Flow Injection Analysis with Immobilized Enzymes in Nonaqueous Media

André R.T.S. Araujo, M. Lúcia M.F.S. Saraiva* and José L.F.C. Lima

REQUIMTE, Departamento de Química-Física, Faculdade de Farmácia da Universidade do Porto, Rua Aníbal Cunha, 164, 4099-030 Porto, Portugal

Abstract: Biocatalysis in nonaqueous media currently constitutes a promising research area which has already demonstrated its potential in numerous and varied applications. Several possibilities exist for the analysis of a wide range of poorly water-soluble analytes present in samples of food, pharmaceuticals, petrochemicals and cosmetic products, among others. The idea of assembling this kind of biocatalysis with flow management approaches clearly seems to be advantageous since it permits confined, precise and reliable analysis within a shorter timeframe while minimising human exposure to organic solvents and toxic effluent production. This article reviews the state-of-the-art of flowing stream systems comprising immobilized enzymes in nonaqueous media. Particular emphasis is devoted to categorising the enzymatic preparations operating in an organic environment: enzyme electrodes and enzyme reactors. The main approaches exploiting the use of immobilized enzymes in water-restricted environments using flow injection analysis for analytical purposes are presented in detail and possible future trends are discussed.

Keywords: Biocatalysis, Organic media, Flow injection analysis, Immobilization, Enzyme electrodes, Enzyme reactors.

1. INTRODUCTION

The potential of enzymes as practical catalysts is well recognized [1]. However, as long as the use of enzymes is restricted to their natural aqueous reaction media, the scope of the biotechnological conversions will therefore be limited by a variety of considerations. Most such compounds are poorly soluble in aqueous media and water, frequently giving rise to unwanted side reactions and degrading common organic reagents. In this sense, most of these problems might be overcome by switching from water to organic solvents as reaction media.

Indeed, recent research work has shown quite remarkably, that many enzymes can function in organic solvents (with or without a small amount of added water) [2] because they remain trapped in the native conformation. This is due to the fact that the water (essential for enzymatic activity) is tightly bound to the enzyme molecules and many remain bound even when the bulk water is replaced by an organic solvent [3]. At present, the belief that enzymes can retain their activity in a variety of nonaqueous media is fully accepted. From a bioanalytical perspective, this growing interest is due to numerous potential advantages in employing enzymes in organic media, namely the: (i) increased solubility of non-polar substrates [4]; (ii) solvent-induced changes in enzyme efficiency or substrate specificity [5]; (iii) enhanced biocatalyst thermostability which may be explained by the fact that proteins in a very low water monophasic organic medium have an increased conformational rigidity [6, 7]; (iv) prevention of microbiological contamination and side reactions and possible reduction of substrate and product inhibition [7]; (v) insoluble nature of most proteins in organic solvents which implies that the enzymes can be rapidly and irreversibly immobilized by simple adsorption from aqueous solutions onto a solid support, without being leached by the solvent and therefore preventing its detachment [8]; (vi) “pH memory” effect as the catalytic activity of the enzymes reflects the pH of the last aqueous solution to which they were exposed [9]. A review dealing with biocatalysis and biorecognition in nonaqueous media was undertaken by Braco [10] in which some approaches developed to exploit enzymes, antibodies or antibody mimics in water-restricted media were briefly discussed.

Despite the above-mentioned advantages, the use of organic solvents does have drawbacks in relation to safety (some are hazardous or explosive and highly volatile) as toxic wastes are produced which make their application in routine procedures difficult.

Automated analytical methods such as flow injection analysis (FIA) [11] have in recent years widely incorporated the use of enzymes as analytical tools for the determination of several compounds in foodstuffs, clinical samples and industrial processes [12]. The advantage of FIA is that virtually any number of additional lines with reagents can be added, almost any type of unit operation can be accommodated within the manifold and practically any type of detector can be used [13]. The fact that it works continuously is particularly advantageous when electrochemical detectors are employed and when dealing with the memory-effect phenomenon, as detailed in the following section. In this context, FIA using enzymes in nonaqueous media combines the unique properties of enzymes such as sensitivity, specificity and speed with the inherent advantages offered by FIA such as versatility, simplicity, repeatability, portability and low cost. This assembly seems to be an excellent candidate for biocatalysis in water-restricted environments. The ease
with which FIA can be implemented (due to the simplicity of its concept) through the use of readily-available equipment and by coupling usual detection systems found in most laboratories, could explain the predominant use of this technique throughout the reports cited. In this sense, the absence of the need for specific equipment and computer programmes to permit operation of the flow system devices as well as data acquisition and processing seems to be behind the exclusive use of FIA among the established flow management methodologies [14-18] when performing biocatalysis in a nonaqueous environment. Moreover, with flow methodologies any human exposure to organic solvents and toxic effluent production are minimized since those procedures are carried out in closed systems and the analyses are performed within a short timeframe and with a very high degree of precision. Consequently, the confirmed advantages over traditional batch procedures (which are usually complicated, laborious and time-consuming methodologies), using significant quantities of organic solvents, complement the use of flow techniques in this kind of analysis.

In all cases, the basic strategy of analytical enzymology in nonaqueous media is based on the use of organic solvents with very low water content as reaction media, instead of conventional aqueous buffers. Therefore, molecules that are poorly soluble in water can be readily dissolved in the carrier solution and therefore, directly determined in the flow system. In line with the above recognized benefits, the number of reports where enzymes have been used for analytical purposes in nonaqueous environments exploiting the flow injection strategy has been increasing, although the predominant amount of work has been carried out in aqueous media.

The following section discusses several developments related to analytical enzymology in organic media exploiting FIA systems, ranging from enzyme electrodes to enzyme reactors.

2. NONAQUEOUS FIA IMMOBILIZED ENZYME MODES

Before exploiting the nonaqueous enzyme immobilization FIA assembly, it should be pointed out that there are important aspects to be considered regarding the use of enzymes in organic media, namely: (i) solvent selection, (ii) enzyme selection, and (iii) enzyme immobilization procedure [4, 8, 19]. Indeed, the solvent used should not severely distort interactions in the hydration shell [20] of the enzyme molecule which is critical for enzymatic activity and should not therefore strip the essential water from the enzyme [21, 22]. Regarding enzyme selection, the enzymes that are theoretically suited can exist in a catalytically active conformation in an unfavourable nonaqueous medium. Finally, the enzyme immobilization procedure allows the enzyme to be fixed in a partially native conformation to a support, preserving it from the potential disturbing effect of the hydration shell by the solvent.

Enzyme immobilization comprises the process of confining the enzyme to a restricted area. This strategy shows some advantages, namely the increased catalytic stability of the enzyme and the possibility of its reutilization, leading to a significant reduction of analysis cost [23, 24]. In this context, it is not surprising to see the scarcity of reports dealing with FIA and solution-phase enzymes operating in nonaqueous media, primarily due to the predictable enzymatic inactivation.

For present purposes, immobilized enzyme systems can be divided into two areas of application. The first area includes the enzyme electrodes (used as the detection devices) where the enzymes are linked to a transducer and the second, the enzyme reactors, where the enzymes are entrapped within inert supports. A schematic representation of a FIA-system coupled to an enzyme electrode as a detector (A) or enzyme reactor (B) is depicted in Fig. (1).

2.1. Enzyme Electrodes as Detection Devices

Enzyme electrodes can be thought of as devices in which an immobilized enzyme is directly held to a part of the sensing system [23]. In fact, this consists of a modification of the surface of conventional electrodes with an immobilized enzyme layer. The linking of the electrodes to FIA manifolds is frequently aimed at overcoming the typical constraints of conventional electrochemical determinations by eliminating the decision-making factors which are attributed to the operators [25]. Additionally, when these electrochemical biosensors operate in an organic medium, there is the possibility of electrical resistance developing in the system and the subsequent need to use deliberately added supporting electrolyte, giving rise to unfavourable signal-to-noise characteristics of the response. Moreover, sample processing and the need for electrode conditioning (particularly in amperometric techniques) are unequivocal drawbacks, making the technique time-consuming and inconvenient when high sample throughput is the objective. Therefore, it is necessary to carry out a physical and/or chemical treatment of the electrode active surface to prevent malfunctions. In this context, the assembly of the enzyme electrodes in flow systems presents several advantages as it: (1) improves the operation of the enzyme electrodes in organic media; (2) permits reduction of sample/electrode contact time; and (3) enables the automatic reconditioning of the electrode surface through the carrier stream.

The analytical applications of enzymes in nonaqueous environments focus on the development of enzyme electrodes as the flow systems detection devices (Fig. (2)).

In fact, this constitutes an integral component which has guided the driving forces of the research as it offers opportunities for the direct analysis of many poorly water-soluble analytes in a variety of inaccessible and challenging sample matrices [10]. Most of the organic-phase biosensors described so far have made use of some “model” enzymes in nonaqueous studies such as horseradish peroxidase and tyrosinase.

The enzymes employed as well as the analytical applications of the resulting electrochemical flow injection detectors are summarised in Table 1. It is important to note that the majority of the flow injection enzyme electrodes developed do not have a specific analytical application with their feasibility being only demonstrated when operating in a nonaqueous medium.

The rapid response of the organic-phase enzyme electrodes can be exploited for high-speed flow injection assays,
Flow Injection Analysis with Immobilized Enzymes in Nonaqueous Media

Current Analytical Chemistry, 2010, Vol. 6, No. 3

Fig. (1). Schematic illustration of a FIA manifold, where a defined volume of sample (S) is injected by means of a rotary injection valve (IV) into a continuous flowing carrier stream (C) through a pump (P), which is subsequently merged with the reagent (R) stream. The ensuing transient generation of product is monitored by a suitable detector, following the stream - A) Enzyme electrodes (Re - Reactor; WE - Working electrode (enzyme electrode); RE - Reference Electrode) or the stream B) Enzyme reactors (ER - Enzyme Reactor; D - Detector), and then expelled by the waste (W).

Fig. (2). Analytical FIA immobilized enzyme applications (%) in nonaqueous environments.

as required in industrial quality control and process monitoring. In this sense, working in nonaqueous media in a flow injection measurement mode permits easy control over the sensitivity and linear range by judicious choice of flow conditions. Apart from the “direct” quantification of hydrophobic analytes, an extension of the linear range has frequently been achieved through the use of organic-phase enzyme electrodes. A short review was made by Wang [26] in which the advantages of the electrochemical enzyme-based detectors together with their performance as flow injection detectors were discussed.

All enzyme electrodes presented in detail here are amperometric in nature. The majority of the electrochemical enzyme-based detectors constructed are made from a variety of electron-conducting carbon allotropes (e.g., carbon paste, graphite, carbon fiber and glassy carbon) due to their easy handling and low cost when compared to other materials used (e.g., gold). On the other hand, the conventional electrodes employed (after enzyme modification) are predominantly attached to the FIA set-ups in the form of “wall-jet” or “thin layer” arrangements (Fig. (3)). In the wall-jet design, the solution spreads uniformly over the working electrode surface creating a diffusion film thickness (Fig. (3a)). On occasion, other configurations have been employed, as will be shown in the following sections.

Due to the larger number of reports dealing with organic-phase biosensors based on horseradish peroxidase (HRP) and tyrosinase (probably related to their intrinsic ability to remain in a catalytically active conformation in this reaction medium compared to those based on other enzymes), they will be discussed separately. Different enzymes immobilization schemes for the organic-phase biosensors operation were adopted and the improved features are discussed.

2.1.1. Peroxidase-Based Biosensors

The first approach to the implementation of an enzyme electrode operating in a mixed nonaqueous-aqueous medium was reported by Wang et al. [27] for the biosensing of or-
Table 1. Analytical Applications of the Electrochemical Enzyme-Based Flow Injection Detectors Operating in a Nonaqueous Media

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Analyte Determined</th>
<th>Organic Media</th>
<th>Analytical Application</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidase</td>
<td>Hydrogen peroxide, 2-butanone peroxide and cumene hydroperoxide</td>
<td>Acetonitrile-buffer (40:60, v/v) mixture</td>
<td>General</td>
<td>[27]</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Hydrogen peroxide and 2-butanone peroxide</td>
<td>Acetonitrile-buffer (90:10, v/v) mixture</td>
<td>General</td>
<td>[28]</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Lauroyl peroxide</td>
<td>Buffer-saturated chloroform solution</td>
<td>Food</td>
<td>[29]</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Hydrogen peroxide and 2-butanone peroxide</td>
<td>Methanol-buffer (10:90, v/v) and Acetonitrile-buffer (10:90, v/v) mixtures</td>
<td>General</td>
<td>[32]</td>
</tr>
<tr>
<td>Peroxidase and glucose oxidase</td>
<td>O-phenylenediamine and 2-aminophenol</td>
<td>Reversed micelles</td>
<td>General</td>
<td>[34]</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>Phenol</td>
<td>Buffer-saturated chloroform solution</td>
<td>Food</td>
<td>[35]</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>Catechol</td>
<td>Acetonitrile with a fixed catechol concentration and water up to 1%</td>
<td>Food</td>
<td>[36]</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>Phenol</td>
<td>Chloroform, 1-butanol and Acetonitrile solutions</td>
<td>General</td>
<td>[37]</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>Phenol and catechol</td>
<td>Heptane, Hexane, Chlorobenzene, Toluene and Chloroform solutions</td>
<td>General</td>
<td>[38]</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>Various phenolic compounds, benzoic acid, thiourea and propyl gallate</td>
<td>Reversed micelles</td>
<td>General</td>
<td>[39]</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>tert-butylhydroxyanisole</td>
<td>Reversed micelles</td>
<td>Food</td>
<td>[41]</td>
</tr>
<tr>
<td>Tyrosinase and peroxidase</td>
<td>p-cresol, phenol, 2-butanone peroxide and lauroyl peroxide</td>
<td>Buffer-saturated chloroform solution and Acetonitrile-water (95:5, v/v) mixture</td>
<td>General</td>
<td>[43]</td>
</tr>
<tr>
<td>Tyrosinase and peroxidase</td>
<td>Phenol and hydrogen peroxide</td>
<td>Acetonitrile-water (96:4, v/v) mixture</td>
<td>Pharmaceutical</td>
<td>[44]</td>
</tr>
<tr>
<td>Tyrosinase and peroxidase</td>
<td>2-butanone peroxide, phenol, thiourea, benzoic acid, diethyldithiocarbamate, hydroxylammonium, 2-mercaptopropanol, dichlorophenoxyacetic acid and dimethylmercury</td>
<td>Acetonitrile-buffer (98:2, v/v) mixture</td>
<td>General</td>
<td>[45]</td>
</tr>
<tr>
<td>Laccase</td>
<td>Catechol, 4-methylcatechol and hydroquinone</td>
<td>Alcoholic solvent-buffer (95:5, v/v) mixture</td>
<td>General</td>
<td>[46]</td>
</tr>
<tr>
<td>Catalase</td>
<td>Hydrogen peroxide</td>
<td>Acetonitrile-buffer (98:2, v/v) mixture</td>
<td>Food</td>
<td>[47]</td>
</tr>
<tr>
<td>Catalase</td>
<td>Hydrogen peroxide</td>
<td>Dimethyl sulfoxide and 1,4-dioxane</td>
<td>General</td>
<td>[48]</td>
</tr>
<tr>
<td>Cholesterol oxidase and cholesterol esterase</td>
<td>Cholesterol</td>
<td>Acetonitrile solution, 2.4 % buffer, 40 % toluene</td>
<td>Food</td>
<td>[49]</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td>Glucose</td>
<td>Acetonitrile and 2-propanol with a 6% and 20% buffer contents, respectively</td>
<td>Food</td>
<td>[50]</td>
</tr>
</tbody>
</table>

Organic peroxides, based on the incorporation of HRP into a carbon paste matrix. Working characteristics and sensitivity were obtained in the presence of an acetonitrile-phosphate buffer (40:60, v/v) mixture, yielding greatly extended linear ranges and stability for hydrogen peroxide, 2-butanone peroxide and cumene hydroperoxide. To circumvent the dissolution problems of the binding materials in the conventional carbon paste biosensors when operating in organic solvents, a bulk of rigid graphite-Teflon matrix was used to immobilize the peroxidase [28]. The electrode coupled to a wall-jet detector cell was applied to determination of the hydrogen peroxide and 2-butanone peroxide operating in an acetonitrile-water (90:10, v/v) environment. A promising poly(ester-sulfonic acid) polymeric film (the Eastman-AQ ionomer) was used to entrap the peroxidase onto the surface of the carbon paste electrode [29]. This offers additional advantages as it is perselective, yields a fast response, has high enzyme loading and is stable in organic media [30]. The developed biosensor was applied to the measurement of the peroxide value (using the long-chain lauroyl peroxide as standard) in vegetable oils, allowing high injection rates of over 120 samples per hour and a detection limit of about 2.5
mg/L lauroyl peroxide. Therefore, the diluted oil samples were injected directly into a buffer-saturated chloroform carrier solution which transported it to the downstream thin-layer glassy-carbon detection system, enforcing the idea of the absence of the need for sample pre-treatment and the possibility to perform the assays in a challenging sample matrix when operating in an organic media. Regarding the working characteristics and sensitivities observed in the presence of organic media using carbon paste electrodes [27], modified carbon microelectrodes with a thin-layer geometry, prepared by immobilizing Arthromyces ramosus peroxidase (ARP) and HRP [31, 32], were applied to the determination of hydrogen peroxide and organic peroxides. They exhibited a fast response time as well as low detection limits. In fact, the improved mass transfer with higher signal-to-noise ratios, fast response time and possibility of working in media with low electrical conductivity are recognized advantages in the use of microelectrodes [33]. Additionally, the role of composite HRP biosensors by coimmobilization with another enzyme has also been assessed [34], thereby benefiting from its inherent advantages. Herein, a glucose oxidase (GOD) - HRP biosensor operating in a medium of reversed micelles was developed for the biosensing of aromatic amines. To construct these biosensors, rigid composite pellets of graphite and Teflon were employed, enabling the coimmobilization of the GOD and HRP by simple physical inclusion. In this case, the in situ generation of the H$_2$O$_2$ needed for enzymatic reaction with the aromatic amines was allowed, thus preventing the inhibitory effect that the presence of a high H$_2$O$_2$ concentration in solution has on HRP activity. In situ H$_2$O$_2$ generation was performed by oxidation of glucose catalyzed by GOD. The behaviour of this biosensor (in a wall-jet cell arrangement) was evaluated in a flow injection system using reversed micelles as the carrier, formed with ethyl acetate, a 5% of phosphate buffer (pH 7.0) containing glucose and sodium dioctylsulfosuccinate. Linear calibrations curves were obtained for $o$-phenylenediamine and 2-aminophenol over the 0.4-6.0 x 10$^{-6}$ M concentration range and detection limits of 9.6 x 10$^{-8}$ M and 1.0 x 10$^{-7}$ M were achieved, respectively.

2.1.2. Tyrosinase-Based Biosensors

The first organic-phase tyrosinase electrode was reported by Wang et al. [35] and was applied to the determination of phenol content in olive oils. Herein, the tyrosinase was adsorbed onto the graphite surface. Diluted oil samples were injected directly into a buffer-saturated chloroform carrier and the designed wall-jet detector provided effective flow injection operation with a detection limit of 4x10$^{-7}$ M phenol and sample frequency of 60 per hour. Other work on a tyrosinase biosensor operating in very low water-containing organic solvents as reaction medium was reported by Mannino et al. [36], involving the stimulation by increasing water contents (up to 1%) of the biocatalytic activity of the immobilized tyrosinase in the presence of substrate. The biosensor was based on the incorporation of tyrosinase into a ceresin paste matrix. This organic-phase water biosensing scheme was applied for indirectly measuring the moisture content of food products such as butter, margarine and lyophilized products, benefiting from the amplification imparted by water to the organic-phase enzymatic activity. Herein, the thin-layer detector was employed, connected to an acetonitrile carrier solution containing a fixed substrate concentration. The standard solutions were then prepared before use by adding the appropriate amount of water in the carrier phase and the increasing moisture content therefore induced biocatalytic conversion of the cathecol by the tyrosinase. This indirect biosensing approach is an alternative to the Karl-Fisher titration method. A tyrosinase-based biosensor, conceived using a self-gelatinizable graft copolymer of poly(vinyl alcohol) with 4-vinylpyridine (PVA–g-PVP) as an immobilization matrix to a glassy carbon surface, was optimized and the effect of some organic solvents on the biosensor response was assessed [37]. This immobilization approach appears as an alternative among existing immobilization techniques, avoiding some loss of enzyme activity and the excessive consumption of tyrosinase while also possibly increasing the useful life of the electrode. An extended linear range for phenol in hydrophilic organic solvents was stated, due to the mentioned solvent-induced changes in the biocata-
lytic efficiency and subsequently in the apparent Michaelis-Menten constant when operating in nonaqueous media [5] and the possibility of its application in monitoring the organic reaction process was promised. Recently, a microelectrode array-based dual-phase biosensing system incorporating immobilized tyrosinase, was developed for the detection of phenolic compounds in both the aqueous and organic phases [38]. Herein, the tyrosinase was immobilized onto the platinum disc microelectrode using a very thin layer of hydrophilic polyurethane polyethylene oxide colloidal dispersion and the cell containing the enzyme was encapsulated with a hydrophilic dialysis membrane. It should be stressed that the use of the microelectrodes instead of electrodes of conventional size was of utmost importance in obtaining short equilibrium times and higher signal-to-noise ratios as well as low detection limits. This system could operate in several organic solvents such as heptane, hexane, chlorobenzene, toluene and chloroform. The sensitivity, linear range and response time were all shown to be very much dependent on the relative hydrophobicity of the organic solvents and substrates (phenol and catechol) investigated as they affected the extraction of the analyte into the hydration layer (formed on the surface of the dialysis membrane) before the biochemical reaction took place.

Reversed micelle systems represent another kind of media that has been exploited for tyrosinase-based biosensors operation. A tyrosinase electrode, coupled to a wall-jet arrangement cell, prepared by adsorption of the enzyme on the graphite surface with the above-referred Eastman AQ polymer immobilization approach, was used to measure various phenolic compounds [39]. In this case, reversed micelles were formed using ethyl acetate as organic solvent, dioctyl sulfosuccinate as emulsifying agent and a 4% of phosphate buffer of pH 7.4 as aqueous phase. The content of aqueous phase needed for enzyme hydration and consequently enzyme functioning, is easily controlled and optimized [40]. The possibility of using these media as the carrier in flow injection analysis and for on-line determination of phenolic compounds as well as for enzyme inhibition measurements was demonstrated. For the latter application, the study of the inhibition process was carried out using benzoic acid, thiourea or propyl gallate as inhibitors of tyrosinase and phenol as the substrate. Soon after, a similar tyrosinase biosensor operating in a medium of reversed micelles similar to that described above [39] was applied to the determination of the antioxidant tert-butylhydroxyanisole (BHA) in commercial biscuits [41]. The BHA was extracted from the samples using ethyl acetate, the continuous phase of the reversed micelles and then concentrated before being diluted with the reversed micellar system prior to injection into the reversed micelle flowing stream. A detection limit of 0.03 mM of BHA was achieved. In fact, this reaction media [42] allows the enzymatic determination of compounds scarcely soluble in water and furthermore the continuous phase of this system (organic solvent) used for the extraction of the BHA is subsequently used to form an appropriate reversed micellar medium in which the analytical measurements are carried out efficiently and thereby dramatically simplifying the analytical procedures and preventing any possible analyte losses.

The scope of the tyrosinase and peroxidase-based organic-phase biosensors expands their applicability towards many important poorly water-soluble analytes. A carbon fiber modified enzyme electrode for on-line biomonitoring of organic streams has been reported [43]. Both peroxidase and tyrosinase were immobilized by simple adsorption onto an electrochemically-pretreated carbon fiber surface. The resulting enzyme-based detectors respond very rapidly to dynamic changes in the concentration of organic peroxides, phenolic compounds in flowing chloroform and acetonitrile solutions. The flow injection behaviour is characterized by the good degree of precision achieved, detection limit of 2-4 x 10^{-5} M and high sample throughput (60 samples per hour). In addition, these organic-phase biosensors were also used for assaying challenging pharmaceutical products. The tyrosinase or horseradish peroxidase were entrapped onto the glassy carbon electrode using the polymeric film Eastman-AQ immobilization approach and offered reliable determination of phenol or peroxide in different antibacterial formulations [44]. These experiments were performed using an acetonitrile solution (containing 4% v/v water) as carrier. Herein, the pharmaceutical formulations were dissolved in the carrier thereby avoiding the need for on-line solvent extraction of the analyte. The thin-layer electrochemical detector constructed enabled rapid and reproducible analysis of the cited pharmaceutical products. On the other hand, the study of inhibitors and the solvent-induced changes in the inhibition mechanism have aroused some interest. As previously constructed [44], tyrosinase or peroxidase electrodes were used for the first time to monitor on-line various inhibitors, such as thiourea, benzoic acid, diethyldithiocarbamate, hydroxylammonium sulphate, 2-mercaptoethanol, dichlorophenoxycetic acid and dimethylmercury, by decreasing the flow-injection peaks of the 2-butanol peroxide and phenol substrates used as the maximum reference signal [45]. These thin-layer enzyme electrode arrangements in a flow-injection system operating in an acetonitrile (containing 2% (v/v) phosphate buffer) medium permitted highly sensitive (probably due to changes in the reaction velocity in this organic media) and rapid measurements to be made of enzyme inhibition with different linear ranges and sensitivities for each inhibitor, as well as the study of the kinetics and mechanism of the enzyme inactivation involved.

2.1.3. Other Enzyme-Based Biosensors

Development of the electrochemical enzyme-based detectors has been extended beyond the “model” enzymes HRP and tyrosinase. Wang et al. carried out a study on the biocatalytic activity of lacase [46]. The above cited Eastman-AQ polymeric film was used for entrapping laccase onto the glassy carbon, by covering its surface with the mixed polymer-enzyme solution. Experiments were performed in a mixed alcohol solvent/buffer solution (95:5 % v/v) and the thin-layer enzyme electrode was shown to respond rapidly to low concentrations of catechols and hydroquinone, allowing a sample frequency of 60 per hour and detection limit for hydroquinone of 6x10^{-7} M. In recent years, a significant number of reports on other enzyme electrodes have emerged. For this purpose enzymes such as catalase, cholesterol esterase and cholesterol oxidase as well as glucose oxidase were selected, showing their potential use for biocatalysis in nonaqueous environments. A biosensor based on an immobilized catalase enzyme in organic-phase solution was reported [47]. The catalase was immobilized by glutaraldehyde on a
natural protein membrane in a thermostatically-controlled thin-layer enzyme cell. The sample loop of the injector which was connected to the thin-layer amperometric cell with glassy carbon measuring electrode was replaced by the enzyme cell. The sample was directly placed into the enzyme cell and, only after a stopped-flow period required to complete the enzymatic reaction, it was injected into the flow system. The developed method was applied to the indirect monitoring of the water content in oily food samples such as butter and margarine, by adding a fixed hydrogen peroxide standard concentration to each treated sample solution. By simply varying the buffer content (0-1.5 %) of the standard solutions, a calibration curve was performed using 2% buffer in the acetonitrile carrier solvent. Subsequently, a biosensor for the detection of hydrogen peroxide in dimethyl sulfoxide (DMSO) and 1,4-dioxane was developed [48]. The catalase was entrapped in polyacrylamide gel and the entrapped catalase gel was secured to the platinum electrode using cellulose and polytetrafluorethylene (PTFE) membranes coupled to a cell of wall-jet arrangement. A decreased sensitivity was observed in the case of the organic solvents when compared to the aqueous solution, probably due to some degree of enzymatic inactivation. Due to mass transport limiting conditions across the membranes, it was possible to monitor higher concentrations of hydrogen peroxide (mM), namely when a hydrophobic PTFE membrane was used. The throughput of this enzyme electrode was measured at 10 injections per hour which seems to be a noteworthy improvement when compared to the same measurements carried out by batch methodologies. Additionally, Adányi and Váradi [49] developed a measuring set-up for the determination of cholesterol content in organic media. It allowed the determination of free and total cholesterol in food samples such as lard, butter and pasta. For the former, cholesterol oxidase (COD) was used while for the latter, a bi-enzyme cell containing immobilized cholesterol esterase (CE) and COD was used. The immobilization approach and analytical procedure of the FIA system were both similar to that described above [47]. Experiments were carried using as carrier an acetonitrile solution, 2.4 % buffer and 40 % toluene while the linear measurement range for the hydrophobic substrates cholesterol oleate and cholesterol was 0.1-0.5 mM. To conclude, a glucose oxidase-based biosensor was developed for the determination of glucose content as the model system in organic media [50]. For this purpose, GOD was immobilized in a similar manner and operated in an identical manifold to that in the above-mentioned reports [47, 49]. Additionally, it could operate in several organic solvents. The selected solvents were acetonitrile and 2-propanol with 6% and 20% buffer contents respectively, as they permit the highest signals. Linear relationships were obtained up to 0.5 or 1.0 mM glucose in acetonitrile or in 2-propanol, respectively. This biosensor was also used to measure glucose content in oily food samples with a frequency of 50-60 samples per hour.

2.2. Enzyme Reactors

In continuous flow systems, a flowing stream of carrier and co-reactants move through the enzyme reactors. Herein, the enzymes are immobilized within inert support materials, mainly porous glass beads [51] before being packed in appropriate reactors thereafter. These devices when applied in the flow injection mode offer not only the selectivity, economy and stability gained by immobilization but they also ensure complete recovery and repeated usage of the same enzyme preparation and hence, a fixed degree of turnover from cycle to cycle is maintained.

In contrast to the enzyme electrodes, little attention has been devoted to enzyme reactors operating in water-restricted conditions, which only represent 30 % of the analytical applications (19% using non-covalently immobilized enzyme reactors and 11% by the use of covalently immobilized ones) (Fig. 2). The number of applications of enzyme reactors in nonaqueous flow injection methodologies is paradoxically lower than would be expected. In fact, with the use of enzyme reactors, higher or similar sampling rates could be achieved compared to using the organic-phase enzyme electrodes as detection devices. Furthermore, the non-covalent immobilization procedures of the enzymes to the support are simpler and non-time consuming against common procedures. The use of immobilized enzyme reactors reflects the aforementioned remarkable benefits offered in nonaqueous media enzymology operation and from their assembly with flow injection systems. This combination permits the direct and rapid analysis of compounds poorly soluble in water, in addition to low reagent consumption and effluent production and most importantly, the enhanced stability of the enzymatic flow reactors.

2.2.1. Non-Covalently Immobilized Enzyme Reactors

Due to the insoluble nature of the enzymes in most organic solvents used, simple adsorption to the phase boundary of the inert support is often sufficient to reach the immobilization. The non-covalent immobilization method relies on non-specific physical interaction between the enzyme protein and the surface of the matrix. The common way to prepare this immobilized enzyme is to mix the aqueous solution of the enzyme with the porous support and remove the water under a stream of cool air, leading to everything in the aqueous solution being deposited on the support.

The first approach to implementation of a flow enzyme reactor application in nonaqueous media was proposed by Braco et al. [52] in which the feasibility of a non-covalently immobilized reactor was shown. A bienzymatic reactor, resulting from the co-immobilization of horseradish peroxidase and cholesterol oxidase operated in a water-saturated toluene medium containing p-anisidine. The resultant coloured oxidized p-anisidine was then monitored spectrophotometrically. Both the cholesterol oxidase and horseradish peroxidase were physically retained onto controlled pore glass beads to form the bioactive material. With this flow COD-HRP reactor, a linear response up to 1.8x10^{-4} M cholesterol, detection limit of 1.0 μM cholesterol and sampling throughput of 60 samples per hour were achieved. Following this, a room-temperature phosphorimetric (RTP) sensor with the same bienzymatic reactor for cholesterol determination in food samples (eggs and butters) was developed [53]. Oxygen consumed was followed via the changes in the RTP of an oxygen-sensitive metal chelate retained on an anion-exchange resin. Two spatial configurations for the enzyme reactor and the RTP sensor were evaluated: one in which the biocatalytic beads inserted into a thermostatically-controlled reactor, were physically separated from the RTP sensing phase (packed into the flow cell) and the other in which the
biocatalytic beads and RTP sensing beads were closely packed into the flow cell. Operating in a mixture of hexane/5% (v/v) chloroform saturated with aqueous buffer, the developed system showed both a higher linear response (up to 4 x 10^{-3} M) and detection limit (5 x 10^{-3} M cholesterol) compared to the previous method, with the approx. 2 min response time being due to the lower carrier flow rate employed in establishing the substrate contact with the enzymatic reactor and subsequent RTP response. More recently, Piñeiro-Avila et al. made use of the non-covalently immobilized reactor in samples from the food industry [54, 55] and environmental field [56], using identical manifold and spectrophotometric detection to that in the above-cited report [52]. A non-covalently immobilized horseradish peroxidase reactor [54] was implemented for the determination of peroxides in a phosphate buffer (pH 7.0)-saturated toluene containing p-anisidine environment. This method yielded a detection limit of 0.9 μM for hydrogen peroxide, 2.6 μM for tert-butyl hydroperoxide and 2.0 μM for benzoyl peroxide with a maximum sampling frequency of 60 per hour and was applied to olive oil and margarine samples. Thereafter, by co-immobilization with the cholesterol oxidase, the flow methodology was applied to the direct determination of free cholesterol in liposoluble foodstuffs such as animal greases and cod liver oil and for total determination after a simple microwave-assisted saponification step [55]. A good linear relationship up to 1.8x10^{-3} M cholesterol, a detection limit of 1 μM and sampling throughput of 60 samples per hour were provided by the method. A similar flow COD-HRP reactor was applied to the determination of cholesterol in sediments [56] after extraction with a chloroform-methanol mixture and, preconcentration and dissolution of the extracts in the buffer-saturated toluene containing p-anisidine carrier. This allowed the determination of both free and total cholesterol following microwave-assisted saponification of the extract. The linear range was up to 1.8x10^{-4} M with the detection limit being 2.0 μM of cholesterol. The present method can also “discriminate” the cholesterol from the cholestanol due to the analytical sensitivity difference achieved, creating the possibility of both compounds being accurately determined in the same sample.

2.2.2. Covalently Immobilized Enzyme Reactors

Covalent bonding on the surface of an inert matrix offers a stable immobilization approach for preparations to be used in continuous flow reactors, in contrast to the non-covalent immobilization that can result in preparations containing enzymes susceptible to being washed out by the flow. The strong covalent bonds resist harsh treatment which ensures additional stability of the resulting enzymatic preparations [57]. The terminal amino acid residues of the enzyme are attached to chemically-activated supports such as cellulosic, synthetic polymers, glass or others. One of the most widely used methods involves alkylamino glass and glutaraldehyde. In addition to the stability of the enzymatic preparations, the covalent attachment of enzymes to an inert matrix takes advantage of exposed reactive groups on the enzyme surface [58]. However, these immobilization procedures are complicated and time consuming when compared to those non-covalently attached.

An approach to the implementation of a covalently immobilized flow enzyme reactor application in chloroform media was proposed a few years ago [59] and applied to the determination of trace amounts of atrazine in pre-treated corn samples. The tyrosinase was chemically immobilized on controlled pore glass beads (CPG) using glutaraldehyde as cross-linking agent and packed in a glass microcolumn. The developed procedure was based on inhibiting the tyrosinase-catalysed oxidation of catechol in chloroform saturated in phosphate buffer with spectrophotometric detection of the o-quinone formed. A linear relationship from 1.0-7.0 mg L^{-1} and detection limit of 0.5 mg L^{-1} atrazine were achieved with a sample throughput equivalent to 12 samples per hour.

A thermal assay probe (enzyme thermistor) has been used to demonstrate the feasibility of employing water/organic solvent mixture with the enzyme thermistor set-up for enzyme-activity measurements [60, 61]. Indeed, the temperature response of the enzyme thermistor is dependent on the enthalpy change of the reaction and of the heat capacity of the system. Since the specific heat of organic solvents is up to three times lower than that of water, it should be expected that the sensitivity of the thermal assay probe could be increased by performing the assays in organic media. For instance, GOD and catalase were coimmobilized as well as the peroxidase onto CPG and the lipase column was prepared after non-covalently attaching onto Celite [60, 61]. The resultant beads were then packed in columns and placed in the thermal probe of the enzyme thermistor unit. It was noticed that rather small amounts of alcohols (around 5 % (v/v)) in the buffer approximately doubled the temperature response for GOD and catalase and the heat production by peroxidase was higher in toluene saturated with buffer. The glyceryl tributyrate determinations were more than twice as sensitive when operating in cyclohexane benefiting from the good solubility of the substrate in this reaction medium. Moreover, a lower operational stability of the Celite-lipase column when compared to the CPG immobilization approach was reported. Additionally, it was immobilized the β-lactamase and α-chymotrypsin enzymes onto CPG and CPG or Eupergit C, respectively [61]. For the former, with the addition of the 5 % (v/v) ethanol in buffer, an increase of the response of more than twice was obtained for the determination of penicillin. For the latter, a linear temperature response was found on increasing concentrations of amino acid esters when operating in a dimethylformamide (DMF)- buffer (10:90, v/v) mixture as reaction medium. Recently, a similar enzyme thermistor was proposed [62] in which the peroxidase is immobilized in a Teflon column in a way similar to the previous CPG immobilization approach. A thermometric procedure to detect the binding retinoic acid-HRP conjugate to retinol binding protein (RBP) was developed and the method enabled butanone peroxide (BP) to be detected in the organic phase and hydrogen peroxide in the aqueous phase. The stability of HRP in the organic phase was used to study the stability of a retinoic acid-HRP conjugate bound to immobilized RBP, constituting a scheme for the design of a thermometric retinol biosensor. Several organic solvents (acetone, acetonitrile, methanol and 2-butanol) with a water content of 1% were used for detection of BP in the flow injection analysis mode. A linear range between 1 and 50 mM BP
was obtained in all organic solvents with a precision of 5-7% while the magnitude and nature of the thermometric response differed in each organic solvent.

3. CONCLUSIONS AND FUTURE TRENDS

Biocatalysis with immobilized enzymes in nonaqueous media with flow injection analysis has been exploited regarding the potential of this assembly for analytical determinations, resulting in fast, confined, precise and reliable assays. The several approaches herein described have been applied to assaying a wide range of poorly water-soluble analytes from inaccessible and challenging sample matrices, emanating from the fields of both quality control testing and industrial process control. Enzymatic preparations operating in nonaqueous media predominantly consist of the enzyme electrodes, used as detection devices and enzymes entrapped within inert supports. In fact, regarding the working characteristics of the mentioned enzyme electrodes, one possible strategy that could overcome some of the operational deficiencies experienced in those enzyme electrodes is based on the multi-site detection concept [63] which consists of changing the position of the flow-through detector by means of an injector-commutator. In this sense, the detector relocation between two different streams permits efficient electrode re-conditioning (circumventing the adsorption effects to the electrode active surface and the subsequent need for physical or chemical treatment of its surface) and allows the composition and flow rate of the conditioning/washing stream in the second channel to be selected irrespective of the carrier stream, besides the reduction in sample/electrode contact.

FIA has been the only flow methodology used to perform biocatalysis in a nonaqueous environment due to the beauty of its simplicity and its world-wide acceptance as an analytical tool. In this context, apart from FIA, the use of more recent flow methodologies such as sequential injection analysis [14], multicommutation [15], multisyringe [16], lab-on-valve [17] and multipumping [18] is expected to carry out biocatalysis in a nonaqueous environment, following the ongoing technological advances regarding the equipment and computer support for the overall control of the flow system devices and for the processing and acquisition data. Indeed, due to its inherent characteristics, these new solution handling techniques could permit the development of more versatile and computer-controlled systems, allowing the assessment of distinct analytical strategies without the need for physical reconfiguration of the flow set-up. In addition, they could be well-suited to toxic organic solvents, minimize the consumption of reagents and organic solvents used, and consequently, the amount of toxic effluent produced. In view of the favourable characteristics involved in using these modes of flow analysis, we foresee other innovations exploiting these features for nonaqueous biocatalysis.

Moreover, one can explore enzymes not only in relatively simple organic solvents and reversed micelle systems but also in a variety of other environments, namely ionic liquids. In fact, enzymatic catalysis in ionic liquids [64] is steadily becoming a new trend in biochemistry and chemical technology as they represent environmentally friendly substitutes for volatile organic compounds because of their negligible vapour pressures at room temperature, often being highly stable. Additionally, they can act as solvent or catalyst and are capable of undergoing multiple solvation interactions with many types of molecules. Therefore, on the basis of their high ability to dissolve a wide variety of substrates together with the custom-synthesized solvent properties of the ionic liquids, further on-line analytical applications of enzymatic procedures in those reaction media are envisaged using flow injection methodologies [65].

ACKNOWLEDGEMENTS

One of us (A.R.T.S.A.) thanks FCT and FSE (III Quadro Comunitário de Apoio) for the Ph.D. grant (SFRH/BDE 23029/2005).

REFERENCES
