Evaluation of an optimized enzymatic biosensor for ethanol used in apple storage management with low oxygen stress

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Abstract

Ethanol has been proposed to be one of the target molecules to monitor the dynamic controlled atmosphere (DCA) technique during apple storage, measured in the squeezed juice or in the air of the storage chamber. One of the proposed commercial sensors for ethanol in apple juice is based on amperometry, after a two-step enzyme-based reaction that involves a diaphorase and an alcohol-dehydrogenase. Even though this method has been reported to overestimate ethanol, this difference is fairly fixed and it is industrially used to check the correct application of the treatment and to set the gas composition protocols when the maximum acceptable ethanol is reached. During the 2018 harvest, the ethanol concentration in juices measured with the commercial sensor appeared much higher than those usually reported in precedent years, particularly for the lower concentrations. Laboratory experiments suggested that differences between years could be due to the presence of a secondary enzyme activity present in the commercial diaphorase employed. In order to increase the sensitivity and accuracy, it has been evaluated the performance of the biosensor emploting a further diaphorase. The performances of both sensors were compared with those obtained with a gaschromatophy mass spectrometry approach after head space extraction (HS-GC-MS) in which the mass spectra was acquired in selected-ion monitoring mode. Samples belonging to 'Red Delicious' cv. were picked up at different temporary points from industrial storage rooms following the application of low oxygen stress. The new biosensor reduced 97% the mean difference respect to the values obtained with the GC-MS method. The difference between sensors was even clearer for samples with concentrations up to 100 mg/L, that could be used as a discriminating value for the evaluation of the technique success in 'Red Delicious' apple juice. The increased sensitivity of the sensor allowed a more accurate monitoring of the DCA at industrial conditions, limiting the risks linked to a false positive on the monitoring during storage.

Keywords: Low oxygen stress, dynamic controlled atmosphere, apple scald, physiological disorders, Red Delicious

Introduction

The apple market shows currently an increasing production trend and a stable or even decreasing demand in Europe (https://ec.europa.eu). To meet the customer demands, it is necessary to extend in time the supply of fruits with the absence of any defect for as long as possible. For

some cultivars, nowadays this is possible with the control of the storage conditions and precisely, with the application of low levels of oxygen. This conservation technique for apple storage is used to limit ethylene biosynthesis and respiration rate that results in controlling the appearance of some physiological disorders as superficial scald (Zanella, 2003; Lurie and Watkins, 2012). This technique is based on the reduction in the storage room of the concentration of oxygen at concentrations between 0.4% -1.0%, maintaining the level of CO_2 at values of 0.8-0.9% thus promoting fermentation that result in the production of ethanol, which even at low concentration, can reduce the appearance of superficial scald (Scott et al., 1995). However, it also entails certain risks as tissue damages or the developing of off-flavours (Wright et al., 2015). The application of initial or repeated low oxygen stress (ILOS and RLOS respectively) during storage has been effectively applied for the long storage of some cultivars (Mditshwa et al., 2018; Fadanelli et al., 2013; Zanella, 2003). It is however necessary to periodically raise the oxygen concentration of the room up to 0.9% -1.1% to favour the absorption of the ethanol produced. The basis of these dynamically control atmosphere treatments (DCA) is that a certain level of the ethanol biochemically produced under this conditions is beneficial for fruit quality (Zanella & Struz, 2012).

This technique permits to both slow down the decay of quality during storage and after shelf life and to guarantee the containment of superficial scald (Fadanelli et al., 2009), that in severe cases can lead to the loss of the entire production. In order to avoid the formation of anomalous tastes, it is necessary however to control the extent of the low oxygen conditions. To control the process, it can be monitored the production of ethanol. Depending on the cultivar, ethanol values should remain within certain minimum and maximum values to ensure the success of the technique (Fadanelli et al, 2013).

The RLOS has been applied for many years in Trentino (Italy) for Red Delicious storage. The monitoring of ethanol has been always performed enzymatically in apple juice with a biosensor based on the activity of a diaphorase and subsequently an alcohol dehydrogenase. During the 2018 harvest, ethanol concentration appeared much higher than values usually reported in previous years during DCA monitoring of commercial storage chambers, especially for lower contents. This was critical to evaluate the correct application of the RLOS, and in particular, for varieties with an intrinsic low ethanol production. Lab experiments suggested that the high values found would be the result of secondary enzyme activities present in the production lot employed for the commercial biosensor.

In order to increase the accuracy and the sensitivity, it has been evaluated the performance of a new industrial diaphorase. Values of ethanol measured with both industrial enzyme kits were compared to those obtained with a gas chromatography-mass spectrometry (GC-MS) approach after head space extraction.

Material and methods

Between October and December 2018, samples of Red Delicious *cv*. have been used to evaluate the analytical performances of the enzyme biosensors (Senzytec, Tectronik, Italy) for ethanol measurement in apple juice, as proposed by Fadanelli et al. (2013).

123 samples from industrial storage rooms were analysed with the commercial biosensor, coming from the Trentino production area (Italy) in orchards located at variable altitudes (250-900 meters above sea level) of the Non and the Adige valleys. Further 55 samples of the same production areas were tested with the new biosensor in which a different diaphorase was used. The samples were representative of the production of the storage room. The industrial conservation differed by the storage conditions (temperature: 0.8-1.2°C, relative humidity: >95%; CO_2: 0.8-0.9% v/v; O_2:0.4-1.0% v/v) and treatment with 1-MCP (treated, n=65; untreated, n=113). Each sample was composed by ten apples, squeezed with the use of a manual press. Juices were analysed with the corresponding enzyme biosensor within one hour, then analysed with the HS-GC-MS approach, after microbiology stabilization (Na3N; 100 mg/L).

Headspace analysis (HS-GC-MS) of ethanol in apple juice was carried out using an Agilent Intuvo 9000 fast GC system coupled with an Agilent 7000 Series Triple Quadrupole MS. The GC autosampler (PAL RSI 85 systems, CTC Analytics AG, Zwingen, Switzerland) was equipped with a 2.5 ml gas-tight headspace syringe and a syringe heater.

For the analysis, 9 mL of apple juice and 3.5 g of NaCl were placed into a 20 mL glass vial with a teflon-coated septum. The vial was then vortexed vigorously for 3 min. The quantification was performed spiking a blank sample with an ethanol standard solution (10 g/L in water) at four concentration levels (10, 50, 100, 500 mg/L).

The samples and the calibration curve were placed in the sample tray of the autosampler and were analysed by means of the headspace procedure. Automated headspace sampling conditions were as follows: the vial was conditioned for 5 min at an incubation temperature of 40 °C with constant stirring at 250 rpm. The temperature of the syringe heater was set to 100 °C and the syringe was flushed with He before and after each extraction. Headspace (150 µl) was sampled at 12 mL/min and injected in split mode (1:20) into a DB-Wax Ultra Inert capillary column (20 m, 0.18 mm id × 0.18 µ m film thickness) with He as carrier gas (at a flow of 0.8 mL/min). The oven temperature was programmed starting at 35°C for 2.5 minutes, raised to 150°C by 30°C/min, and finally raised to 240°C by 35°C/min. The injector and transfer line were set both at 250°C, while source temperature was set at 230°C.

Mass spectrometer was equipped with an electron impact ionization source (70 eV, 50 µA) and the acquisition was performed in selected-ion monitoring mode (SIM) by recording the

abundance of the ions m/z 45 $[M]^+$ and m/z 46 $[M]^+$. Dwell time was set at 20 ms and solvent delay was set for 0.5 min.

Data acquisition and analysis were performed using the MassHunter Workstation software supplied by the manufacturer.

Statistical analysis was performed with Rstudio 4.0 (RStudio, MA; USA)

RESULTS AND DISCUSSION

The distribution parameters of ethanol determined with the HS-GC-MS approach are represented in figure 1, split by the dataset used in the commercial (n=132) and the new (n=55) ethanol biosensor evaluation. The analysis of the distribution parameters (Mann-Whitney U-test; p=0.94) confirms that the datasets employed for the evaluation of the two enzyme pools can be considered comparable, thus allowing the confrontation of the results of the two enzyme kits.

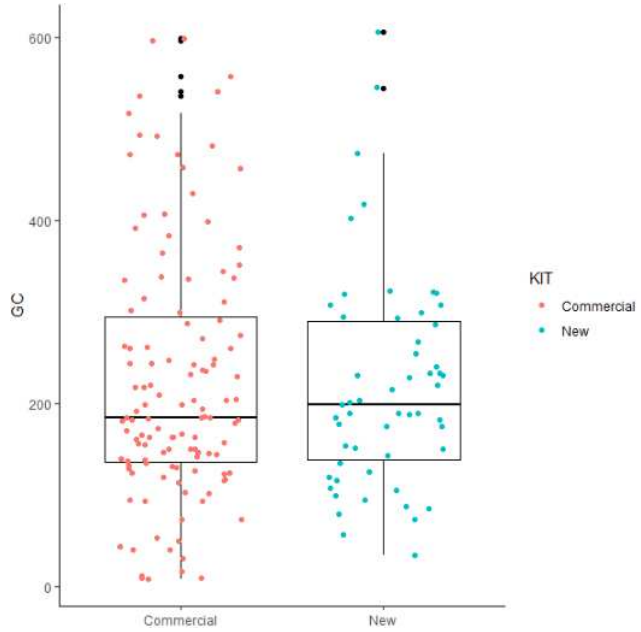

Table 1. Distribution of the ethanol concentration (mg/L) measured in apple juice samples with the HS-GC-MS method of the commercial (n=123) and the new (n=55) biosensor datasets.

The ethanol concentration measured for the two datasets is shown in figure 2, comparing results against the reference method. It can be observed that the responses of the enzymatic approach fits linearly for both with a good regression coefficient (>0.80), confirming the results of Fadanelli et al., (2013) that reported the use of ethanol concentration in the squeezed apple juice for

the monitoring of the success of the DCA. The linear regression shows however that both biosensors present a different sensitivity depending on the diaphorase used and the intercept of the commercial and new biosensor were substantially different: -140 mg/Land 15 mg/L respectively. Previous studies have reported the overestimation of ethanol with the biosensor (Fadanelli et al., 2013). Authors concluded however, that for the operational control of the success of the DCA, the advantages in terms of cost and simplicity of the enzymatic analysis could overcome the lack of sensitivity as far as differences between methods appear to be rather constant and the accuracy of the enzymatic sensor was good enough. Nevertheless, with the commercial biosensor, low values of ethanol with the HS-GC-MS approach correspond to concentrations always over 100 mg/L. This is critical for the monitoring the adequateness of the DCA application and to set the following oxygen stress in RLOS. Therefore, low values of ethanol with the commercial biosensor could be considered as a false positive. The application of the new biosensor has permitted to enhance the precision of the method and to reduce the risk of false positives.

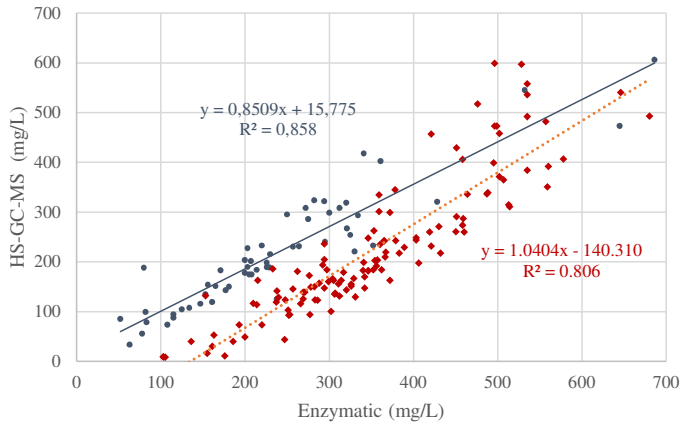

Figure 1. Comparison of the ethanol concentration with the commercial (♦) or new (●) biosensor and the HS-GC-MS reference method.

To verify the accuracy of the method, it has been calculated the difference of the concentration obtained for every sample of each database between the enzymatic analysis and the reference HS-GC-MS method. The calculated value is shown in figure 3, sort by the ethanol concentration obtained with the HS-GC-MS method thus, the higher the sample number, the higher the concentration of ethanol. Positive values indicate an ethanol overestimation of the biosensor and, vice versa, negative values an underestimation. Both sensors tend to overestimate ethanol in apple juice samples even though, the tendency is less clear with the new biosensor that underestimated ethanol in 27% of the samples, mainly at high ethanol concentration. Meanly, the overestimation determined by the commercial is 126±61 mg/L and it was differentiated (Mann-

Whitney U-test; $p < 0.05$) respect to the new pool of enzymes, for which the average concentration respect to the reference method was 19±48 mg/L (n=55). Reliable values of low concentrations has been reported to be crucial for a good management of the RLOS technique (Fadanelli et al., 2013).

For both datasets the difference between analytical methods applied tend to be rather constant, confirming the considerations of Fadanelli et al. (2013), regarding the applicability of this technique on monitoring the ethanol concentration in the squeezed juice. However, "high" ethanol concentration (approximately over 320 mg/L with the HS-GC-MS method in both datasets), the tendency change, possibly indicating a saturation of the enzymatic pathway.

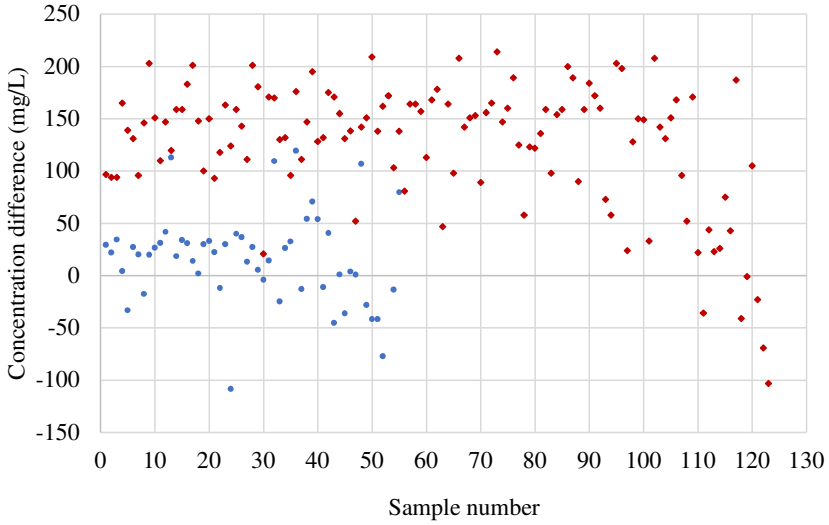

Figure 3. Difference between the concentration of ethanol determined with the comercial (♦) or the new (●) biosensor and the HS-GC-MS method, sorted by the HS-GC-MS sample concentration.

Conclusions

The results obtained for the biosensor tested with the new diaphorase has permitted to enhance the accuracy respect to the former, noticeable at low concentrations. This is particularly important for the management of those varieties producing low quantities of ethanol during DCA. Besides, reliable values are needed for the correct management of further stress periods with RLOS technique. For high ethanol concentration, both biosensors show a negative trend of the difference with the HS-GC-MS approach, suggesting a saturation of the enzymatic pathways that lead to a reduced signal.

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