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RESEARCH PAPER

Further development on DMFC device used for analytical purpose: real applications in the pharmaceutical field and possible in biological fluids

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Abstract The analytical research devoted to the utilization of the direct methanol fuel cell (DMFC) for analytical purposes has been continued. The research reported in this paper concerns two points, one of which was the possibility of improving the features, from the analytical point of view, of a catalytic fuel cell for methanol and ethanol, by introducing an enzyme, immobilized into a dialysis membrane small bag, in the anodic area of the fuel cell. This objective has been fully achieved, particularly using the enzyme alcohol dehydrogenase, which has increased the sensitivity of the method and reduced dramatically the response time of the cell. The second point concerned the opportunity to determine two particular antibiotics having an alcohol functional group in their molecule, that is, imipenem and chloramphenicol. Also, this goal has been reached, even if the sensitivity of the method is not so high.

Keywords Direct catalytic fuel cell · Immobilized enzyme · Improvement performances · Imipenem and chloramphenicol determination

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Introduction

Recently, some publications [1–4] described the possibility to use a catalytic direct methanol fuel cell (DMFC) as analytical tool for methanol analysis, but the effective application in real matrices was poor.

Also, our research group developed a new analytical method for methanol and ethanol determination, using a commercial DMFC device (originally constructed for the purpose of obtaining energy from methanol or ethanol) for analytical purposes [5]. In addition, the response of the cell to other types of alcohols different from methanol and ethanol was also evaluated. In the present paper, it was experimentally established that, although the sensitivity to other more complex alcoholic molecules is not high, it is still sufficient to use the fuel cell for the determination of particular types of alcoholic compounds. the interest to apply this device to the analysis of specific real samples, for instance pharmaceutical compounds, containing an alcoholic functional group is so accrued. In the first research [5], we demonstrated that, using a small commercial and inexpensive DMFC device, it was possible to check the ethanol content of several alcoholic beverages [6, 7], in a very similar way to what it is possible to do it using for instance a common enzymatic biosensor [5, 6]. The more recent step of the research concerning analytical applications in real samples, reported in the present paper, was to apply the same fuel cell for the determination of species of pharmaceutical interest, i.e., particular antibiotics, containing an alcohol functional group, such as imipenem (a β-lactam antibiotic), or chloramphenicol, another type of not β-lactam antibiotic.

As the only inconvenience that was already observed in a previous paper [5], comparing for analytical purpose of the DMFC device to the ordinary enzymatic sensors, was the measurement time (two or three more times longer, using the fuel cell, than that it is necessary if an ordinary enzymatic

sensor is used), in the present research, we tried to reduce the measurement time, shortening the response time of the fuel cell, by associating a suitable enzyme to the catalytic fuel cell, which contributes to accelerate the oxidative process taking place in the fuel cell. In fact, although the global reactions that take place in the catalytic fuel cell to the methanol or ethanol placed in the anodic area of the cell are well known:

$$CH_3OH + H_2O \rightarrow 6\,H^+ + 6\,e^- + CO_2$$

$$C_2H_5OH + 3H_2O \rightarrow 12H^+ + 12\,e^- + 2CO_2$$

really, the reaction mechanism is more complex and it consists of several enzymatic steps [8–10], the first of which takes place the oxidation of alcohols to aldehydes; therefore, it is in these first steps that is likely that an oxidase or alcohol dehydrogenase enzyme can produce its catalytic effect, accelerating the course of the total oxidation reaction process, which occurs in the fuel cell.

Experimental

Materials

The hydroalcoholic solutions were obtained by diluting with distilled and deionized water-fixed volumes of ethanol (CAS: 64-17-5), 96 % purity, supplied by Sigma-Aldrich (Milan, Italy). The enzymes used for measurements with the "fuel cell" were the alcohol dehydrogenase (from *Saccharomyces cerevisiae* E.C.1.1.1.1, CAS: 9031-72-5), the catalase (from bovine liver E.C. 1.11.1.6, CAS:9001-05-2), the alcohol oxidase (from *Candida boidinii* E.C. 1.1.3.13, CAS:9073-63-6) and the aldehyde dehydrogenase (from *S. cerevisiae*, E.C. 1.2.1.3, CAS:9028-88-0), all supplied by Sigma-Aldrich (Milan, Italy).

The standard antibiotics solutions, used in the measurement by fuel cell, were obtained by diluting with distilled water, known weight of imipenem (CAS: 74431-23-5) (see Fig. 1), purchased from VWR International (Milan, Italy) and chloramphenicol (CAS: 56-75-7) (see Fig. 2), supplied by Sigma Aldrich (Milan, Italy).

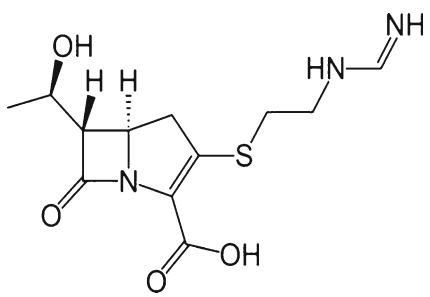

Fig. 1 Imipenem

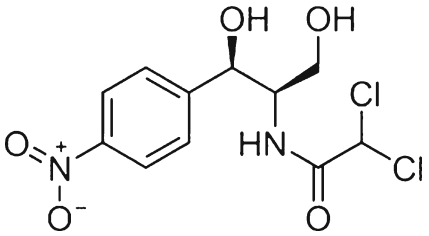

Fig. 2 Chloramphenicol

Fuel cell apparatus

For the fuel cell measures, a DMFC H-TEC Model F111 (Fig. 3), weighing about 100 g, was obtained from the Fuel Cell Store (College Station, TX, USA). The electrode area was about 4 cm^2 and maximum generated power 10 mW. The fuel cell frame was made in Plexiglas®, while the electrode end plate was made in Pt-Ru black catalyst assembled with Nafion™ membrane. For potentiostatic format measurement, Palmsens mod. EmStat potentiostat was used; connected on fuel cell, the current supplied to the cell was recorded and collected with data interface by PSTrace Software ver. 4.6 to Compaq Presario PC.

Fuel cell measurement and calibration curves for methanol and ethanol

Using the fuel cell working in potentiostatic format mode [1, 4, 5, 11], the supplied current (SC) through the cell was measured. The potentiostat Palmsens mod. EmStat was used, connected to a PC with PSTrace ver. 4.6. Software, for acquisition and data processing. The fuel cell anode was connected to EmStat as a working electrode, while the fuel cell cathode was connected to EmStat as the reference and counter electrode. Before the current measurement, the EmStat automatically measured the open circuit voltage (OCV) [11, 12] value for a time of about 200 s, and then the anode potential was set to a value of the optimized applied potential (OAP) [3, 4, 11], experimentally established in a previous paper [5] (i.e., OCV minus 100 mV). In all cases, the fuel cell before the measurement was carefully washed with 0.5 % water-ethanol (or methanol) solution and then several times with distilled water. Subsequently, the fuel cell was filled with the solution to be analyzed (2 mL) and closed to prevent evaporation of the alcohol, and then the measurement can begin after conditioning the system for about 60 s. The fabrication of a calibration curve was carried out using water-alcohol solutions containing increasing percentages of methanol, or ethanol, added time by time to the fuel cell, and then the SC by the cell was each time recorded [3–5, 11] (see Electronic Supplementary Material (ESM, Fig. S1)); the current supplied after 60 min, when it had reached a stationary state value, was lastly read time by time. The current variations thus obtained have been reported as a function of the increasing concentration of the

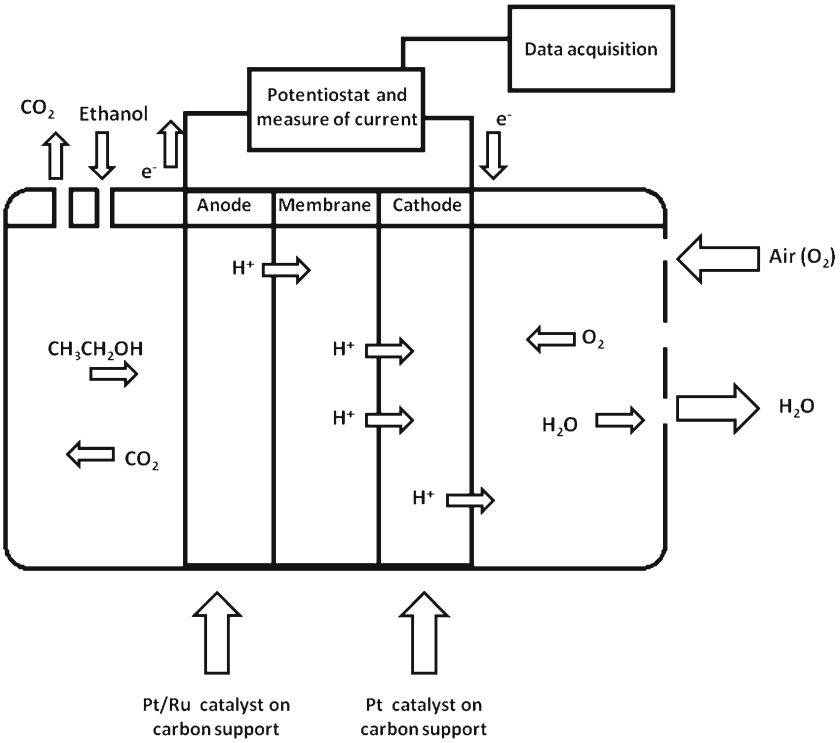

Fig. 3 Scheme of direct methanol fuel cell

tested alcohol. The straight-line equations for methanol and ethanol (so obtained) were found in a previous paper [5], but for the safety of reader, the equation data have been shortly collected also in the Table 1. In the same table, the R^2 values of the straight lines were reported, and the main analytical data of the calibration curves for methanol and ethanol, thus obtained, have also been summarized. Lastly in the ESM (Fig. S2), selectivity data to several more complex alcohols are shown as histograms.

For the measurements in the presence of each of the tested enzymes, a weighed quantity of the enzyme (for instance 5 mg of alcohol dehydrogenase) was placed on a very small dialysis membrane cylindrical bag, together with a drop of phosphate buffer. After positioning cautiously into the dialysis membrane bag a rigid plastic stick, a sort of cylindrical stiff bag is so obtained, which was sealed at the top, inside of which was contained the mush of the enzyme. The bag was placed into the anode area of the fuel cell (see Fig. 4) before the measurement. The successive measurement format was then the same as described above for the non-enzymatic fuel cell.

Results and discussion

Application of not enzymatic fuel cell to check antibiotics with an alcoholic functional group

The amperometric format for the fuel cell measurements was optimized in previous paper [5], in which also all problems concerning the cross-over [1, 3, 5],

Table 1 Main analytical data, for methanol and ethanol determination, using the DMFC

Method	Linearity range Slope value Correlation coefficient Pooled SD %		LOD (M)		Time of measurement	
	Methanol	Ethanol	Methanol	Ethanol	Methanol	Ethanol
Fuel cell SC potentiostatic format at (OAP)	$(1.0 \times 10^{-3}$–$2.0 \times 10^{-1})$ 21.8 (±0.78) $R^2 = 0.9912$ Pooled SD % = 7.2	$(1.0 \times 10^{-3}$–$4.0 \times 10^{-2})$ 17.8 (±0.95) $R^2 = 0.9888$ Pooled SD % = 6.8	8.0×10^{-4}	8.0×10^{-4}	~60 min	~60 min

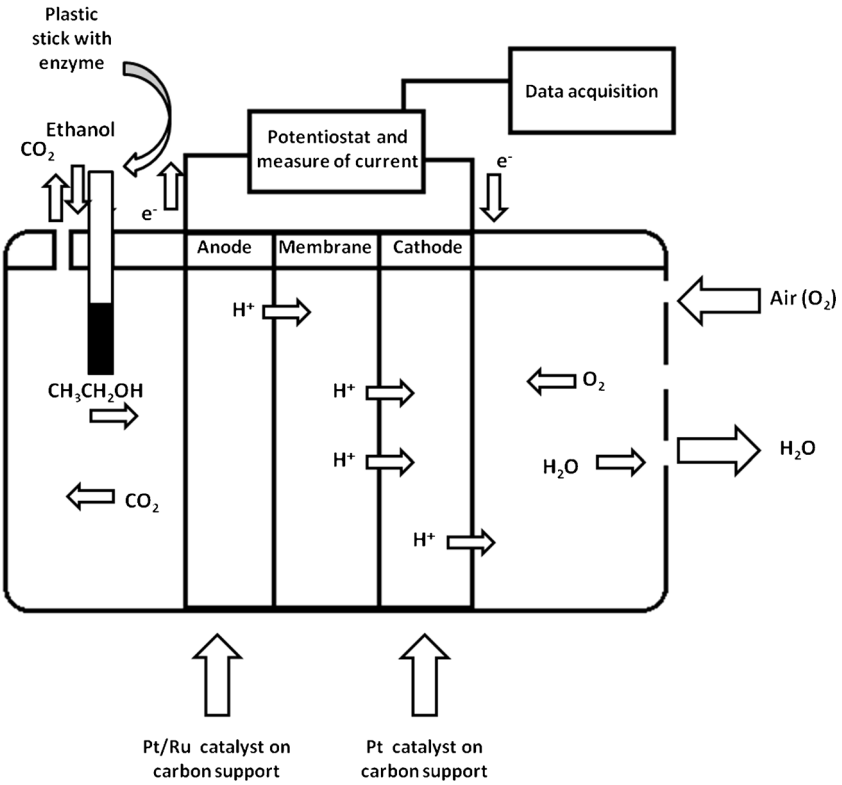

Fig. 4 Scheme of enzymatic DMFC

thermostatation [8, 9], and life time significance [5, 6] joint to the used fuel cell, were explored and discussed.

After fuel cell was analytically characterized and its measurement format pointed out, the fuel cell was applied first of all to check the ethanol in several alcoholic beverages [5], then to determine more complex organic molecules containing an alcohol functional group, in particular two antibiotics, containing in their structural formula an alcoholic functional group, i.e., imipenem and chloramphenicol (Figs. 1 and 2).

Also in this case, as described in the previous section, measures were made at different concentrations of imipenem or chloramphenicol in the fuel cell, recording the current supplied, using the same format employed for methanol or ethanol determination, described in "Fuel cell measurement and calibration curves for methanol and ethanol." The current variation values, read at the steady state, in this case after 90 min, for two tested antibiotics and their trends, have been respectively shown in the ESM (Figs. S3 and S4), while calibration curves for chloramphenicol and imipenem, obtained plotting the data of the recorded current variation of the steady state vs the antibiotic concentration in a semilogarithmic scale, were displayed in Figs. 5 and 6; however, the main analytical data, i.e., straight-line equations, R^2 values, LOD, and the linearity ranges, for these two antibiotics, are collected in Table 2. Lastly, examples of the current trend of recorded current variation, as a function of the time for several aqueous solutions, at different concentrations of two antibiotics, thus obtained, have been shown in Fig. 7a, b. The cause of the logarithmic trend response of the catalytic cell to these two antibiotics can be primarily identified, in the cross-over effect of the analyte (and most probably of smaller oxidation products derived from it), which has been demonstrated can begins to manifest particularly at higher concentrations if the analyte was ethanol or methanol, as it was discussed in our previous work [5] and was explained in papers of other authors reported in

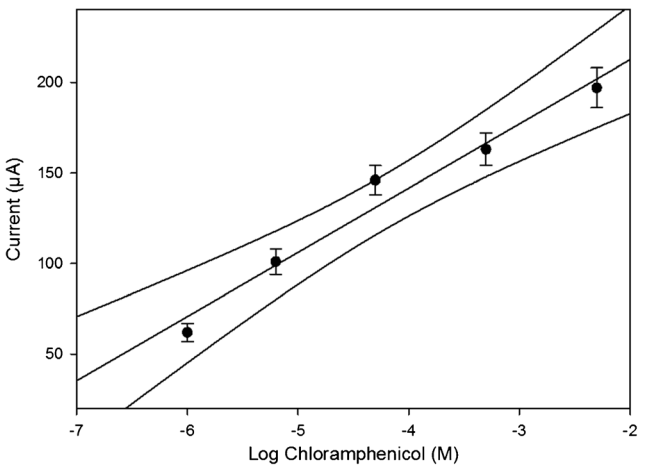

Fig. 5 Calibration curve for chloramphenicol. SC vs chloramphenicol concentration. Potentiostatic format at OAP

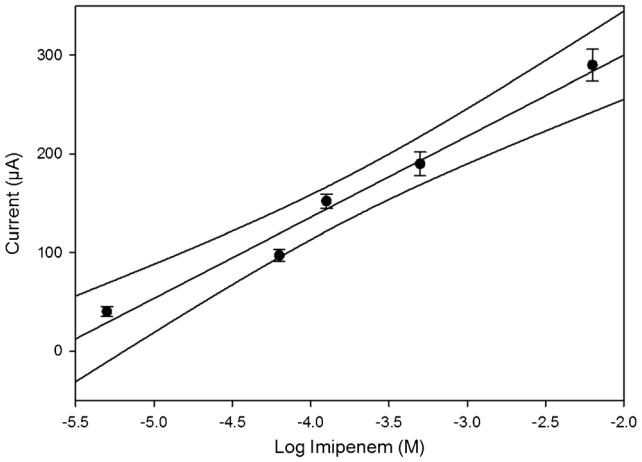

Fig. 6 Calibration curve for imipenem. SC vs imipenem concentration. Potentiostatic format at OAP

the literature [1, 3, 4]. It is clear that the cross-over is also dependent from the analyte under examination. In the case of antibiotics considered evidently, it seems that this effect already begins to appear at quite low concentrations, thus limiting the amplitude of the linearity range. However, another explanation should be that a contribution to the loss of linearity at more high concentrations, it may, at least in part, be due to the development of CO_2, which can begin to accumulate at the anode [5].

Data reported in Table 2 demonstrated that, using fuel cell method, it was possible to analyze these two particular type of antibiotics, one of which (imipenem) cannot be actually well analyzed through classic immunological, immunosensor, or SPR methods that are instead suitable for chloramphenicol [13–19], or other antibiotics [20–25], as its specific antibody is not still commercially available. The linearity range of fuel cell method for both these antibiotics, in semilogarithmic scale, is only about three decades, i.e., shorter if compared, for instance, to immunological [13–15] or HPLC [26–30] methods reported in the literature for chloramphenicol or other antibiotics [20, 21], but the LOD value (see Tab.2) is still sufficiently low, so that the method is suitable for antibiotics determinations also in food, drugs, and environmental real samples [20–25].

Improvement of fuel cell analytical performances, using different enzymes

The second research point stressed in the present research was to carry out several tests, by measuring again the current supplied by DMFC device vs ethanol increasing concentration, although also using enzymes, such as catalase, or alcohol oxidase, or alcohol dehydrogenase, or alcohol dehydrogenase plus aldehyde dehydrogenase (and NADH as cofactor in the latter cases), inserted inside the anodic section of the fuel cell and contained in the small dialysis bag, immersed in the ethanol hydroalcoholic solution (see Fig. 4) and checking if the abovementioned enzymes actually are able to shorten the response time of the device, speed up the ethanol breakdown process and increase the sensitivity of the device, and therefore to enhance the analytical performance of the fuel cell.

The response of the enzymatic cell, increasing ethanol concentration and the calibration straight lines for ethanol of the DMFC device containing time by time different enzymes, is displayed in Fig. 8, parts (a), (b), (c), and (d), respectively, while all the corresponding calibration equations and main analytical data are collected in Table 3. Finally, examples of polarization and power curves [31, 32] of the fuel cell, in the presence and absence of each of the tested enzymes, are showed in the ESM (Fig. S5 and S6, respectively).

For what concerns the measures in presence of each of the tested enzymes (after all, the idea of associating an enzyme to a fuel cell is not entirely new [33]), the comparison of the calibration curves equations for ethanol, in the absence and in the presence of one enzyme of those, reported in Table 3, shows that the sensitivity (as slope value of calibration curve) is always greater in the presence of one of the tested enzyme, instead of its absence

Table 2 Main analytical data for chloramphenicol and imipenem determinations, using DMFC. SC using potentiostatic format at OAP

Method: Fuel cell SC potentiostatic format at (OAP)	Analytical data		LOD (M)		Time of measurement	
	Chloramphenicol	Imipenem	Chloramphenicol	Imipenem	Chloramphenicol	Imipenem
Regression equation (Y = μA., X = M)	$Y = 35.3\ (\pm 4.5) \log X + 281\ (\pm 45)$	$Y = 37.6\ (\pm 3.3) \log X + 481\ (\pm 27)$	9.0×10^{-7}	5.0×10^{-6}	~90 min	~90 min
correlation coefficient	$R^2 = 0.9765$	$R^2 = 0.9820$				
Linear range (M)	$(1.0 \times 10^{-6}$–$5.0 \times 10^{-3})$	$(6.0 \times 10^{-6}$–$6.0 \times 10^{-3})$				
Pooled SD %	5.9	6.0				

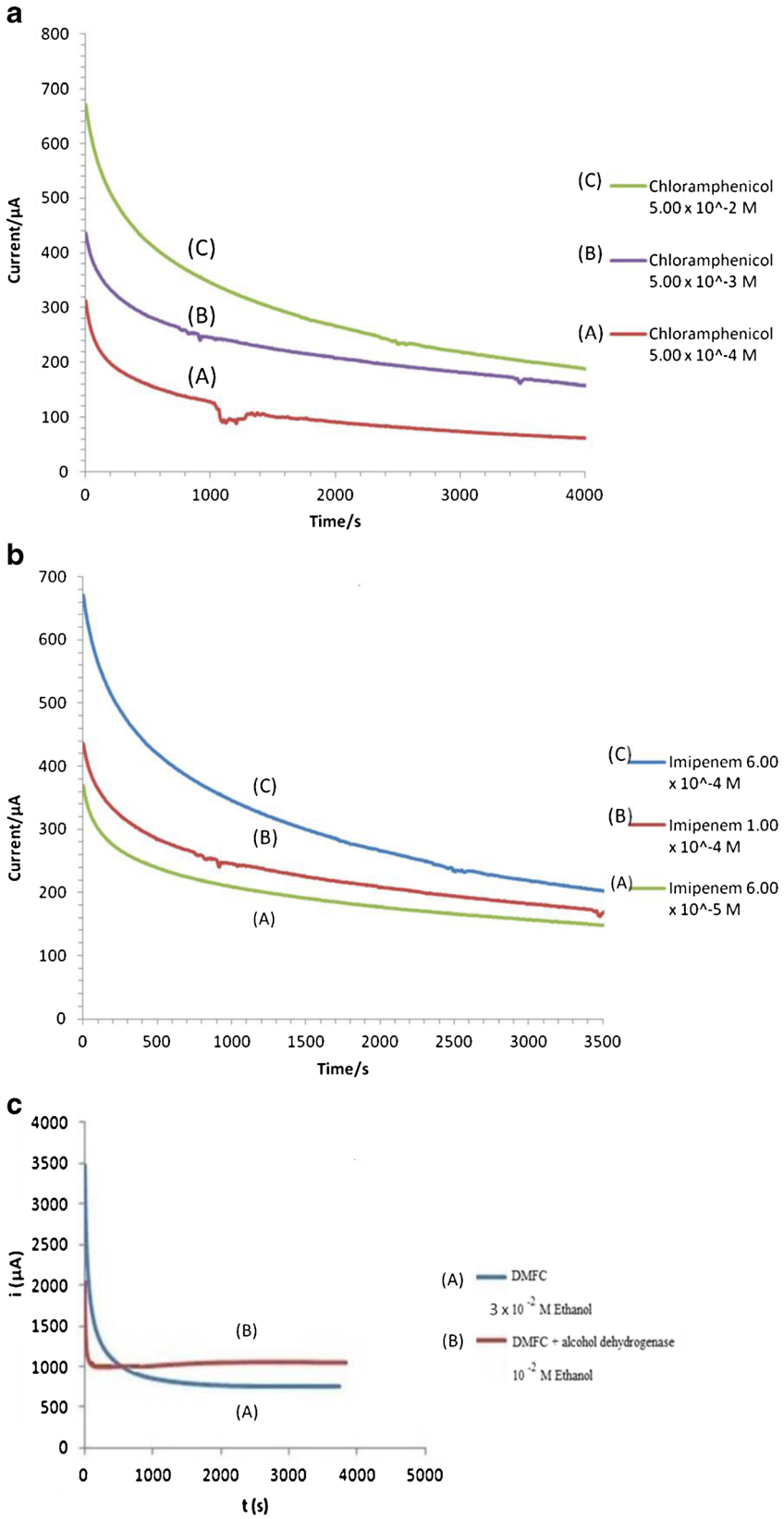

Fig. 7 Supplied current trend of the fuel cell vs time, using potentiostatic format polarized at OAP: **a** at different increasing chloramphenicol concentrations, **b** at different increasing imipenem concentrations, **c** for ethanol solutions (*A*) in the absence and (*B*) in the presence of alcohol dehydrogenase enzyme

and the sensitivity increases in the order: alcohol dehydrogenase plus aldehyde dehydrogenase ≈ alcohol dehydrogenase >> alcohol oxidase > catalase > fuel cell without the enzyme. This trend is also in full agreement

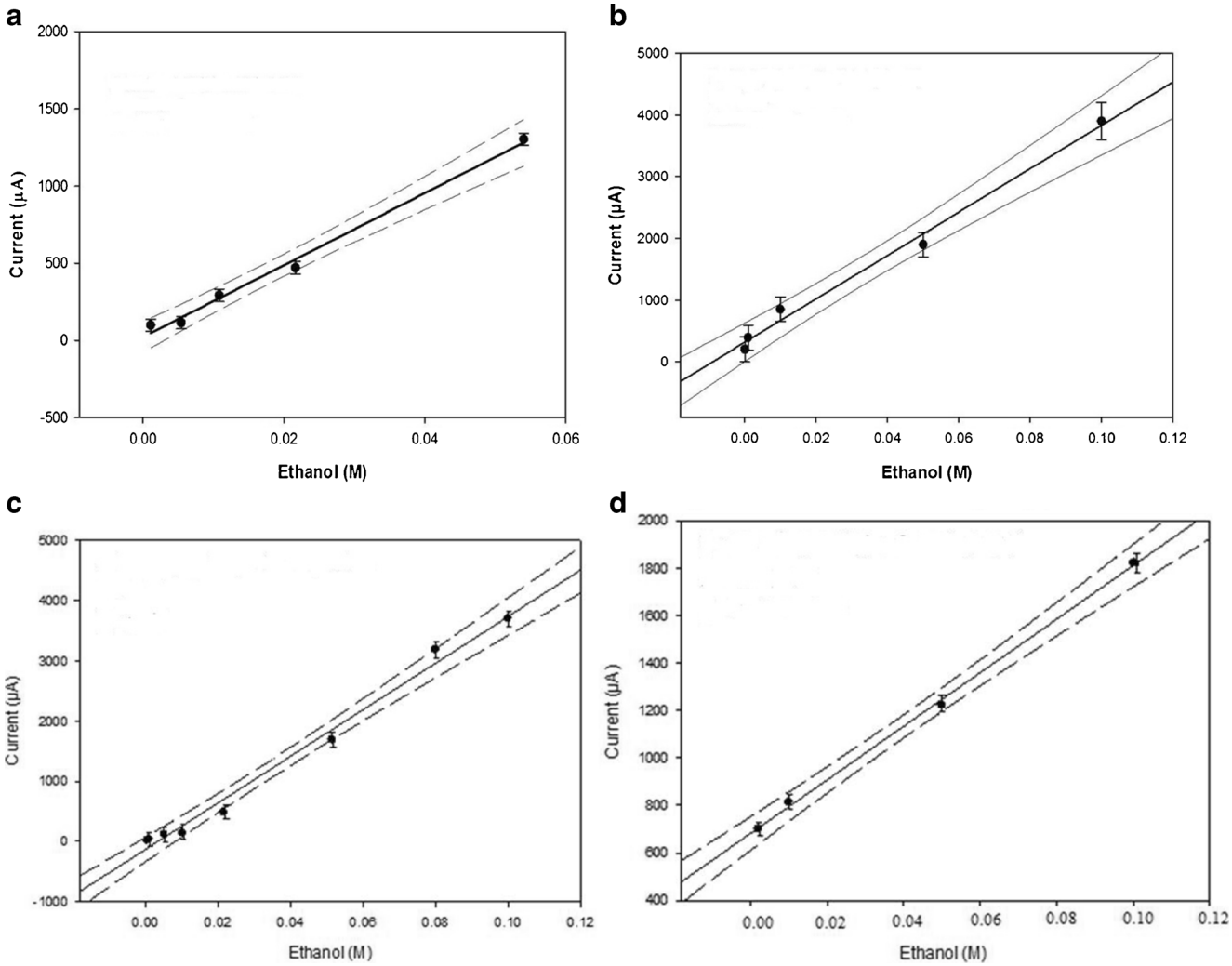

Fig. 8 Calibration curve for ethanol as SC vs. ethanol concentration: **a** in presence of catalase, **b** in presence of alcohol oxidase, **c** in presence of alcohol dehydrogenase, and **d** in presence of alcohol dehydrogenase and aldehyde dehydrogenase

with trends of polarization and power curves shown in the ESM (Figs S5 and S6).

Really, from the point of view of the sensitivity, the enzyme alcohol dehydrogenase, it is the most efficient. The aldehyde dehydrogenase addition in excess to the alcohol dehydrogenase improves the sensitivity, but negligibly. On the other hand, a certain quantity of the NADH is always present as impurity in the commercial alcohol dehydrogenase enzyme [34]. It is even more interesting also to observe how the presence of an enzyme drastically shortens the response time (see the example in Fig. 7c) and then reduces the measurement time of the fuel cell to only about 20 min, i.e., a time that is comparable (or sometimes even lower) to that of the ordinary enzymatic amperometric biosensors for ethanol [6, 35]. A more wide analytical comparison, between the conventional enzyme-biosensing methods and the present method based on DMFC device, has been carried out extensively in our previous work [5], in which it is shown as former methods have in general a linearity range shifted to the concentrations of at least two decades lower, compared to the second one, the same thing can be said for the LOD; nevertheless, it can be observed as the lifetime of the latter is extremely longer (more than two months) compared to the one of the first conventional methods [5]. It is clear moreover that referring for instance to the determination of ethanol in alcoholic beverages, it is well known that there are also good methods, but "outdated", such as that based on distillation [36] or, on the contrary, exist more recent methods based on gas [37, 38], or liquid (HPLC) [39, 40] chromatography, of course with excellent performances from the point of view of the LOD, selectivity, and other analytical features. But it is also clear that in this case, we can no longer speak of simple methods, inexpensive and fast enough, such as sensor or/and

Table 3 Comparison of main analytical data for ethanol analysis, by DMFC, using potentiostatic format, with (or without) different enzymes

Method	Equations of calibration curves for ethanol R^2 Linearity range (M)	LOD (M)	Response time (min)
Fuel cell (SC) potentiostatic format polarized at OAP	$Y = 17.77\ (\pm 0.95)\ X + 0.07\ (\pm 0.02)$ $R^2 = 0.\ 9888$ (Y = mA; X = M) $(1.0 \times 10^{-3}$–$4.0 \times 10^{-2})$ M	8.0×10^{-4}	~60
Catalase enzymatic Fuel cell (SC) potentiostatic format polarized at OAP	$Y = 23.33\ (\pm 3.04) X - 0.02\ (\pm 0.01)$ $R^2 = 0.9936$ (Y = mA; X = M) $(5.0 \times 10^{-4}$–$5.0 \times 10^{-2})$ M	5.0×10^{-4}	~20
Alcohol oxidase enzymatic Fuel cell (SC) potentiostatic format polarized at OAP	$Y = 35.04\ (\pm 2.60) X + 0.31\ (\pm 0.03)$ $R^2 = 0.9901$ (Y = mA; X = M) $(5.0 \times 10^{-4} - 1.0 \times 10^{-1})$ M	5.0×10^{-4}	~30
Alcohol dehydrogenase enzymatic Fuel cell (SC) potentiostatic format polarized at OAP	$Y = 38.71\ (\pm 1.68) X - 1.36\ (\pm 0.83)$ $R^2 = 0.9888$ (Y = mA; X = M) $(5.0 \times 10^{-4} - 6.0 \times 10^{-1})$ M	2.0×10^{-4}	~20
Alcohol dehydrogenase plus aldehyde dehydrogenase Fuel cell (SC) potentiostatic format polarized at OAP	$Y = 40.50\ (\pm 3.21)\ X + 0.25\ (\pm 0.04)$ $R^2 = 0.9940$ (Y = mA; X = M) $(2.0 \times 10^{-4} - 2.0 \times 10^{-1})$ M	5.0×10^{-4}	~20

biosensor methods [5–7, 41, 42], or like the method described in the present and in our previous work [5], instead of good methods, which however can be applied only in the laboratory and using not cheaper apparatus.

Conclusions

In conclusion in this research, using an enzymatic DMFC device, we have reached the main goal that we had prefixed, concerning the drastic reduction of the measurement time by the fuel cell used for analytical purposes, enhancing at the same time its sensitivity. Lastly, it has been demonstrated as the fuel cell can be useful to determine also other organic molecules, which contain an alcoholic function (although with a much lower sensitivity than methanol or ethanol) in real matrices, which do not contain high concentrations of possible alcohol interfering compounds. It is precisely the case for example of antibiotics contained in injectable solutions, checked in the present research. Lastly, owing the low cost, the very low encumbrance of the cell and measurement apparatus [5], the high sensitivity and short response time achieved by the enzyme alcohol dehydrogenase addition in the fuel cell, this small enzymatic DMFC can be also proposed as a device able to check the ethanol concentration in the test for the measurement of the alcoholic level in breath test for serum, saliva, and sweat analysis of drivers [41–43]; of course, further experimental research will be needed before it can be said that the latter application is actually possible.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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