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Ferrocene-Mediated Carbon Paste Electrode Modified with D-Fructose Dehydrogenase for Batch Mode Measurement of D-Fructose

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Abstract

A mediated modified carbon paste and renewable surface electrode for fructose amperometric measurement based on D-fructose dehydrogenase (FDH) was prepared and optimized. Commercially available ferrocene (FcH) and hydroxymethyl ferrocene ($FcCH_2OH$) were used as mediators. The substituted FcH showed better linearity and higher sensitivity. The influence of different experimental parameters was studied for optimum analytical performance. The final FDH-modified electrode showed good analytical performance for batch mode measurements of fructose.

Index Entries: Renewable surface electrode; modified carbon paste electrode; fructose dehydrogenase; fructose.

Introduction

The use of D-fructose dehydrogenases (FDHs) (EC 1.1.99.11) in conjunction with amperometric electrodes is certainly the most interesting method for D-fructose measurement. Studies of various biosensors for the detection and quantification of fructose have been published, but probably the most selective and rapid determination of fructose was obtained when membrane-bound FDH was used in the presence of mediators *(1–7)*. It was also reported that FDH might directly exchange its electrons with the electrode surface in the absence of mediators either in the solution or at the electrode surface *(8,9)*. Several types of material were explored for the

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design of enzyme-modified electrodes including solid graphite, platinum, gold, and carbon paste *(10–12)*. The use of carbon paste appears very attractive for biosensor fabrication and development *(13)*. Indeed, bulk-modified carbon paste offers the advantage of confinement of mediators, enzymes, and stabilizers into the carbon paste and quite often provides fast exchange rates at the surface of the electrode.

Although ferrocene (FcH) has been used often as mediator for the construction of biosensors, it is surprising to observe that it has been used only minimally in the case of biosensors for fructose measurement *(14,15)*. To our knowledge, no account of the assembly of FDH, FcH, and carbon paste is reported. We present in this article the first report on the preparation of biosensors based on carbon paste modified with the FDH enzyme and FcH itself or its derivatives as mediators.

Despite the extensive utilization of carbon paste in the construction of enzyme electrodes and the many investigations about the influence of various experimental parameters on the electrochemical response, the repeatability of the renewable surface electrode has barely been considered. A major goal of the present study was to present the analytical results for FDH-modified carbon paste electrode used in batch mode that implies the renewal of the electrode surface before each assay prior to the chronoamperometric measurement at 20 s *(16)*. The analytical performances of the FDH electrode were investigated for various experimental parameters such as mixing time and rates of enzyme, mediator, or pasting liquid. The long-term stability was also examined and discussed in the presence and absence of surfactant added into the carbon paste.

Materials and Methods

Reagents

FDH (EC 1.1.99.11) from *Gluconobacter* sp. from Toyobo-Japan (FCD-301) was used as received and without further purification. Bovine serum albumin (BSA) (cat. no. 63 309; Fluka), glutaraldehyde Grade II 25% aqueous solution (cat. no. G-6257; Sigma, St. Louis, MO), FcH and hydroxymethyl ferrocene ($FcCH_2OH$) (Stream Chemicals), graphite powder (cat. no. 16858; Carbone Lorraine), and paraffin oil (cat. no. 7161; Merck) were used as received. All solutions were prepared with 0.1 *M* phosphate buffer (pH 7.2) containing 0.1% Triton X-100 (Sigma).

Electrode Preparation

FDH-modified carbon paste electrode was prepared by dissolving FDH in a distilled water solution of BSA. The amount of BSA was fixed so that the final ratio in the carbon paste was 5%. FDH and BSA were crosslinked with a 0.25% solution of glutaraldehyde. The resulting mixture was stirred at room temperature for 10 min. The carbon powder was then added and the mixture was allowed to dry under vacuum. The modified

carbon paste was obtained by mixing in a mortar the resulting dried modified carbon powder with a given amount of paraffin oil in which the mediator had already been dissolved. The paste was compacted into an insulating cartridge to obtain the final electrode.

Procedure

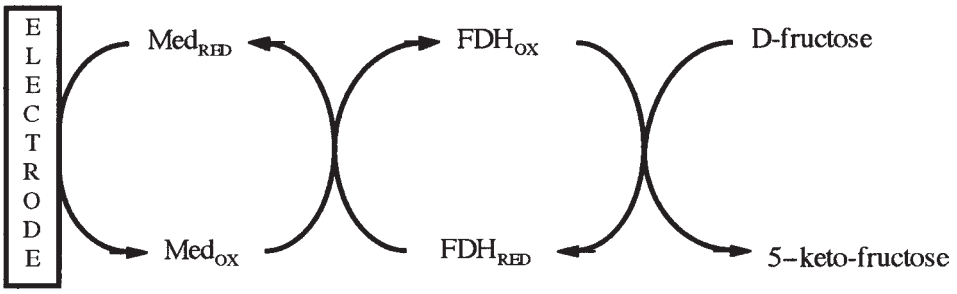
Cyclic voltammetry and chronoamperometric experiments were performed with an EG&G Instruments potentiostat (Model 273). A three-electrode configuration was used with the modified carbon paste as working electrode, a reference saturated calomel electrode (SCE), and a platinum wire acting as the auxiliary electrode.

All measurements concerning the study of analytical performances of the composition of the electrode were made by using the Multicapteur MC2 from Dosivit, France. The Multicapteur MC2 is a biosensor made up of an electronic unit (potentiostat) and a mechanical handle, which uses the enzyme-modified carbon paste cartridge as the working electrode and a concentric platinum quasi-reference electrode. The renewable surface is obtained before each measurement by automatically activating the piston of the handle. This piston is electronically controlled and pushes a known amount of enzyme-modified carbon paste out of the cartridge. A new electrode surface is obtained by polishing the end of the cartridge on the surface of clean paper. All measurements with an FDH-modified electrode were performed at a fixed potential of 0.7 V vs quasi-reference platinum electrode. This potential corresponds to 0.5 V vs SCE. The measurement time is automatically fixed and set up at 20 s. After each measurement, the reference electrode was cleaned and the surface of the working electrode was renewed. No change of the potential of the quasi-reference electrode was noted during the very short chronoamperometic experiment.

Results and Discussion

The electroenzymatic oxidation of D-fructose by FDH mediated by ferricinium cation (FcH^+) mainly involves three consecutive steps to generate the catalytic current. As illustrated in Scheme 1, fructose is detected and oxidized at the electrode surface via FDH. FDH_{ox} and FDH_{red} are the oxidized and reduced forms of the enzyme, respectively. The electrooxidation cycle of fructose can be achieved by electrochemical regeneration at the electrode surface of the mediator that continuously provides the enzymatically active form of FDH.

Initial experiments were performed with FDH-modified carbon paste electrode containing 2 U/mg of enzyme and 0.15% FcH as mediator. The electrochemical behavior of such an electrode was examined by cyclic voltammetry. Figure 1 displays typical voltammograms obtained at a scan rate of 2 mV/s of the FDH-FcH-modified carbon paste electrode in the absence and presence of fructose in the buffer solution (0.1 *M* phosphate, pH 7.2). In the absence of fructose, the voltammogram shows the classical



Scheme 1. Fructose oxidation at a carbon paste electrode modified with FDH and mediator.

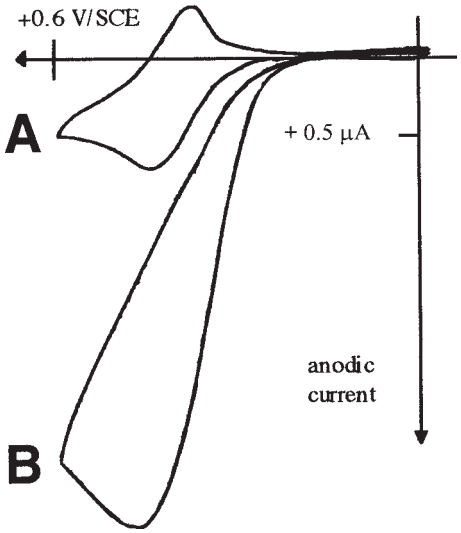


Fig. 1. Cyclic voltammograms of FDH-modified carbon paste electrode (2 U of FDH/mg, 0.12% FcH, and 23.66% paraffin oil) in absence **(A)** and presence **(B)** of D-fructose (30 m*M*). Scan rate: 2 mV/s, 0.1 *M* phosphate buffer, pH 7.2.

pair of anodic and cathodic peaks consistent with the reversible electrochemical behavior of FcH. The addition of fructose in buffer solution shows an increase in the anodic current representative of the catalytic oxidation of fructose by FDH via FcH at the electrode surface.

The electrochemical response of the FDH electrode was also examined using the chronoamperometric technique. Figure 2 illustrates typical chronoamperometric curves recorded in the absence and presence of various amounts of fructose. The potential of the working electrode was set up at 0.5 V vs SCE (equivalent to 0.7 V vs quasi-reference platinum electrode). The addition of fructose in the solution provoked the increase in the catalytic current, showing that such an electrode assembly may be used for fructose quantification.

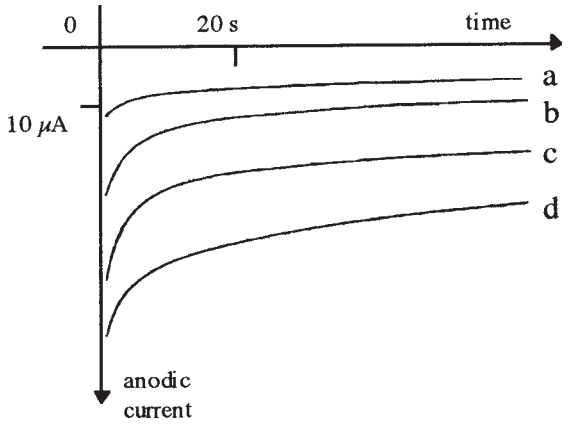


Fig. 2. Chronoamperometric curves. Current measured with enzyme-modified carbon paste electrode containing 2 U of FDH/mg, 23.66% paraffin oil, and 0.12% FcH. Phosphate buffer without (a) and with 1.2 (b), 6.1 (c), and 25.7 m*M* (d) D-fructose. Working potential: 0.5 V vs SCE.

To improve the analytical performance of the electrode, the composition of the modified carbon paste was investigated, and then the effect of the pasting oil was studied both on the electrochemical response of the electrode and on its shelf life. All these investigations were achieved in batch mode measurement using the Multicapteur MC2.

As described in the Materials and Methods section, the measurement procedure consisted of renewing the electrode surface before each assay. The renewal of the surface in an identical way is therefore necessary to obtain repeatable and accurate measurements. The repeatability of the measurements can also strongly be affected by other factors. It mainly depends on the homogeneity of the distribution of both enzyme and mediator in the bulk of the electrode. In a previous article, we demonstrated that the repeatability as well as the sensitivity of modified carbon paste electrode depends on the ratio of the pasting liquid and on the oil:carbon ratio *(18)*.

To improve the electrode repeatability, the homogeneity of the distribution of each component into the modified carbon paste must be considered. Two elements must be considered during the preparation process of the FDH-modified carbon paste electrode. First, the modified graphite powder obtained after the mixing of the enzyme with the carbon and after the drying step must be very homogeneous. Second, the homogeneity of the oil in the bulk of the modified carbon paste must be as perfect as possible. From an experimental point of view, the first point is relatively easy to achieve, but the second is more critical because it involves the intimate mixing of two different phases that may affect the enzyme activity. Therefore, we examined the influence of mixing time on the electrochemical response. Figure 3 shows a series of calibration curves recorded under the same experimental conditions but with various mixing times. It is obvious that significant deactivation results from an extended mixing time leading

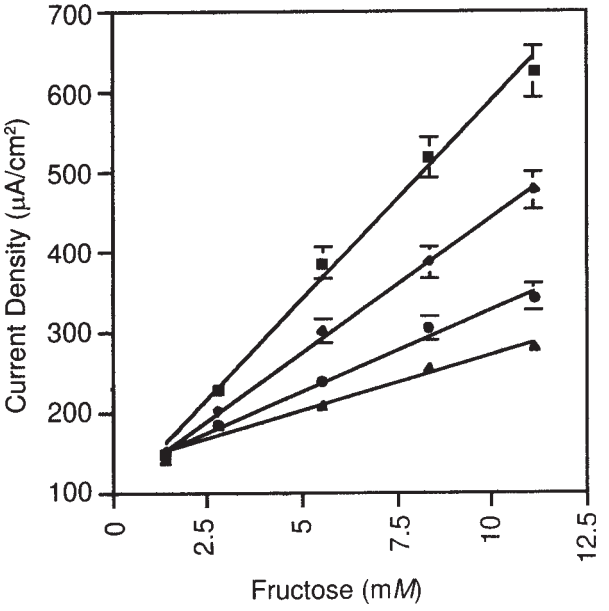


Fig. 3. Effect of mixing time (■, 5; ◆, 10; ●, 15; and ▲, 20 min) on the electrochemical response of the carbon paste electrode modified with FDH (7.1 U/mg), paraffin oil (23.66%), and FcH (0.12%).

Table 1
Effect of Mixing Time on Analytical Performances of FDH-Modified Carbon Paste Electrode[a]

Mixing time (min)	Sensitivity[b] (μA/[m*M*·cm²])	Linear range[b] (m*M*)	Repeatability[b] (RSD %)
5	50	1.5–11	7–10
10	30	1.5–11	2–5
15	15	1.5–9	1–3
20	10	1.5–4	1–3

[a]FDH (7.1 U/mg); FcH (0.15%), and paraffin oil (23.64%).
[b]RSD is calculated from 25 measurements of standard sample (5 m*M*).

to a gradual decrease in the linear range as well as in the sensitivity. Table 1 summarizes the effect of mixing time on the analytical performances of the FDH electrode. It shows that the relative standard deviation is inversely proportional to the mixing time. This indicates that the improvement of the homogeneity of the modified carbon paste is accomplished to the detriment of the electrode sensitivity and of its linear range. The mixing time of 10 min appears to be the best compromise between high sensitivity and good repeatability.

We previously showed for the lactate oxidase–modified carbon paste electrode *(18)* that because of its solubility in water and in paraffin oil, the

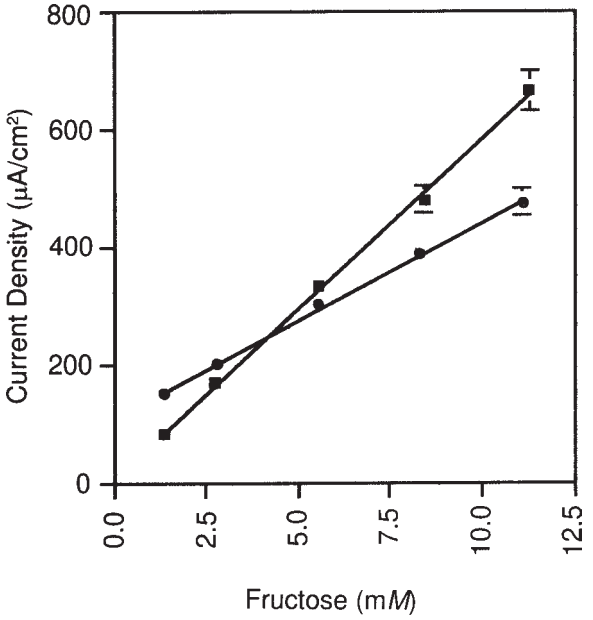


Fig. 4. Calibration plots of FDH-modified carbon paste electrodes containing (●) 0.12% FcH and (■) 0.12% FcH_2OH.

use of $FcCH_2OH$ as a redox mediator *(18,19)* leads to an improvement of both sensitivity and linearity range of modified electrodes used in batch measurement mode. This arises from the fact that sensitivity and linearity range depend on the amount of mediator in the vicinity of the electrode surface in both aqueous and pasting liquid phases *(14–16)*. This does not exclude, as suggested by one of the referees of this paper, that the difference is a result of $FcCH_2OH$ reacting more efficiently with FDH. Figure 4 shows the calibration curves obtained with the FDH electrode modified with 0.12% FcH and 0.15% $FcCH_2OH$, respectively. In the latter case the sensitivity was greatly improved.

The effect of the ratio of $FcCH_2OH$ in the carbon paste was examined by measuring the slopes of the calibration curves obtained with the FDH electrode modified with two different amounts of $FcCH_2OH$. Figure 5 shows that the linear range decreased when the mediator ratio decreased from 0.15 to 0.09%. At a lower concentration of fructose (<6 m*M*), the effect of mediator ratio on the sensitivity was insignificant whereas for higher fructose concentrations the electrode became more sensitive when the $FcCH_2OH$ ratio increased.

The amount of enzyme in the modified electrode also has an important effect on sensitivity and linear range as well as on long-term stability. Figure 6 shows the calibration curves for three different amounts of FDH (6.3, 8.3, and 10.12 U/mg of paste). The sensitivity increased with an increase in the amount of FDH in the carbon paste whereas the linear range seemed to be independent at least for the ratios of FDH >6.3 U/mg of

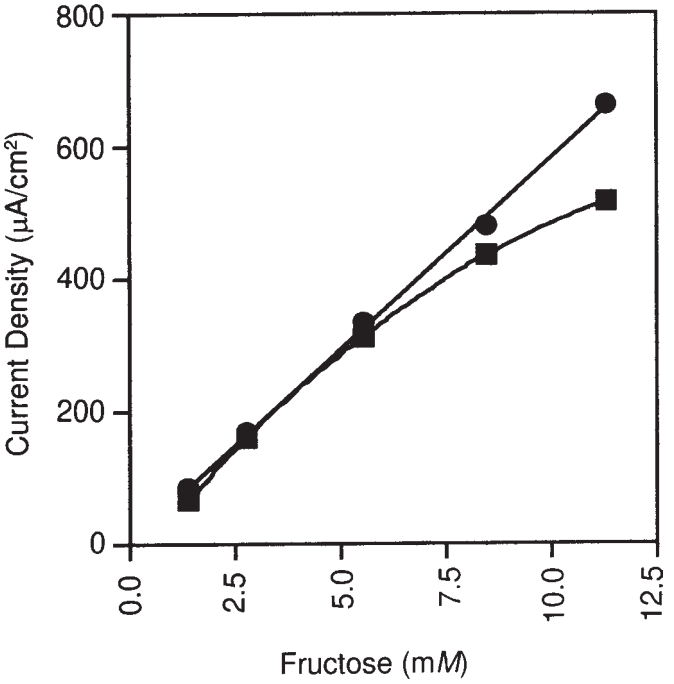


Fig. 5. Calibration plots of FDH-modified carbon paste electrodes (6.3 U/mg of FDH, 23.66% paraffin oil) containing 0.092% (■) and 0.12% (●) $FcCH_2OH$.

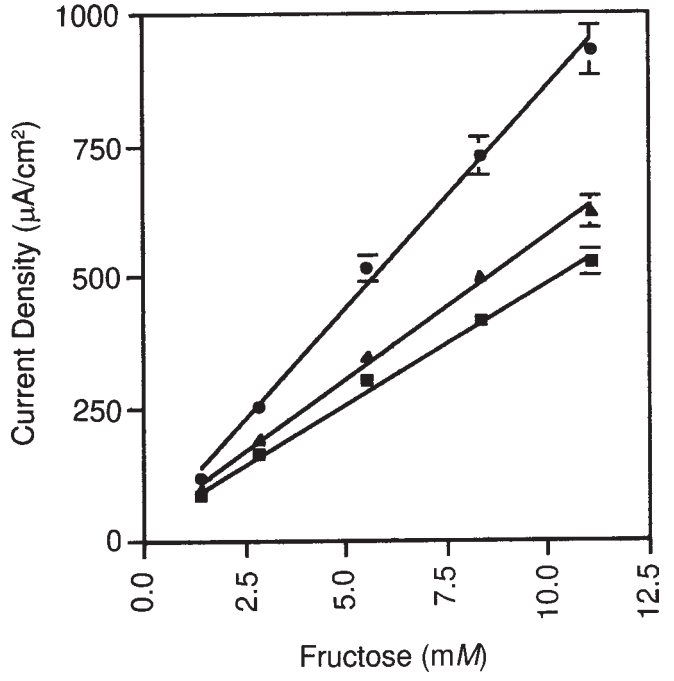


Fig. 6. Calibration plots of carbon paste electrodes modified with 0.12% $FcCH_2OH$; 23.66% paraffin oil; and (●) 10.12, (▲) 8.3, and (■) 6.3 U of FDH/mg of paste.

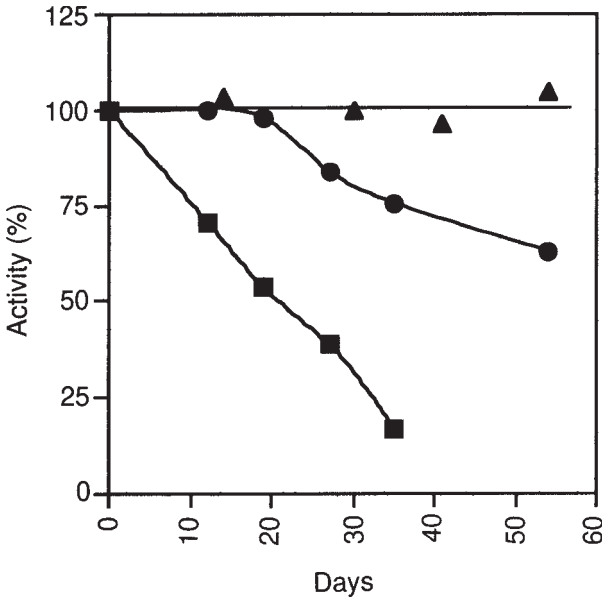


Fig. 7. Shelf life of (■) classical FDH-electrode, (▲) FDH-modified graphite powder, and (●) FDH electrode containing 10% Triton X-100.

paste. For economical reasons, the ratio of 6.3 U/mg of paste was adopted for the following studies.

The ratio of pasting oil in FDH-modified carbon paste electrode was also examined. Higher sensitivity was obtained when the amount of pasting liquid was decreased. The effect of oil on the electrochemical response of the enzyme-modified carbon paste electrode was complex. Because of the hydrophobic environment of the electrode surface, the kinetics of both the electron transfers *(20)* and the enzymatic reaction may be affected. It is well known that increasing the amount of oil into the carbon paste favors the sluggish electron transfer at the electrode surface *(21,22)*. This will lead to a diminution of the response and thus to a decrease in the sensitivity. When the ratio of oil decreases from 23.7 to 16.7%, the sensitivity of the electrode increases (from 56.7 to 72.1 μA/[m*M*·cm^2]). This may be owing to the influence of the hydrophobic environment, which could influence either the enzyme kinetic or the contact between the mediator and FDH.

Long-term stability of the FDH-modified carbon paste electrode was investigated. The sensitivities obtained by calculating the slope of the calibration curve at different days were compared. The electrode was kept at 4°C when not used. Twenty days after the preparation of the electrode, the value of the sensitivity was only half of the initial one (Fig. 7). The sensitivity dropped quickly and the response of the electrode became insignificant after 1 mo. In parallel, the FDH-modified graphite powder was kept at 4°C under the same conditions as the FDH carbon paste, and its activity was measured by preparing, the same day, a carbon paste electrode. The results indicate that the presence of the oil in the carbon paste electrode caused the

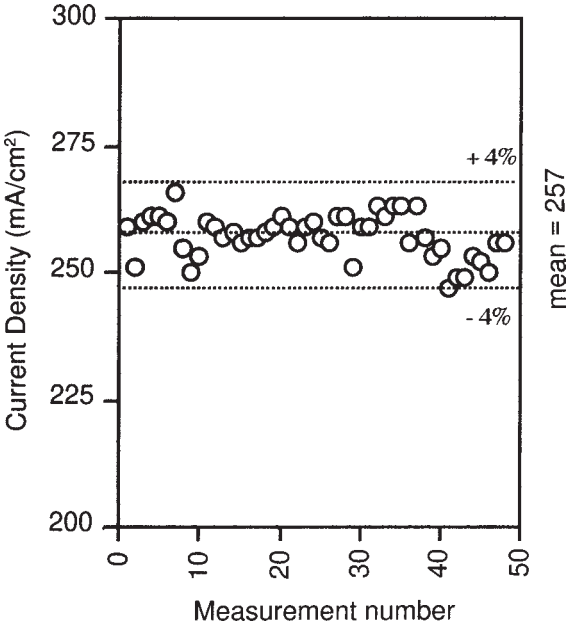


Fig. 8. Repeatability test of FDH electrode containing 10% Triton X-100 (4.56 m*M* fructose solution in 0.1 *M* phosphate buffer at pH 7.4).

deactivation of FDH. FDH is a membrane-bound enzyme and its catalytic site is known to be located in the lypophilic region *(23,24)*, and thus it can be soluble in the pasting liquid. This solubility causes the denaturation of the enzyme or prevents contact with the substrate solution or the mediator. Removing the oil from the deactivated paste with dichloromethane does not restore the enzyme activity after evaporation of the solvent.

To diminish the hydrophobicity both inside the carbon paste and at the surface of the electrode, we added 10% detergent (Triton X-100) in the bulk of the electrode. Triton X-100 is known to be beneficial for FDH activity when it is present in solution. The addition of Triton X-100 into the FDH-modified carbon paste electrode also seems beneficial for its activity. The stability of the Triton X-100 FDH-modified electrode remained nearly constant for 20 d; then the activity dropped but remained high (60%) for more than 2 mo (Fig. 7).

The combination of the different parameters that affect the electrode activity and stability permitted the optimization of the mixture of FDH-carbon paste electrode: 8.3 U/mg of enzyme, 0.15% of hydroxymethyl FcH, 16.7% paraffin oil, and 10% Triton X-100. The repeatability of such an electrode has been evaluated: it shows good results (Fig. 8) that are adequate for industrial applications.

The fructose biosensor has been evaluated on real fruit juice samples and compared to the standard spectroscopic enzyme method using Boehringer Mannheim analysis kits (Table 2). The fruit juice samples were diluted 100 times for the electrochemical assays and 1000 times for spectrophoto-

Table 2
Compared Results of Fructose Analysis
in Fruit Juices Obtained with Amperometric Biosensor
and Standard Method Using Soluble Enzymes with UV Detection

Product	FDH electrode (g/L)	Enzymatic kit (g/L)	Difference (%)
Grape juice	67.5	66.4	+1.7
Apple juice	62.4	63.7	–2.0
Orange juice	36.3	36.2	+0.3

metric enzyme analyses. The comparative values were obtained in a single shot measurement for both techniques in order to meet industrial routine customs. Indeed, the accepted value must be obtained from the first measurement because the majority of industrial users cannot afford to spend time obtaining a mean value from a set of different results.

For both techniques dilution of the sample is needed to fit it into the linear range. For the fructose biosensor, the factor of 10 for the linearity limits of fructose concentrations is adequate to meet the requirement of end users. The results in Table 2 show a good correlation between the two methods; however, the biosensing procedure is much faster.

Conclusion

The work developed in this study shows that carbon paste may be compatible with an unstable enzyme such as FDH and that FcH or $FcCH_2OH$ can be used as an electron transfer mediator. The stability of the FDH-modified electrode was tremendously increased by the addition of detergent in the bulk of the electrode. The decrease in the amount of pasting liquid also had a beneficial effect on the sensitivity and the stability of the electrode. The combination of these different parameters allowed us to prepare a stable, sensitive, and repeatable electrode for fructose measurement usable for the construction of a fructose biosensor that can meet the industrial needs for routine assays.

Acknowledgments

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