed. Flavor maturation is the rate-limiting factor in beer maturation.[12] Measuring the acetoin concentration during the production process of wine and beer can serve for quality management as it has a negative effect on the beer flavor if it exceeds a certain concentration level, and in addition, unnecessary maturation time can be avoided.[13,14] Typical acetoin concentrations in red wine and beer are in the range of 100–200 and 10–50 $\times$ 10^{-6} M, respectively.[13,15,16] Apart from its significance in alcoholic beverages, acetoin detection plays also an important role in biotechnological applications, where it can be used as an indicator for the metabolic activity of bacteria during fermentation processes.[17] In addition, the production of acetoin itself is

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of great interest because it is widely used in food production, as chemical raw material, and as a precursor in the synthesis of liquid fuels by microorganisms.[18,19]

Until now, standard methods for the detection of acetoin are colorimetric techniques, such as the Voges–Proskauer test[19,20] and gas chromatographic (GC) methods.[21,22] Although gas chromatography enables the precise detection of different acetoin concentrations, it is time-consuming because samples must be prepared, equipment must be available at the company, whereby costs arise, and trained staff is needed. At the same time, in fermentations some decisions on the ongoing process must be made fast, whereby rapid and accurate concentration determination is desirable.[23]

Acetoin detection is still an ongoing research topic.[14,24–26] However, most studies are based on GC methods, having no possibility of on-line determination. A biosensor could avoid the named drawbacks of this technique by enabling on-site measurements with fast response time. Our group recently developed a biosensor for the detection of acetoin that is based on an enzyme-modified electrolyte–insulator–semiconductor (EIS) sensor and characterized it in buffer solutions with regard to its sensor performance under laboratory conditions. First "proof-of-concept" experiments could even demonstrate its feasibility in real test samples, such as buffer samples spiked with white wine.[27] As enzymatic receptor layer, a novel acetoin reductase from *Bacillus clausii* DSM 8716^T is used.[28,29] In the current work, the storage stability of the capacitive acetoin field-effect biosensor was further studied with a specific focus on the detection of acetoin in real samples of beer, red wine, and fermentation broths for the first time.

2. Experimental Section

2.1. Materials

Acetoin, nicotinamide adenine dinucleotide (NADH), glutaraldehyde, glycerol, hydrochloric acid (HCl), sodium hydroxide (NaOH), and sodium chloride (NaCl) were purchased from Sigma-Aldrich (Darmstadt, Germany). Tris buffer was acquired from Carl Roth (Karlsruhe, Germany) and diethyl ether from AppliChem (Darmstadt, Germany). For measurements in beer samples, Premium Pils from Bitburger (Bitburg, Germany) was used. Measurements in red wine were performed in samples from a dry red wine (Tempranillo, 2016, Spain). For measurements in fermentation broth, samples from two in-house fermenters producing subtilisin proteases with *Bacillus subtilis* DB104 were provided. The fermentation samples were stored at −20 °C until they were analyzed.

2.2. Sensor Fabrication

The acetoin biosensor is based on a pH-sensitive EIS sensor with Ta_2O_5 as the transducer material. EIS sensors have been previously used for various research studies.[30–36] Ta_2O_5 was chosen as a pH-sensitive transducer material due to its nearly Nernstian pH sensitivity, chemical stability, and low drift and hysteresis.[37,38] As a semiconductor, p-doped silicon (p-Si, 5–10 Ω cm) was used with an ≈30 nm thick SiO_2 insulating layer, which was thermally oxidized in O_2 atmosphere at 1000 °C. A 30 nm tantalum (Ta) layer was deposited on the SiO_2 by electron-beam evaporation. Afterward, a ≈60 nm Ta_2O_5 layer was grown by thermal oxidation of the Ta at 520 °C. Removing the rear-side SiO_2 by an etching process in hydrofluoric acid enabled electron-beam evaporation of a thin Al layer (300 nm) as rear-side contact. The contact layer was additionally tempered for 10 min at 400 °C in N_2 atmosphere. After that, the wafer was cut in 10×10 mm^2 chips. Before the EIS chips were applied for electrochemical measurements and enzyme immobilization, they were cleaned in an ultrasonic bath for 10 min with acetone, isopropanol, and deionized water, respectively.

2.3. Enzyme Immobilization

The enzyme acetoin reductase was produced as described in Muschallik et al.[28] It was solved in 10×10^{-3} M tris-HCl buffer with 150×10^{-3} M NaCl and stored at −80 °C. To obtain comparable measurement results, a constant enzyme activity of 400 U mL^{-1} was selected. Therefore, the enzyme activity was verified by photometric analysis (Ultrospec 2100 pro, biochrom, Holliston, Massachusetts, USA) and diluted with 10×10^{-3} M tris-HCl plus 150×10^{-3} M NaCl buffer prior to each sensor experiment. The enzyme was immobilized on the Ta_2O_5 transducer surface by means of crosslinking. Thus, glutaraldehyde as a crosslinker was mixed with glycerol (10 vol%) for higher membrane flexibility. By blending the prepared enzyme solution with the glutaraldehyde–glycerol mixture in a volumetric ratio of 1:1.5, the final enzymatic membrane cocktail was achieved. On each EIS sensor chip, enzyme membrane solution with ≈13 units was drop coated and dried for at least 6 h at room temperature. Afterward, the enzyme-modified sensor was stored at 4 °C overnight. To avoid condensation, silica gel was added to the storage box.

2.4. Acetoin Detection in Fermentation Broth via Gas Chromatography

Fermentation broth samples were percolated with a syringe filter (pore size of 0.4 µm) to remove cell debris. The samples were then extracted with diethyl ether (1:2), mixed, and centrifuged for 1 min at 13 000 rpm. Subsequently, the organic phase was concentrated by a factor of 6. These samples were analyzed with a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) with a flame ionization detector (carrier gas helium with a linear velocity of 40 cm s^{-1}) and a Hydrodex-γ-DIMON (25×0.25 mm^2 ID, Macherey-Nagel, Düren, Germany) chiral chromatographic column. The injector and detector temperature were 215 and 245 °C, respectively. For data collection and processing, the Shimadzu GC System software was used. Retention time was 9.7 min for (*R*)-acetoin and 11.7 min for (*S*)-acetoin. The acetoin concentration in the fermenter samples was calculated from the peak areas using a racemic acetoin standard.

2.5. Functioning of the Capacitive Acetoin Biosensor and Measurement Setup

For electrochemical measurements, the (enzyme-modified) sensor was mounted into a home-made measurement chamber and

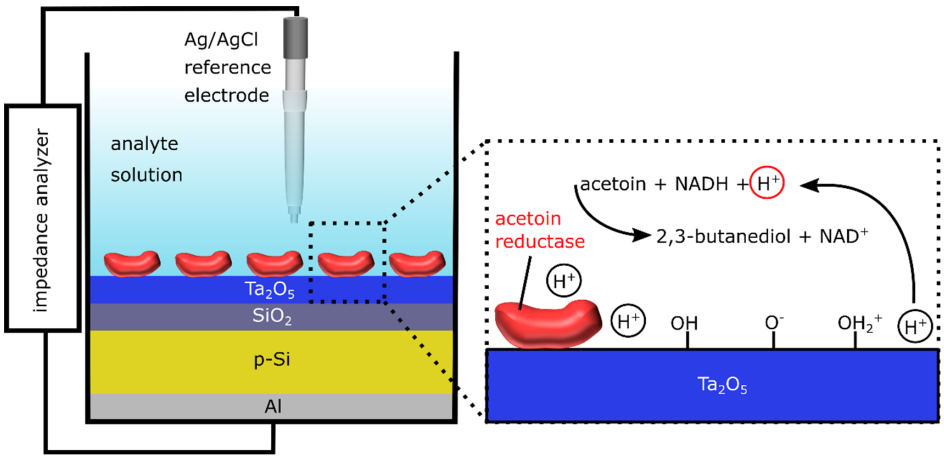

Figure 1. Schematic layer structure of the Al/p-Si/SiO_2/Ta_2O_5–enzyme EIS sensor with measurement setup (left), and enzymatic reduction of acetoin catalyzed by the enzyme acetoin reductase (right).

sealed with an O-ring, whereby ≈0.5 cm^2 of the sensor surface was exposed to the analyte solution. The sensor was connected to an impedance analyzer (Zahner Zennium, Zahner Elektrik, Kronach, Germany) by a Ag/AgCl reference electrode (filled with 3 M KCl, Metrohm, Filderstadt, Germany), which was dipped into the analyte solution and by a rear-side contact, which was connected to the Al layer, as schematically shown in **Figure 1**.

The field-effect-based EIS sensor detects changes in H^+-ion concentration near the pH-sensitive Ta_2O_5 transducer layer. The immobilized acetoin reductase on the Ta_2O_5 catalyzes the reduction of racemic acetoin to (*R*,*R*)- and *meso*-2,3-butanediol, while NADH serves as a cosubstrate, which is oxidized to NAD^+ as shown in Equation (1).

$$\text{acetoin} + \text{NADH} + \text{H}^+ \xrightarrow{\text{acetoin reductase}} 2,3\text{-butanediol} + \text{NAD}^+ \quad (1)$$

The local pH change, induced by consumed H^+ ions during the enzymatic reaction, shifts the surface potential of the Ta_2O_5 layer. By that, the width of the space-charge region in the semiconductor near the insulator–semiconductor interface is influenced, and therewith the overall capacitance of the EIS biosensor. Thus, different acetoin concentrations in the analyte solution lead to proportional capacitance changes of the EIS sensor, i.e., a shift of the capacitance–voltage curve in the depletion region.[37]

All sensors were electrochemically characterized by capacitance–voltage (*C*–*V*) and constant capacitance (ConCap) measurements. Leakage-current measurements served as quality control of the SiO_2–Ta_2O_5 gate oxide. For *C*–*V* measurements, a direct current (DC) voltage was applied between the reference electrode and the Al rear-side contact, which was varied in 100 mV steps in the range of −1.5 to +2 V. In addition, an alternating current (AC) voltage of 20 mV with a frequency of 120 Hz was superimposed to measure the capacitance. In the ConCap mode, the capacitance of the EIS biosensor is kept constant at a working point set in the depletion region of the previously recorded *C*–*V* curve (≈60% of the maximum capacitance) and the acetoin concentration–dependent potential changes at the Ta_2O_5 surface can be recorded in real time. All electrochemical measurements were performed in a dark Faraday cage at room temperature.

For acetoin measurements, a low-capacity 0.2×10^{-3} M tris-HCl buffer, together with 150×10^{-3} M NaCl as ionic strength adjuster, was used with varying acetoin concentrations from 10 to 90×10^{-6} M. Red wine, beer, and fermentation broth were diluted with 0.2×10^{-3} M tris-HCl plus 150×10^{-3} M NaCl to lower the buffer capacity of the samples. Different acetoin concentrations were spiked to these solutions, reaching from 1 to 500×10^{-6} M. In all measurement solutions, 500×10^{-6} M NADH was used as cosubstrate. Before starting the experiments, the pH value was controlled with a pH-glass electrode (Mettler Toledo, Gießen, Germany) and adjusted to pH 7.1 with HCl and NaOH, if necessary.

3. Results and Discussion

3.1. Electrochemical Characterization of Bare EIS Sensors

Before modification with acetoin reductase, the bare EIS sensors were electrochemically characterized to evaluate their pH-sensor functionality. The results of leakage current, *C*–*V*, and ConCap measurements performed with bare EIS sensors are summarized in the Supporting Information.

3.2. Storage Stability of the Acetoin Biosensor

Apart from long-term stability, in general the storage stability of a biosensor is of great importance when long-term applications are of interest.[39] Therefore, the storage stability of three fabricated acetoin biosensors was examined over a period of 21 days. ConCap measurements were performed every 7 days in buffer solution (0.2×10^{-3} M tris-HCl + 150×10^{-3} M NaCl + 500×10^{-6} M NADH, pH 7.1) with different acetoin concentrations between 10 and 90×10^{-6} M with each sensor. When not in use, the sensors were stored at 4 °C.

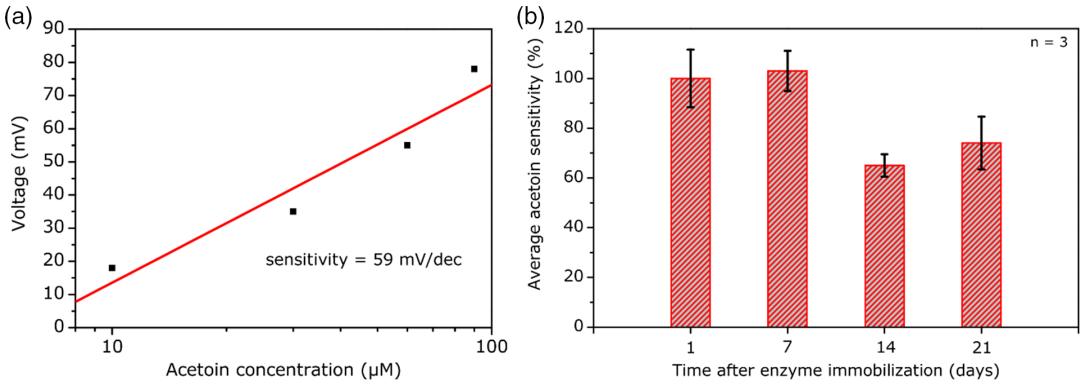

Figure 2. a) Exemplary calibration curve of an acetoin biosensor evaluated from ConCap measurements, 1 day after enzyme immobilization. b) Bar chart diagram showing the average acetoin sensitivity of $n = 3$ acetoin biosensors performed periodically at day 1, 7, 14, and 21 after enzyme immobilization. Analyte solution: 0.2×10^{-3} M tris-HCl + 150×10^{-3} M NaCl + 500×10^{-6} M NADH (pH 7.1) with varying acetoin concentrations from 10 to 90×10^{-6} M.

Figure 2a shows an exemplary calibration curve, evaluated from a ConCap measurement with an acetoin biosensor 1 day after enzyme immobilization in buffer solution with acetoin concentrations from 10 to 90×10^{-6} M. The sensor showed an average acetoin sensitivity of 59 mV dec^{-1}, which is in good agreement with the results achieved in Molinnus et al.[27] The average acetoin sensitivity of three individual acetoin biosensors at different measurement days compared to their initial sensitivity value of the first measurement is shown in the bar chart diagram in Figure 2b. The mean acetoin sensitivity on the first day after enzyme immobilization is set as 100%.

During the second acetoin concentration measurement, on the seventh day after enzyme immobilization, the biosensors observed no loss in sensitivity. There was even a slight increase in the average acetoin sensitivity (≈3%), which is, however, in the range of the standard deviation and can be explained by ordinary fluctuations. On day 14 of storage, the sensor still exhibited 65% of its original acetoin sensitivity, and on day 21, the sensor displayed 74% of its initial sensitivity. The decrease in acetoin sensitivity could be caused by, for example, leaching effects of the enzyme membrane and/or a decrease in the enzymatic activity, both induced by washing steps of the sensor and also representing slight fluctuations in the overall acetoin sensitivity. Despite these satisfying results, further measurements after day 28 could not be performed because the enzyme membrane was detaching from the sensor surface subsequently. Further experiments to optimize immobilization and storage conditions could help to comprehend such phenomena, and additionally should improve the storage stability. Nevertheless, even after 3 weeks, the immobilized enzyme still possessed high activity and the acetoin sensors retained their functionality, enabling the detection of different acetoin concentrations in the range of 10–90×10^{-6} M in buffer solution.

3.3. Acetoin Detection in Real Samples

In this study, the novel biosensor was mainly applied for acetoin measurements in beer, red wine, and fermentation broth samples. As the measurement principle is based on a local pH change generated by the enzymatic conversion of acetoin to 2,3-butanediol, as described earlier, a low buffer capacity of the analyte solution is preferred. As beer, red wine, and fermentation broth exhibit a high buffer capacity (e.g., for red wine in the range of 40×10^{-3} M),[40] it is necessary to lower the buffer capacity: For this reason, the samples were diluted with 0.2×10^{-3} M tris-HCl plus 150×10^{-3} M NaCl, adjusted to pH 7.1 (to ensure working at the pH optimum of the enzyme[27]), and 500×10^{-6} M NADH was added. For acetoin measurements in beer and red wine samples, a dilution of 1:20 was used and for fermentation broth samples, a dilution of 1:50 was applied.

3.3.1. Acetoin Detection in Alcoholic Beverages

In red wine and beer samples, ConCap measurements with three individual acetoin biosensors were conducted, respectively. The diluted samples were spiked with acetoin concentrations between 1 and 500×10^{-6} M. **Figure 3** shows an exemplary ConCap curve recorded in beer (a) and red wine (b) samples. In all samples, spiked acetoin concentrations from 1 to 500×10^{-6} M could be clearly detected, resulting in distinct signal shifts. Obvious steps were found for each concentration. More interestingly, the signal strengths achieved in the different samples differ only slightly from each other, showing that the developed biosensor is independently applicable for various alcoholic beverages. Although the signal steps for different acetoin concentrations are similar in beer and red wine samples, the absolute voltage values in the ConCap measurement mode are different, which is because the sensors were fabricated from two different wafers as well as different working points have been selected for ConCap measurements. Nevertheless, despite the complex compositions of the samples, whereby multiple disturbing substances should be included, the acetoin biosensor maintains its functionality.

The dilution ratio of the alcoholic sample can be regulated accordingly: For example, if a red wine sample contains an acetoin concentration of 200×10^{-6} M, after a 1:20 dilution there is still a concentration of 10×10^{-6} M left, which can be recorded by the developed acetoin biosensor considerably, having a sensor signal of about 40 mV.

Figure 3c overviews the mean calibration plots of three individual acetoin biosensors per sample (beer or wine) for acetoin

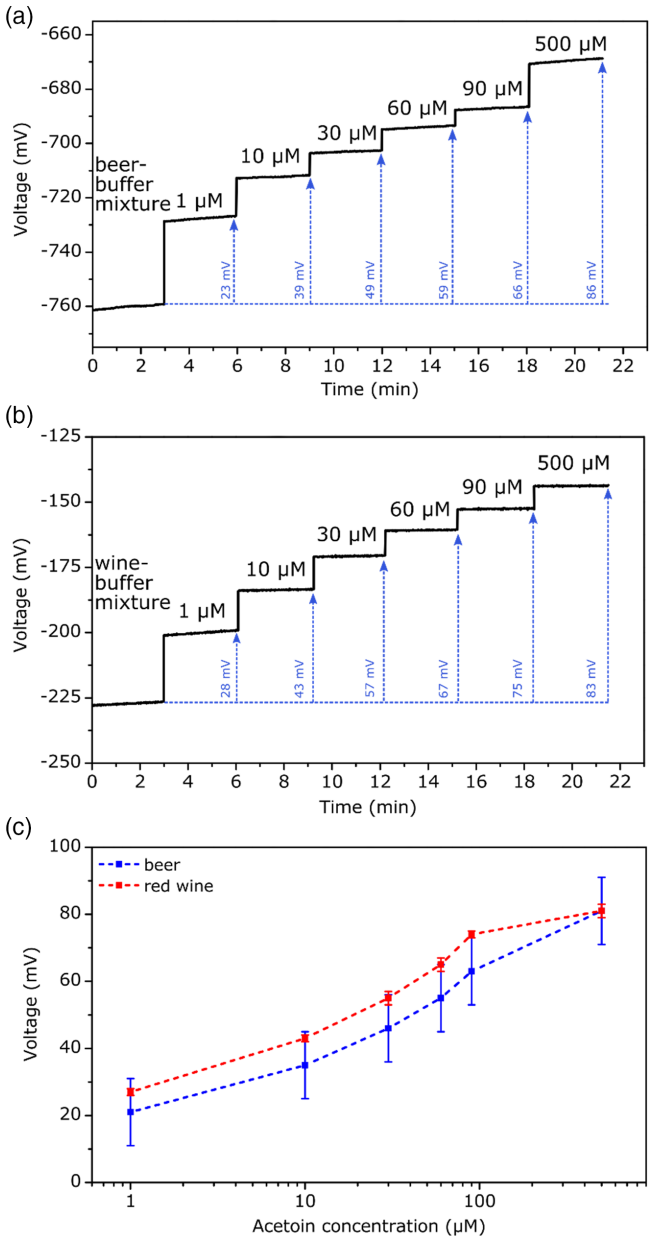

Figure 3. ConCap curves recorded in a) beer–buffer mixture (buffer: 0.2×10^{-3} M tris-HCl + 150×10^{-3} M NaCl + 500×10^{-6} M NADH) and b) red wine–buffer mixture, both with a ratio of 1:20, pH 7.1. c) Mean calibration plots with standard deviations ($n = 3$) obtained with the acetoin biosensor measured in the beer–buffer solution (blue) and red wine–buffer solution (red), respectively. The acetoin concentrations were in the range of 1–500×10^{-6} M.

concentrations between 1 and 500×10^{-6} M, respectively. For both alcoholic beverages, an s-shaped calibration curve can be seen that is typical for electrochemical biosensors, with a linear concentration range from 10 to 90×10^{-6} M. Here, the sensors showed a mean acetoin sensitivity of 28 ± 0.8 mV dec^{-1} in beer and of 32 ± 1.2 mV dec^{-1} in red wine, respectively. The mean acetoin sensitivity is somewhat smaller than in case of acetoin measurements in buffer solutions (see Figure 2). On the other hand, no additional sample pretreatment has been performed for the beer and wine samples, where further containing substances can influence the biosensor signal behavior.

3.3.2. Acetoin Detection in Fermentation Broths

In addition to acetoin measurements in alcoholic beverages, the possible application of the acetoin biosensor in biotechnological fermentation processes was studied as acetoin is a crucial parameter in many fermentations, such as industrial fermentation processes of *Bacillus* sp. For this purpose, samples from two different in-house fermenters producing subtilisin proteases with *B. subtilis* DB104 were taken. Here, the acetoin concentration was determined in advance to the sensor measurements by gas chromatography as an additional reference method. As described in Section 2.4, the samples had to be concentrated by a factor of 6 due to low acetoin concentrations in the samples. Three measurements were performed, each by taking two different samples 1 and 2. For sample 1 and sample 2, $388 \pm 53 \times 10^{-6}$ M (*R*)-acetoin and $460 \pm 91 \times 10^{-6}$ M (*R*)-acetoin were determined, respectively. The (*S*)-acetoin concentration was below the detection limit in both samples and could not be quantified. This could be caused by further enzymes, which are also able to convert (*S*)-acetoin, existing in the fermentation broths.

For electrochemical acetoin measurements, the fermentation broth samples were diluted with buffer solution in a ratio of 1:50, leading to an (*R*)-acetoin concentration of $\approx 7.8 \times 10^{-6}$ M in sample 1 and of $\approx 9.2 \times 10^{-6}$ M in sample 2, respectively. To study the feasibility of the developed biosensor to detect naturally formed acetoin in fermentation broths, ConCap measurements were performed first in buffer solution and afterward in buffer–broth mixture. **Figure 4**a shows two exemplary ConCap curves recorded in sample 1 and sample 2, respectively. For sample 1, a signal shift of 46 mV and for sample 2 a signal shift of 62 mV were obtained. As expected, the signal strength increases with higher acetoin concentration. The results achieved with three individual sensors and three individual GC measurements for each sample are compared in Figure 4b. For sample 1, an average signal shift for the biosensor of 48 ± 3.6 mV was recorded. For sample 2, it amounted to 63 ± 2.9 mV. The mean signal shifts correlate well with the mean acetoin concentrations measured via GC, where the particular concentrations are $388 \pm 53 \times 10^{-6}$ M (sample 1) and $460 \pm 91 \times 10^{-6}$ M (sample 2), respectively. This way, the developed biosensor can be also successfully applied to detect the naturally formed acetoin during fermentation processes validated in this "proof-of-concept" experiment.

For a better understanding of the acetoin sensitivity in fermentation broth, similarly as for acetoin determination in alcoholic beverages, ConCap measurements were performed with different spiked acetoin concentrations between 1 and 500×10^{-6} M (see Figure 4c). Again, the biosensor showed distinct steps for varying acetoin concentrations. The corresponding calibration curve can be seen inset in Figure 4c, resulting in an acetoin sensitivity of 32 mV dec^{-1} in the concentration range from 10 to 90×10^{-6} M. The signal shifts for the different acetoin

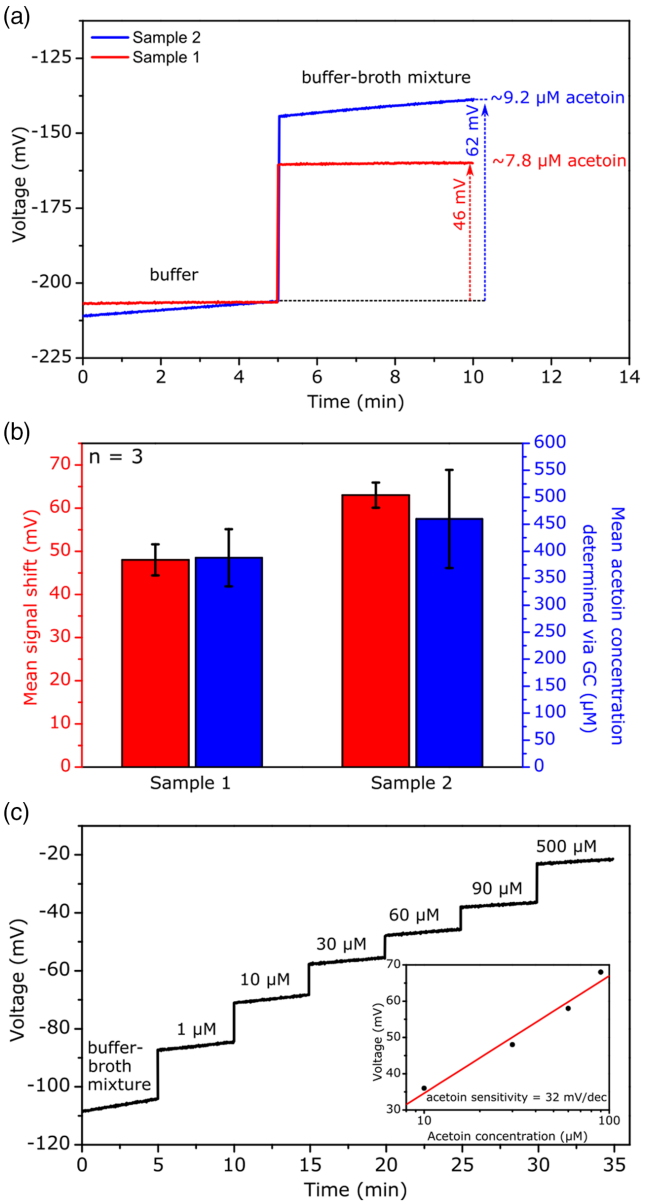

Figure 4. a) ConCap curves recorded in buffer solution (0.2×10^{-3} M tris-HCl + 150×10^{-3} M NaCl + 500×10^{-6} M NADH, pH 7.1) and fermentation broth–buffer mixture with a dilution ratio of 1:50 from two different samples (sample 1 (red) and sample 2 (blue)). b) Mean signal shift from three individual biosensors measured in diluted samples (1:50) from two different fermenters (sample 1 and sample 2) and corresponding acetoin concentrations determined via gas chromatography. c) ConCap curve recorded in fermentation broth–buffer mixture (1:50) spiked with different acetoin concentrations between 1 and 500×10^{-6} M. The inset is showing the corresponding calibration curve in the acetoin concentration range from 10 to 90×10^{-6} M.

concentrations are fully comparable to those obtained in beer and red wine samples.

The rather complex fermentation broth was not filtrated before electrochemical acetoin measurement, i.e., all ingredients, including the proteases remaining in the solution, being examined. Thus, the results also underline the high potential of the acetoin biosensor for applications in industrial fermentation processes such as the production of industrial enzymes for application in laundry detergents. In addition, in comparison to measurements with the GC, the biosensor offers a simple on-line measurement tool without any time-consuming and error-prone sample preparation.

4. Conclusion

Acetoin detection is still a current topic in analytical sciences. One possible approach for on-line measurements of acetoin is represented by recent biosensors. In this work, a novel acetoin biosensor was investigated for its storage stability and applied for acetoin measurements in beer, red wine, and fermentation broth samples. The sensors showed a storage stability of at least 21 days and retained ≈74% of their initial acetoin sensitivity. The acetoin biosensor was systematically examined in real samples and showed repeatable measurement results: In beer samples, the sensor exhibited a mean acetoin sensitivity of 29 mV dec^{-1} and in diluted red wine 32 mV dec^{-1}, both in the linear concentration range from 10 to 90×10^{-6} M. In fermentation broths, the biosensor was able to detect different concentrations of acetoin, which was naturally formed during the fermentation process. Spiked acetoin concentrations from 1 to 500×10^{-6} M could be detected: The average acetoin sensitivity in the linear concentration range (10–90×10^{-6} M) amounted to 32 mV dec^{-1}, again.

The reproducible results achieved in different sample types demonstrates the high potential of the developed biosensor for application in various industrial processes, such as production of alcoholic beverages or enzymes for laundry detergents. Future studies will mainly focus on further stabilizing the enzyme activity in the long term by variation of the immobilization procedure, where one successful strategy might be given by utilizing *tobacco mosaic virus* (TMV) particles as enzyme nanocarriers. The application of TMV particles could lead to better long-term stability of the biosensor and a broader linear detection range, due to high-density enzyme immobilization of the acetoin reductase on the surface of the TMV nanotubes and the resulting optimized diffusion conditions for substrates to the active centers of the enzymes (see, e.g., Poghossian et al.,[33] Bäcker et al.,[41] and Koch et al.[42]). Moreover, the sample-taking procedure should be further adapted to the biosensor setup to minimize disturbing factors such as foam formation during measurement. Future experiments also plan to have access to real beer and wine samples from different stages of the fermentation (brewing) process.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

Keywords

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