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Evaluation of different mediator-modified screen-printed electrodes used in a flow system as amperometric sensors for NADH

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Abstract

This work presents a comparative study between two different methods for the preparation of mediator-modified screen-printed electrodes, to be used as detectors in a reliable flow injection system for the determination of the nicotinamide adenine dinucleotide (NADH) coenzyme. The best strategy was selected for the final development of compact biosensors based on dehydrogenase enzymes. For the first immobilisation strategy, different redox mediators were electropolymerised onto the SPE surface. The second immobilisation strategy was carried out using polysulfone–graphite composites, which were deposited by screen-printing technology onto the screen-printed electrode (SPE) surface. Both methods achieved an effective and reliable incorporation of redox mediators to the SPE configuration. Finally, a flow system for ammonium determination was developed using a glutamate dehydrogenase (GlDH)-Meldola's Blue (MB)-polysulfone-composite film-based biosensor.

The stability of the redox mediators inside the composite films as well as the negligible fouling effect observed on the electrode surface improve the repeatability and reproducibility of the sensors, important features for continuous analysis in flow systems. Furthermore, the optimised bio/sensors, incorporated in a flow injection system, showed good sensitivities and short response times. Such a good analytical performance together with the simple and fast sensor construction are interesting characteristics to consider the polysulfone-composite films as attractive electrochemical transducer materials for the development of new dehydrogenase-based SPEs.

Keywords: SPE; Flow injection analysis; Polysulfone; Redox mediators

1. Introduction

Despite the wide range of available dehydrogenase enzymes, dehydrogenase-based biosensors have not been as widely developed as might be expected. The reason can be found in the fact that they relay on the amperometric detection of the NADH cofactor, process that involves some drawbacks. Actually, the development of a reliable system for the amperometric detection of NADH has been during years one of the main research subjects in electrochemical sensors. The problems related to the electrochemical oxidation of NADH involve high overpotentials [1] and the subsequent formation of byproducts that foul the electrode surface (i.e. dimerisation of $NAD^{\bullet}$ radicals and other oxidation products) [2,3]. As a result, low selectivity and stability are obtained. Much work has been related to the use of redox mediators [4]. These compounds allow the construction of more sensitive, selective and stable biosensors, since they permit to lower the overpotential and so prevent the electrode fouling [5]. However, the soluble, or partially soluble, mediating species may diffuse away from the electrode surface towards the bulk solution, especially when the sensor is used in multiple analyses, e.g. in a continuous flow system. In fact, sensors used as detectors in FIA systems are exposed to flowing buffer and, consequently, special attention must be given to the mediator immobilisation. If the mediator leaks from the sensor, significant current loss will occur and therefore the lifetime of the sensor will be considerably reduced. Different strategies to incorporate redox mediators into the electrochemical system involve either adding the mediator to the solution [6,7] or immobilising it within or on the electrode, producing compact chemically modified sensors [8,9]. The latter is achieved following different methodologies, such as the dispersion of the mediator in the bulk of a composite electrode [10,11] or its immobilisation on the electrode surface by physical adsorption [12,13], covalent attachment [14,15],

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electropolymerisation [16,17], gel entrapment [18] or cross-linking [19]. Other immobilisation techniques involve the use of a polymeric film, where the mediator is covalently attached [20–23] or physically entrapped [24–26]. The chosen strategy will depend on the specific characteristics of the working system and the sensor performance under them, as the activity of the immobilised molecules depends on the immobilisation method, as well as, in the case of using an immobilising matrix [27], on its surface area, porosity and hydrophilic character.

In previous studies [28,29], the behaviour of different redox mediators, incorporated to the system by five different strategies was compared: in solution, incorporated inside a composite matrix, adsorbed or electropolymerised onto the electrode surface and incorporated inside polysulfone-composite films deposited on the electrode surface. This work was carried out with cylindrical-configuration electrodes based on epoxy–graphite composites. These new polysulfone-composite membranes were prepared by mixing polysulfone with graphite powder.

In the work reported here, two previously optimised immobilisation strategies have been used to prepare mediator-modified SPEs. Results show that electropolimerised mediators and mediator-modified polysulfone-composite films overcome one of the most critical points in the manufacture of thick-film sensors, such as the adhesion of the sensing layer to the transducer layer. The excellent stability of electron mediators immobilised inside polysulfone-composite materials together with the fact that they can be easily incorporated to the sensors through an additional layer in the screen-printing process, allow a massive production of disposable mediator-modified electrodes. Furthermore, the leakage absence and the negligible surface fouling ensure the feasibility to consider SPEs based on polysulfone-composite films as electrochemical detectors for flow injection systems. Finally, it has been demonstrated the usefulness of these sensors for the development of dehydrogenase-based biosensors to be used in FIA systems, showing as an example the performance of GlDH-mediator-polysulfone SPEs.

2. Experimental

2.1. Reagents

Polysulfone Ultrason nature 3120 was obtained from BASF. NADH, Meldola's Blue (MB), *o*-phenylenediamine (*o*-PDA), glutamate dehydrogenase (GlDH, EC 1.4.1.3 from bovine liver, 42 units/mg prot.) and α-ketoglutarate were purchased to Sigma. 3,4-dihydroxybenzaldehyde (3,4-DHB), *p*-benzoquinone (*p*-BQ) and *N*,*N*-dimethylformamide (DMF) were bought to Aldrich and dichlorophenolindophenol (DCPIP) to Fluka. Ammonium chloride, sodium dihydrogen phosphate and potassium chloride were purchased from Panreac. A platinum sheet (Ref. PT000251, Goodfelow, England) with 99.95% purity and 0.125-mm thick, a fibreglass support (Ariston), silver conductive resin 410E and the proper hardener (Epoxy Technology, Billerica, MA, USA) and epoxy diacrilate (Ebecril 600, UCB Chemicals) have been used for the construction of a planar-configuration platinum electrode. Clear polyester sheets, 0.5-mm thick, were used as support for the SPEs. SPEs have been prepared by successive layer printing with inks supplied by Acheson: silver ink (Electrodag 418 SS), graphite ink (Electrodag 423 SS) and insulating ink (Electrodag 451 SS).

All solutions were prepared using deionized water obtained from a Milli-Q purification system, and they were de-aerated prior to their use.

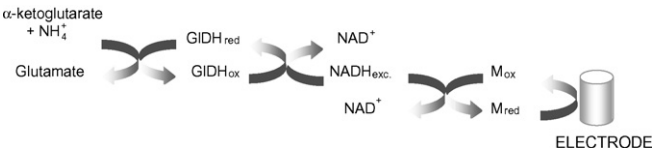
2.2. Electrochemical measurements

Cyclic voltammetry was carried out using a multipotentiostat, AUTOLAB model PGSTAT10 (Eco Chemie) in a conventional electrochemical cell of 10-mL volume. Amperometrical studies were performed on a LC-4C potentiostat (BAS, USA) in a flow injection system. All experiments were carried out at room temperature, in a conventional three-electrode system with a planar-configuration platinum electrode or a SPE as working electrode. The auxiliary electrode was an epoxy–graphite composite electrode in a tubular configuration, and the reference electrode was a double-junction Ag/AgCl electrode (Orion 92-02-00), with a commercial inner filling solution (Thermo Orion 900002) and 0.1 M KCl as outer filling solution. The three electrodes were disposed along the flow system using methacrylate supports. The flow injection system consisted of a Gilson (Minipuls 3) peristaltic pump, interconnecting Teflon tubing (0.8 mm inner diameter) and a sample injection valve.

2.3. Electrode preparation

Glass fibre with photolithographed cooper tracks was used as conductive support for the construction of the planar-configuration platinum electrode. For this electrode, with an active surface area of 24 mm^2, the electric contact between the cooper and the platinum was achieved using a conductive silver resin, which allows the welding of both materials and, finally, the electrode was encapsulated with an epoxy resin.

A DEK 248 screen-printing system (DEK, UK) was used to fabricate the SPEs (with a geometrical area of 24 mm^2). The electropolymerisation process used for the preparation of the mediator-modified SPEs was previously described for conventional electrodes [28]. Briefly, the composite SPE surface was pre-treated by dipping it in phosphate buffer solution and performing cyclic voltammograms between −0.2 and +0.1 V for 10 min at 0.05 V s^{-1} [16,30]. The aim of this pre-treatment was to enhance the reproducibility of the electrode surface characteristics. The second step was the electropolymerisation of the mediator on the SPE surface, by applying a constant potential to the electrode immersed in a redox mediator solution. The applied potential, as well as the mediator concentration and the electropolymerisation time, varied depending on the used mediator. Finally, the electrode surface was activated by recording 10 cyclic voltammograms at 0.05 V s^{-1}, using potential windows that depended on the mediator. On the other hand, for the preparation of the polysulfone–graphite composite-modified SPEs, a 7.5 wt% polysulfone solution was prepared in dry DMF. Once the solution was homogenised, 150 μL of this solution were mixed with 30 mg of graphite and 1.5 mg of redox mediator



Scheme 1. Sequence of reactions for the determination of ammonium.

(MB, 3,4-DHB, *p*-BQ, *o*-PDA or DCPIP). A thin film of this mixture was screen-printed onto the SPE surface. Immediately after printing, it was precipitated by phase inversion achieved by immersing the electrode in cold water (approximately 4 °C) or in a 0.477 units of GlDH/μL aqueous solution, depending on the kind of sensor to be prepared [31,29]. This process leads to a controlled phase change of the polysulfone-composite (cast onto the electrode surface) from liquid to solid. By controlling the initial state of phase transition, the membrane morphology can be controlled (i.e. porosity, thickness). Then, the polysulfone-composite SPEs were thoroughly rinsed with double-distilled water prior to use. The SPEs without enzyme were stored in air at room temperature, while the ones with enzyme were stored dried at 4 °C.

2.4. Analytical procedure

Cyclic voltammetry at a scan rate of 0.1 V s^{-1} was used to electrochemically characterise the evaluated SPEs. From these results, the appropriate working potential for each electrode was determined.

Electrochemical experiments were performed using two different background electrolyte solutions, depending on the assay: 0.05 M phosphate buffer solution with 0.05 M KCl at different pH or a solution containing 2.5 mM α-ketoglutarate and 0.2 mM NADH, freshly prepared in phosphate buffer at pH 7.3. These solutions, pumped at 0.9 mL min^{-1} flow rate, were used as carrier solutions in the flow injection system. Stock solutions of ammonium (1 M) and NADH (0.01 M) were prepared using phosphate buffer at pH 7.3 and 6.5, respectively. NADH solution must be freshly prepared prior to use. For the evaluation of both NADH sensors and GlDH-modified biosensors in flow system, 100 μL of various NADH or ammonium solutions, respectively, were injected once a stable baseline was reached. The applied potential (versus SCE) for the oxidation of NADH was dependent on the used mediator (Scheme 1).

All the measurements were based on the current peak height, calculated as the difference between the maximum current value after the injection and the current value for the baseline due to the carrier solution. Several calibration curves were performed with each electrode under the same conditions in order to study the evolution of the slope value, since this parameter will give information about the degree of alteration of the electrode surface.

For all the experiments at least three replicates were done, and the results given are the averages of all the measurements with the corresponding relative standard deviations (R.S.D.s).

3. Results and discussion

3.1. Evaluation of non-modified platinum planar-configuration electrode and SPEs used as NADH sensors in a flow injection system

In order to demonstrate the improved features of SPEs over platinum electrodes, the fouling effect on both surfaces was evaluated by measuring the current intensity after successive NADH injections. Results demonstrated that the SPEs slightly improved the repeatability compared to the platinum transducer, showing also a higher sensitivity value (i.e. the sensitivity values obtained from 10 successive calibration curves were 310 μA M^{-1} with a R.S.D. = 5% for a platinum electrode and 900 μA M^{-1} with a R.S.D. = 4% for a SPE). Even more important, the R.S.D.s for both electrodes were not only due to random oscillations among measurements, but they were more likely attributed to the decrease of sensitivity after successive calibration curves as a consequence of the fouling process of their surface. The SPEs slightly improved this loss of sensitivity, fact that agrees with previous studies carried out with platinum and graphite electrodes [32]. However, none of both is reliable for measuring NADH oxidation with time, as the SPEs lost around 40% of the signal for 20 injections of a 2×10^{-4} M NADH solution in different days, and the platinum electrode lost 60% (results not shown).

3.2. Development and characterization of redox mediator-modified screen-printed electrodes used as NADH sensors in a flow injection system

3.2.1. Amperometric studies

After choosing SPEs as working electrodes, the response to NADH oxidation was evaluated with calibration curves for

Table 1
Calibration parameters of NADH oxidation performed with SPEs based on electropolymerised mediators or mediator-modified polysulfone-composite films[a]

Redox mediator	Electropolymerised mediator		Mediator-modified polysulfone-composite film	
	E_w (V vs. SCE)	Sensitivity (μA M^{-1})	E_w (V vs. SCE)	Sensitivity (μA M^{-1})
MB	0.100	96 ± 29	−0.100	1009 ± 38
3,4-DHB	0.300	263 ± 17	0.350	727 ± 25
p-BQ	0.325	79 ± 8	0.175	753 ± 24
o-PDA	0.450	152 ± 12	0.300	640 ± 21
DCPIP	0.320	73 ± 13	0.150	761 ± 27

[a] Sensitivity values come from calibration curves where each point is the average of seven experimental data. The evaluated NADH concentration range was between 2×10^{-5} and 5×10^{-4} M. The carrier electrolyte solution was de-aerated 0.05 M phosphate buffer with 0.05 M KCl at pH 6.5, pumped at 0.9 mL min^{-1} flow rate.

Table 2
Evaluation of the repeatability of 20 successive injections of different NADH concentrations into a de-aerated carrier solution, using electropolymerised mediators or mediator-modified polysulfone-composite films

Redox mediator	E_w (V vs. SCE)	Current intensity (nA)	R.S.D. (%)
Electropolymerised mediator			
MB[a]	0.100	14	4
3,4-DHB[b]	0.300	61	3
p-BQ[b]	0.325	45	1
o-PDA[b]	0.450	33	5
DCPIP[a]	0.320	41	3
Mediator-modified polysulfone-composite film			
MB[b]	−0.100	206	1
3,4-DHB[b]	0.350	146	1
p-BQ[b]	0.175	151	2
o-PDA[b]	0.300	139	1
DCPIP[b]	0.150	159	1

[a] Injections of 100 μL of 5×10^{-4} M NADH.
[b] Injections of 100 μL of 2×10^{-4} M NADH.

Table 3
Evaluation of the reproducibility of five successive calibration curves obtained by injecting different NADH concentrations into a de-aerated carrier solution, using electropolymerised mediators or mediator-modified polysulfone-composite films

Redox mediator	E_w (V vs. SCE)	Sensitivity (μA M^{-1})	Sensitivity R.S.D.[a] (%)
Electropolymerised mediator			
MB	0.100	96	1
3,4-DHB	0.300	264	2
p-BQ	0.325	80	3
o-PDA	0.450	152	5
DCPIP	0.320	75	3
Mediator-modified polysulfone-composite film			
MB	−0.100	1035	1.5
3,4-DHB	0.350	695	0.4
p-BQ	0.175	751	0.7
o-PDA	0.300	640	1.3
DCPIP	0.150	769	0.7

[a] Reproducibility of the slope of five calibration curves. The evaluated NADH concentration range was between 2×10^{-5} and 5×10^{-4} M.

the electrodes with electropolymerised mediators or mediator-modified polysulfone-composite films. Each point of the calibration curves is the average of the current intensity for a known NADH concentration after seven successive injections. All the electrodes showed linear calibration curves for the evaluated NADH concentration range, from 2×10^{-5} to 5×10^{-4} M. Table 1 shows that best sensitivity values were achieved with electrodes prepared using mediator-modified composite films. However, since the main drawback of non-modified electrodes is the fouling of the electrode surface by NADH oxidation byproducts, it was required a more thorough study. With this purpose, repeatability and reproducibility were evaluated for all the electrodes. The repeatability of the measurements was evaluated by measuring the current intensity values due to 20 successive injections of a known NADH solution. The reproducibility was evaluated by measuring the current intensity values due to injections of increasing NADH concentrations using the same electrode (calibration curves), in order to obtain the sensor sensitivity, and repeating it with the same electrode several times. The reproducibility of the sensitivity values (sensitivity R.S.D.) was done as the operational stability for the developed sensors.

Table 2 shows the results of repeatability for all the mediator-modified SPEs. The mediator presence, especially for composite films-based sensors, improves the R.S.D. compared to the results with a non-modified SPE (R.S.D. = 6%, $n = 20$). Table 3 shows the better reproducibility of the sensitivity values obtained with mediator-modified SPEs, pointing out how each electrode behaves after being used in successive calibration curves. The slight improvement of the reproducibility for the composite films-based sensors could be attributed to the different effect of the absorbed mediator. In electropolymerised mediator-based sensors, there is always a significant amount of adsorbed mediator that has a direct influence on the electrochemical characteristics. On the contrary, for the electrodes prepared with polysulfone, the amount of mediator still adsorbed after thoroughly rinsing the electrodes prior to use, can be neglected compared to the total amount of mediator retained in the polysulfone film. Thus, the electrochemical characteristics of these electrodes will mainly be dependent on the mediator entrapped inside the film. Furthermore, the minimisation of the electrode fouling for the electrodes based on polysulfone films, shown as an improvement of the reproducibility of the sensitivity values, could be explained from the better contact achieved between the mediator and the graphite particles.

Another important parameter is the response time, especially in flow systems, since it gives valuable information about the ability of the flow system to perform continuous measurements. As included in Table 4, all the mediator-modified SPEs had response times ($t_{95\%}$) from 12 to 30 s. All the electrodes showed response times that notably improved the response time ($t_{95\%}$), respect to non-modified electrodes, when a 2×10^{-4} M NADH solution was injected (>50 s).

Best sensitivity, repeatability and reproducibility were achieved using SPEs based on mediator-modified polysulfone-composite films. Moreover, the electrodes based on electropolymerised mediator were produced one by one through a process that requires about 30 min, while the electrodes based on polysulfone-composite films can be mass-produced by screen-

Table 4
Dynamic characteristics of the response obtained using electropolymerised mediators or mediator-modified polysulfone–graphite films[a]

Redox mediator	Response time ($t_{95\%}$, s)	
	Electropolymerised mediator	Mediator-modified polysulfone-composite film
MB	30 ± 1.2	17 ± 1.0
3,4-DHB	18 ± 0.4	20 ± 0.5
p-BQ	12 ± 1.3	19 ± 1.5
o-PDA	13 ± 0.4	20 ± 0.6
DCPIP	22 ± 2.8	26 ± 1.9

[a] Injections of 100 μL of 2×10^{-4} M NADH. Each given value is the average of three experimental values. The carrier electrolyte solution was de-aerated 0.05 M phosphate buffer with 0.05 M KCl at pH 6.5, pumped at 0.9 mL min^{-1} flow rate.

printing. As a result, mediator-modified polysulfone-composite film-based SPEs were chosen for the development of an ammonium biosensor, based on glutamate dehydrogenase enzyme (GIDH), to be implemented in a flow system.

3.2.2. Storage stability of the mediator-modified SPEs

The storage stability was calculated from the sensitivity of calibration curves obtained in different days with electrodes belonging to the same sheet, i.e. prepared at the same time. Consequently, firstly it was necessary to study the variability among electrodes belonging to the same sheet (36 electrodes/sheet). It was calculated from the values of current intensity for four electrodes belonging to the same sheet for 20 successive injections of a 2×10^{-4} M NADH solution. The R.S.D. for electrodes based on polysulfone-composite films varied from 4 to 9%, depending on the mediator. Since all mediators follow a heterogeneous catalysis mechanism, which implies that current intensity is proportional to both the surface area and the mediator coverage, it is inferred that the differences in both parameters, due to intrinsic limitations of the construction process, will notably increase the variability among the electrodes belonging to a same sheet. However, the high reproducibility observed may be due to the fact that the amount of mediator inside the polysulfone-composite membrane is high enough to ensure that small variations in mediator coverage do not induce important changes in the electrical signal. Once the inherent variability among NADH calibration curves for electrodes from the same sheet was known, it was possible to evaluate the storage stability. It was possible to conclude that the sensitivity values were affected only for the electrodes prepared with *p*-BQ and DCPIP during the first 15 days in a way that could not be attributed to the variability among the sheet.

3.3. Development, characterization and implementation of an ammonium biosensor based on GlDH-mediator-polysulfone–graphite SPE in a flow injection system

The behaviour of mediator-polysulfone-composite film-based sensors in front of some electroactive compounds that might interfere with the response of a dehydrogenase-based biosensor was evaluated. No interference was observed from common electroactive compounds, such as ascorbic and uric acids. Taking advantage of this good permselective behaviour, ammonium biosensors were developed using glutamate dehydrogenase (GlDH) enzyme incorporated into polysulfone-composite films placed onto SPEs. Based on previous studies about polysulfone membranes [29], this enzyme can be incorporated in the films during the phase inversion process that causes the precipitation of the polysulfone. Fig. 1 shows five successive calibration curves obtained using a GlDH-Meldola's Blue (MB)-polysulfone-composite film-based SPE in a flow system. MB was chosen as it was the mediator that offered the highest sensitivity. These biosensors showed a linear correlation for the evaluated ammonium concentrations ranging from 5×10^{-5} to 2×10^{-2} M under the working conditions described. The sensitivity to ammonium obtained for these biosensors in a flow system was 15 μA M^{-1} and the sensitivity R.S.D. was

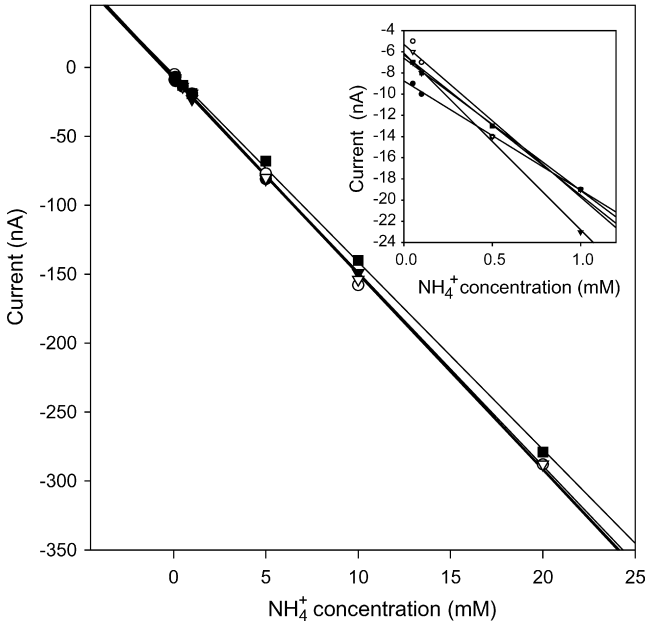


Fig. 1. Five successive calibration curves obtained from injections of different known ammonium concentrations in a flow system. A SPE based on GlDH-MB-polysulfone-composite film has been used as working electrode. De-aerated carrier solution: 0.05 M phosphate buffer with 0.05 M KCl, containing 2.5 mM α-ketoglutarate and 0.2 mM NADH, at pH 7.3. Working potential: −0.1 V vs. SCE. Flow rate: 0.9 mL min^{-1}.

1.9% ($n = 5$ consecutive calibration curves), corresponding to the operational stability. The reproducibility among biosensors belonging to the same sheet, meaning that they were prepared at the same time, was also studied, finding an R.S.D. associated to the slope of the calibration curves obtained with four of these electrodes of 4%. In addition, the storage stability was evaluated, proving that these biosensors can be stored at 4 °C at least for 1 month, showing a lost of sensitivity of 4%, being negligible as it is comparable to the sensitivity R.S.D. for different electrodes of the same sheet. Finally, the response time ($t_{95\%}$) for sample volumes of 100 μL was lower than 30 s, followed by a short recovery time, allowing an analysis time of 1 min. These results prove the usefulness of these biosensors for determining ammonium in flow, being able to be changed each day for a new electrode stored at 4 °C.

4. Conclusions

It has been demonstrated that both electropolymerised mediator-based SPEs and mediator-modified polysulfone-composite film-based SPEs, avoid problems related to the amperometric oxidation of NADH when used as detectors in a flow system, since they lower the high required overpotentials, thus minimising electrode fouling. Furthermore, both mediator-based strategies show good selectivities and low response times (<30 s) compared to the results for non-modified SPEs, indicating better electrocatalytic behaviour and allowing a higher number of NADH determinations in less time. Comparing the results for both types of sensors, it can be concluded that polysulfone-composite film-based sensors show the highest sensitivity, as

well as the best repeatability and reproducibility results. The latter parameter can be directly related to the operational stability, proving the absence of mediator leakage and electrode fouling, essential characteristics for electrodes to be used in flow systems. Finally, having considered the variability among electrodes belonging to a same sheet, it can be noticed that the electrodes based on MB, 3,4-DHB and *o*-PDA-polysulfone films kept 100% of their sensitivity at least during the first 15 days. These good performance characteristics demonstrated the usefulness of the mediator-modified polysulfone-composite films-based SPEs for the development of dehydrogenase-based biosensors and their further implementation in flow systems. Moreover, the simple and fast fabrication procedure allows their mass production. GlDH-MB-polysulfone-composite film-based SPEs have been shown to be reliable biosensors for the amperometric detection of ammonium in solution at a working potential of −0.1 V versus SCE, showing an excellent reproducibility among successive calibration curves (1.9%, $n = 5$). Thus, it can be concluded that a stable flow system for the determination of ammonium has been developed.

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