Sequential injection analysis as a tool for implementation of enzymatic assays in ionic liquids

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**A R T I C L E   I N F O**

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**A B S T R A C T**

An approach based on the use of water/1-butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF4]) mixtures in a sequential injection analysis (SIA) system is presented. The rapid and robust procedure developed was used to evaluate horseradish peroxidase activity in [bmim][BF4] and is intended to be a generic tool for enzymatic assays in ionic liquids.

The horseradish peroxidase activity tests were based on the implementation of the 4-aminoantipyrine (4AAP)/phenol test in the SIA system, using 1-naphtol as substrate. Small volumes (12 μL) of sample, reagents and enzyme were sequentially aspirated to the holding coil before being sent to the spectrophotometric detector (λ = 510 nm), where a coloured product proportional to the enzyme activity was measured.

The results were compared to those obtained when the assay was performed in water/methanol mixtures under the same conditions, to evaluate [bmim][BF4] as an alternative to conventional organic solvents. Comparative evaluation of the enzyme behaviour revealed that the enzyme activity increased significantly when the assay was performed in water/[bmim][BF4] mixtures.

The SIA methodology exhibited good repeatability over the full concentration range (R.S.D. < 3.3%, n = 15) studied, produced approximately 1.7 mL of effluent and consumed approximately 36 μL of solutions prepared in water/[bmim][BF4] mixtures for each analytical cycle.

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1. Introduction

The implementation of procedures involving enzymatic reactions in flow systems has grown with the evolution of enzyme catalysis and use of new reaction media, leading to a large number of enzymatic procedures with a wide range of analytical purposes [1]. Among flow techniques, sequential injection analysis (SIA) has been used for a large variety of analytical determinations including enzymatic reactions, proving itself to be a robust and accurate solution handling approach [2,3]. Its unique mode of operation, based on the forward and reversed flow of well defined zones of sample and reagent solutions through a multiposition valve makes it a very economic tool since it permits the aspiration of precise volumes and an effective utilisation of solutions, in the process drastically reducing the consumption of sample and reagents [4]. Furthermore, the effective computer control of the most relevant analytical parameters at run-time ensures great operational flexibility, which allows the assessment of distinct analytical strategies without physical reconfiguration of the flow set-up and facilitates system optimisation [5]. This is particularly important in procedures that demand precise control of the reaction conditions such as enzymatic reactions, since it guarantees standardised conditions with all assays. These features greatly increase the analytical potential of SIA manifolds, making it a first choice technique and explain why SIA has been increasingly used for the mechanisation of enzymatic procedures that either involve expensive enzymes or small sample amounts, mostly in the biological area. Within this perspective, SIA, due to its particular advantages, can once again become a powerful tool in this area.

The use of water as solvent in enzymatic reactions for years limited the field of application of enzymes in biocatalysis and the productivity of some processes, particularly those involving hydrophobic substrates [6]. As a consequence, the applicability of catalysis in non-aqueous solvents has been tested and discussed and as a result, new reaction media have been suggested [8]. The possibility of working with hydrophobic substances, the decrease in microbial contamination and reduction of side reactions are the
main advantages related to the development of procedures in non-aqueous media [6]. Organic solvents helped to reduce some problems such as the insolvability of hydrophobic compounds and propagation of radicals in aqueous solutions and were shown to be good solvents for enzymes catalysis, in some cases offering benefits related with enzyme stability and selectivity [9,10]. However, organic solvents exhibit known disadvantages such as human and environmental toxicity and high volatility that make their application in routine procedures difficult. These drawbacks can be substantially reduced through implementation of these procedures in closed flow systems but some cases of enzyme inactivation in organic polar solvents have been related, limiting the applicability of these solvents and requiring specific activity studies [11].

As a consequence, researchers have in the last decade embarked upon exploring a new group of compounds, room temperature ionic liquids (ILs), in an attempt to establish their applicability in biocatalysis [12]. Due to their particular characteristics, ILs have emerged as “green” substitutes for conventional organic solvents in enzymatic reactions, sometimes with remarkable results, offering new possibilities for the application of solvent engineering to biocatalytic reactions [13]. Their specific properties such as nonflammability, nonvolatility and good chemical and thermal stability made them a safe alternative to conventional organic solvents [14]. However, the study of enzyme activity in ILs, apart from enzyme availability and reagents toxicity, must consider the high cost of these solvents that hinder their routine utilisation. Moreover, although IL are known as green solvents, the assays must still be performed in order to produce minimal wastes, since its long-term toxicity is not as yet known [15].

Considering the characteristics of enzymatic assays in ILs, its implementation in a SIA manifold, although having never been tried, seems very promising and challenging, since it combines the advantageous features of enzyme catalysis in IL with the versatility and low consumption of SIA. Thus, the present paper describes the implementation of an enzymatic assay, performed in a water miscible IL in a SIA system. The assay was based on implementation of the 4-aminoantipyrine (4AAP)—phenol test [16] using horseradish peroxidase as catalyst, 1-naphthol as substrate and 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF4]) as solvent. This is a very well known reaction catalysed by an enzyme that is very abundant in nature and involved in many biological reactions. Besides this, the peroxidase mediated coupling of phenols is one of the most studied redox processes in biochemistry and highlights the importance of the use of non-aqueous reaction media to aid solubilisation of hydrophobic substances, increasing the field of the studies [17].

The developed work also involved a comparative study of the enzyme activity in methanol, to evaluate ILs as an alternative to conventional organic solvents. The presented approach was intended to provide a fast, robust and economic generic means of evaluating enzyme activity in IL which could additionally represent a basis for the future mechanisation of analytical procedures.

2. Experimental

2.1. Reagents

All solutions were prepared using chemicals of analytical reagent grade and high purity water (milli-Q) with a specific conductivity <0.1 μS cm⁻¹.

The carrier solution of the flow system comprised a Briton and Welford universal buffer solution with pH adjusted between 6 and 12.

Daily, a horseradish peroxidase solution (Sigma, Type VI-A) of 0.1 mg mL⁻¹ was prepared in water from a 1 mg mL⁻¹ stock solution. The stock solution was stored in the refrigerator and remained stable for about 3 days.

A stock solution of 1-naphthol 0.15 mol L⁻¹ was prepared by dissolving the appropriate amount of powder in a water/[bmim][BF4] mixture (1:2), which guaranteed the full solubilisation of the compound. Standard solutions of 1-naphthol were prepared from the stock solution by suitable dilutions in water/[bmim][BF4] mixtures of 1:0.5, 1:1 and 1:1.5, all of which were stable for several weeks. For the comparative studies of enzyme activity, stock and standard solutions of 1-naphthol were prepared as described but by replacing [bmim][BF4] with methanol.

4AAP 0.025 mol L⁻¹ and hydrogen peroxide 0.0175 mol L⁻¹ were prepared daily in water/[bmim][BF4] mixtures of 1:0.5, 1:1 and 1:1.5.

2.2. Apparatus

Spectrophotometric measurements were made in a 6300 Jenway spectrophotometer, set at 510 nm and equipped with a 30 μL flow cell (Helma 178.7110S, Müllheim, Baden, Germany).

The SIA system (Fig. 1) consisted of a Gilson Minipuls 3 peristaltic pump, equipped with PVC pumping tube (1.2 mm i.d.) and a 10-port multiposition Vici Valco selection valve. Manifold components were connected by means of 0.8 mm i.d. PTFE tubing which was also used for the holding and reaction coil (4 and 1 m, respectively).

Analytical system control, including the operation of the peristaltic pump and selection valve, was achieved by means of an Advantech PCL 711B interface card and a Pentium-I-based microcomputer. Software was developed in Microsoft Quick-Basic and permitted the control of flow rate, flow direction, valve position, sample and reagent volume as well as data acquisition and processing. During optimisation, the analytical signals were also recorded on a Kipp & Zonen BD 111 strip chart recorder.

In the evaluation studies on the influence of temperature on horseradish peroxidase, the reaction coil was thermostatized between 30 and 45 ± 0.5°C in a Falc FA950 temperature controller.

2.3. Sequential injection procedure

The analytical cycle established for the implementation of the 4AAP-phenol test in the SIA system is summarised in Table 1. Initially, small volumes (12 μL) of horseradish peroxidase, H₂O₂, 1-naphthol and 4AAP were sequentially aspirated to the holding coil. Then, the flow was reversed and the reaction zone propelled by the carrier solution through the reaction coil, directly to the detector where the product of the reaction was measured. For the comparative assays involving the use of water/methanol mixtures, the procedure was exactly the same in order to conduct a comparison under the same conditions.
3. Results and discussion

This approach based on insertion of water/[bmim][BF4] mixtures in a SIA system to evaluate horseradish peroxidase activity was developed in four steps that involved the implementation of the 4AAP-phenol test in the flow system, evaluation of enzyme activity in [bmim][BF4], study of the influence of media conditions (pH, temperature, concentration of IL) on the activity and comparison of these results with those obtained when the reaction took place in water/methanol.

3.1. Implementation of the 4AAP-phenol test on a SIA system

The 4AAP-phenol test was used as a means to evaluate horseradish peroxidase activity since it is a very well known chemical reaction that fits the purpose of the developed work which does not involve, at this time, an innovative analytical application. The reaction is based on the oxidation of phenol by H2O2 in the presence of horseradish peroxidase and subsequent reaction of the formed radical with 4AAP to form a coloured product, with maximum absorption at 510 nm. The enzymatic reaction was performed in [bmim][BF4], a water miscible IL that has been successfully tested in some enzymatic reactions.

The main problem related with the insertion of IL, namely [bmim][BF4], in a SIA system is its high viscosity that complicates the reproducible aspiration of small IL volumes and its dispersion on an aqueous carrier solution. However, [bmim][BF4] is a water miscible IL, so it is possible to work with mixtures that exhibit less viscosity and can be reproducibly aspirated to the flow system. Nevertheless, while optimising the procedure it proved very difficult, even with water/IL mixtures, to get an effective mixture of the aspirated aliquots in the SIA system. It is important to highlight that, due to the specific characteristics of SIA manifolds, the interdispersion of the aspirated zones in this kind of system is only partial but is essential for development of the chemical reaction. In the studied situation, when the aspirated volumes were between 50 and 150 µL, double peaks were recorded, revealing mixing problems due to different viscosities of the aspirated zones and carrier solution. To solve this problem, the aspirated volumes were reduced to a minimum in order to get a homogeneous reaction zone and measurable analytical signal. The final analytical cycle involved the aspiration of 12 µL of each solution since this volume guaranteed repeatable results and high analytical signals in a single peak shape.

The concentrations of 4AAP and H2O2 were established as 0.025 and 0.0175 mol L\(^{-1}\), respectively. These were maximum concentrations above which no variation in analytical signal was apparent, so that the future analysed changes were undoubtedly related with the change of enzyme activity and not due to the lack of reagents. The aspiration flow rate was 0.67 mL min\(^{-1}\) since it permitted the reproducible aspiration (R.S.D. < 3%) of the chosen small volumes. Due to the high sensitivity of the 4AAP-phenol reaction, the propulsion flow rate was set at 2 mL min\(^{-1}\) in order to decrease the residence time and dispersion of the reaction zone. Flow rates between 0.8 and 1.5 led to an increase in residence time of the reaction zone which could reach 75 s, resulting in a decrease in analytical signals due to the enhanced dispersion.

Among several coils (0.5–1.5 m), a 1 m long figure-eight-shaped reaction coil was chosen to perform the assays since it permitted efficient mixing of the aspirated zones without excessively increasing the dispersion of formed product.

The developed analytical procedure exhibited very interesting characteristics, performing around 60 determinations per hour and producing around 1.7 mL of effluent per analytical cycle. Repeated analysis of samples (n = 15) of different concentrations did not show R.S.D. (%) greater than 3.3, which is very acceptable considering the small aspirated volumes.

3.2. Evaluation of horseradish peroxidase activity in [bmim][BF4]

Under the described set of conditions it was possible to produce 1-naphthol calibration curves up to 0.015 mol L\(^{-1}\), showing that the enzymatic catalytic action is proportional to the amount of 1-naphthol. The evaluation studies of enzyme activity were based on the sensitivity of the determinations.

Once established the initial assay conditions, the effect of pH, temperature and concentration of [bmim][BF4] on horseradish peroxidase activity were studied.

The composition of the buffer carrier solution was changed in order to attain solutions with pH values between 6 and 12 and then study the effect of this parameter on the enzyme activity. These studies were performed at room temperature using a water/[bmim][BF4] mixture (1:0.5) as reaction media. It was observed that enzyme activity increased with pH up to 11.2, above which the analytical signals decreased, being inexistent at pH 12 (Fig. 2). These observations confirmed the results of Sgalla et al. [10] and showed that under these conditions, the maximum activity peak is achieved in conditions distinct from that obtained in aqueous media [18]. This difference could be related with the inhibition of horseradish peroxidase by the F\(^{-}\) anion of [bmim][BF4], which binds to the heme iron of peroxidase under acidic pH conditions [10]. Thus, the enzyme activity in ILs is strongly pH dependent and increases under alkaline conditions.

The effect of temperature on enzyme activity was studied by immersing the reaction coil of the SIA system in a thermostatically controlled water bath. Assays were performed with a carrier solution of pH 11.2 and as before, with a water/[bmim][BF4] mixture (1:0.5) as reaction media. Analytical signals increased very slightly with the temperature of the water bath up to 40 °C and higher temperatures led to a significant decrease in analytical signals, revealing the onset of the process of enzyme denaturation. With the aim of evaluating the influence of concentration of [bmim][BF4] on horseradish peroxidase activity, a study involving

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**Table 1**

<table>
<thead>
<tr>
<th>Step</th>
<th>Position</th>
<th>Time (s)</th>
<th>Flow rate (mL min(^{-1}))</th>
<th>Volume (µL)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.67</td>
<td>12</td>
<td>Aspiration of peroxidase</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0.67</td>
<td>12</td>
<td>Aspiration of H2O2</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>1</td>
<td>0.67</td>
<td>12</td>
<td>Aspiration of 1-naphtol</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>1</td>
<td>0.67</td>
<td>12</td>
<td>Aspiration of 4AAP</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>50</td>
<td>2</td>
<td>–</td>
<td>Propulsion to the detector</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Influence of the pH of the carrier solution on horseradish peroxidase activity in [bmim][BF4].

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the use of water/[bmim][BF₄] mixtures using different proportions (1:0.5, 1:1, 1:1.5) was performed. As previously described in Section 2, these mixtures were used as solvent for the preparation of 1-naphtol, 4AAP and H₂O₂ solutions which were then introduced in the SIA system. It was verified that the sensitivity of the determination as well as enzyme activity, increased with the increase in [bmim][BF₄] concentration until proportions with water of 1:1.5. These observations confirm the excellent activity of horseradish peroxidase in this IL but also its enhancement in the presence of increasing concentrations of [bmim][BF₄]. The implementation of the assay in a SIA system hindered efforts to test more concentrated mixtures due to the increased viscosity of the resulting solutions. However as expected, it was verified that the enzyme activity was totally lost if prepared exclusively in [bmim][BF₄], showing that the presence of water is essential for enzyme activity. This issue has already been discussed regarding other enzymes activity in organic media [19] and very recently in IL [20] and it is accepted that a few water molecules, presumably bound to charged groups on the surface of the enzyme, are required for catalytic function. It is also important to consider the problem of enzyme solubility which also hinders the exclusive use of organic solvents or IL [13]. If it was technically possible to continue increasing the amount of [bmim][BF₄] in the solvent mixture, in the SIA system, the enzyme activity would increase until the amount of water was so small that deactivation of the enzyme occurred.

3.3. Evaluation of horseradish peroxidase activity in methanol

The study of horseradish peroxidase activity in methanolic media was intended to provide information on performing a comparative evaluation of enzyme behaviour in [bmim][BF₄] and methanol, with a view to suggesting the utilisation of ILs as an alternative to conventional organic solvents. On this basis, using the same manifold and the same conditions used in the test involving [bmim][BF₄], an evaluation of horseradish peroxidase activity in methanol was performed. This evaluation comprised the implementation of the 4AAP-pheno test in the SIA system, using water/methanol mixtures as reaction media, the assessment of pH influence, temperature and methanol concentration on enzyme activity and a comparison of the results obtained with those obtained in the presence of [bmim][BF₄].

The 4AAP-pheno test was again implemented in the SIA system, maintaining all of the established conditions except the solvent for the preparation of the solutions, which in this case was methanol. At this point it was important to evaluate whether the optimum enzyme activity would be achieved under the same conditions described in the studies with IL and if the studied parameters would have the same influence on enzyme activity.

To begin, the evaluation of horseradish activity was performed at room temperature and pH 11.2, and using a 1:0.5 water/methanol mixture, which comprised the initial set of conditions for the assays with [bmim][BF₄]. These conditions were chosen in order to conduct the studies in the same direction and perform comparisons step by step. This assay showed that the enzyme activity was maintained but decreased significantly when compared under the same conditions, with the activity exhibited in the presence of [bmim][BF₄] (Fig. 3).

The study of pH influence on the sensitivity of the determination was performed as before by changing the pH of the carrier solution between 6 and 12. It was concluded that the optimum situation in terms of the enzyme activity was achieved at pH 7.5. Effectively, there was an enhancement of horseradish peroxidase activity until this value and beyond which a significant decrease occurred, showing that, in the absence of inactivation phenomena by ILs in acid solutions, the enzyme exhibits maximum activity at pH values similar to those described as the optimum in water [18]. These results are in good agreement with several studies involving peroxidase catalyzed reactions performed in methanol at pH between 7 and 7.5 [21,22].

The influence of temperature on the activity of horseradish peroxidase in methanol was evaluated using a carrier solution of pH 11.2 (Fig. 4). A significant enhancement in enzyme activity up to 40 °C was observed, showing that in this media, temperature performs an important role on enzyme activity, probably due to an increase in stability of the enzyme–substrate complex. Above 40 °C, it was only possible to evaluate the enzyme activity at 45 °C but already with some problems related with the volatility of the solvent which resulted in the formation of air bubbles inside the flow system. Nevertheless, the obtained results showed that the enzyme maintained its activity at this temperature, meaning that under these conditions there is no denaturation of the proteic structure of horseradish peroxidase and confirming the theory of enhanced thermal stability of enzymes in organic media [23].

Solutions of water/methanol 1:0.5, 1:1 and 1:1.5 were prepared and as previously described, tested in the SIA system, in order to evaluate the influence of methanol concentration on horseradish peroxidase activity. As in the case of [bmim][BF₄], an increase in enzyme activity up to proportions of 1:1.5 was observed, showing that a predominantly aqueous media does not provide the best conditions for horseradish peroxidase performance. The results are similar and probably related to the high solubility of 1-naphtol in both [bmim][BF₄] and methanol that results in a preferential partition of the hydrophobic compound into the non-aqueous phase.
As a result, when the non-aqueous phase increases, there is also an increase of substrate in the interface between the organic and aqueous phase, enhancing its contact with the enzyme and consequently, the sensitivity of the determination.

4. Conclusions

This approach based on the insertion of water/[bmim][BF4] mixtures in a SIA system is intended to be a generic tool for the implementation of enzymatic assays in ILs and has allowed us to confirm once again that SIA is an accurate fluids handle technique that minimizes consumption of reagents and production of effluents, thus being an excellent choice for enzymatic assays in general.

Furthermore, it is important to highlight that the presented approach resulted in a fully automatic procedure and represents an evolution relative to the classic methods traditionally used in this kind of study that are subject to constant operator intervention and all the drawbacks and errors associated with this. On this basis, the developed methodology reduced the operator exposure to 1-naphthol and solvents and produced a very small amount of effluent when compared to conventional batch procedures, resulting in increased environmental and human safety. Moreover, the consumption of reagents was dramatically reduced due to the possibility of strictly aspirating the required small solution volumes on a reproducible basis. This fact is of utmost importance considering the high prices of the commercial ILs and makes the assays less expensive and more promising due to the possibility of exploiting all potentialities of this kind of solvent with reduced amounts of solutions.

Moreover, the implementation of the 4AAP-phenol test in the SIA using water/[bmim][BF4] as reaction media, led to a fast, versatile and robust methodology that could also be applied in routine determination of phenols.

Regarding horseradish peroxidase activity, the assays showed that the enzyme has maximum activity at pH 11.2 and 40 °C and maintained its activity in water/[bmim][BF4] mixtures up to 1:1.5. Furthermore, the comparative evaluation of enzyme behaviour under these conditions showed that there is an enhancement in enzyme activity of about 5 times when the assay was performed in water/[bmim][BF4], confirming the thesis that in this media enzymes exhibit improved stability, selectivity and activity and that this solvent can be advantageously used as an alternative to conventional organic solvents [14]. This is probably explained by the polarity of ILs that results in strong charge–charge interactions with the enzyme that may be responsible for its activation. However, it is obvious that this complex issue is related to other parameters, most of them unexplained, that affect enzyme–solvent interactions. Globally, it is accepted that IL generate a stabilizing microenvironment that enhances enzyme activity. Nevertheless, the enormous variety of IL and its tuning nature open up many possibilities in terms of research and do not allow general conclusions and considerations to be taken.

With this work it was possible to yet again confirm the potential and importance of SIA for the mechanisation of biocatalytic procedures by opening a new perspective on the implementation of enzymatic assays in IL, so that in the near future, this kind of strategy could become an important analytical tool for enzymatic procedures that demand increased sensitivity.

References