Automatic flow methodology for kinetic and inhibition studies of reactions with poorly water-soluble substrates in ionic liquid systems


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ABSTRACT

In the present work an automatic generic tool, based on sequential injection analysis (SIA) for kinetic and inhibition studies of reactions with poorly water-soluble compounds in ionic liquid (IL)-containing systems, is described.

The oxidation of the poorly water-soluble phenolic compound, caffeic acid, catalyzed by the mushroom tyrosinase, in different 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF\(_4\)]) / buffer mixtures as reaction media, was investigated. This determination was based on measuring depletion rate of the substrate caffeic acid at its maximum wavelength (\(\lambda_{\text{max}}\) 311 nm).

The influence of several parameters such as substrate and enzyme concentration, temperature, pH, delay times and measurement periods on the sensitivity and performance of the SIA system were studied and the optimum reaction conditions subsequently selected.

The obtained results showed that tyrosinase was active in oxidising caffeic acid in this water-miscible IL and the presence of an impaired tyrosinase activity with increase in [bmim][BF\(_4\)] concentration as an increase in the apparent Michaelis–Menten constant (\(K_{\text{app}}\)) was observed while the maximum reaction rate (\(V_{\text{app}}\)) remained fairly constant. The results were compared to those obtained when the assay was performed in water/methanol mixtures under the same conditions to substantiate [bmim][BF\(_4\)] as an alternative to conventional organic solvents.

Additionally, it was shown that tyrosinase is effectively inhibited by the substrate analogues tested (trans-cinnamic acid and 3,4-dihydroxybenzoic acid) in the IL-containing aqueous system used.

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1. Introduction

Room-temperature ionic liquids (ILs) are usually considered environmentally friendly solvents, possessing almost no vapour pressure and having negligible toxicity as well as high chemical and thermal stability [1]. Thus, they have been established as convenient green solvents, capable of replacing organic solvents as a reaction medium in a number of organic synthetic and biocatalytic reactions [2]. Indeed studies on enzymatic efficiency, enzyme stability and selectivity suggest that ILs represent a promising new class of solvents for performing enzymatic reactions [3–8]. Furthermore, on the basis of ILs' high capacity to dissolve a wide variety of poorly water-soluble substrates, they could effectively replace conventional organic solvents and new analytical applications for enzymatic procedures in these reaction media are envisaged.

However, to fully appreciate the possibilities of applying enzymes in ionic liquids, it is important to understand the behaviour of the enzymes in this reaction media. Some batch kinetic studies on enzymatic catalysis using ILs as reaction media have been undertaken [9–13] to establish not only the ability of ILs to affect reaction rates but also their manner in modifying reaction mechanisms [14]. However, the high cost of these solvents, the need to minimize produced waste and issues centred on unknown, long-term toxicity [15] should not be ignored.

Implementation of these enzymatic assays in ILs in flow manifolds has been shown to be clearly advantageous since it guarantees low consumption of solvent and reagents, is highly versatile and makes it possible to mechanise these enzymatic assays in IL-containing aqueous systems [16]. Sequential injection analysis (SIA) [17] has been regarded as a good option for automation since the technique’s inherent capacity to handle precise volumes as well as the economic characteristics inherent to this technique, are essential for the purposes of this type of work. Moreover, flow techniques represent a source of dynamic and kinetic information as the concentration gradient formed (controlled as a function of space and time) carries the dynamic or physical information via its infinite number of elements with different dispersion values. Hence, different concentration ratios of sample and reagent are possible and the reactions that occur embody the kinetic or chemical information [18]. Thus, the used methodology guarantees both the exact
timing of fluidic manipulations and precise control of the reaction conditions. In addition, reproducibility is guaranteed in all operations, thereby guaranteeing standardized conditions under which all determinations are made. With batch methodologies, it is difficult to ensure reproducibly rigorous time control within all samples which is crucial in controlled kinetic processes such as enzymatic catalysis.

Therefore, the development of an automatic analytical flow methodology based on SIA, for rapid and reliable kinetic studies of the biocatalysis of poorly water-soluble substrates in IL-containing aqueous systems, is an improved option for studying biocatalysis in ILs.

Oxido-reductase enzymes have the capacity to catalyze difficult reactions such as selective oxidations and reductions of organic molecules in ionic liquid reaction systems and their application in oxidative biotransformations is particularly important in the environmental field. Furthermore, oxido-reductases are largely used in organic synthesis, in the production of chiral compounds, modification of ketones, alcohols and aldehydes while also being extremely important in the pharmaceutical area [19]. Due to its specific regioselectivity, tyrosinase has been one of the most synthetically useful oxido-reductases [20], facilitating the formation of various products that are important in areas such as the pharmaceutical and fine chemical industries [21]. This enzyme catalyzes the dehydrogenation of o-diphenols to o-quinones using molecular oxygen [22]. Tyrosinase has been shown to exhibit greater activity in organic solvent media than in aqueous medium [23]. Moreover, due to their poor water solubility, the phenolic derivative substrates are normally dissolved in an alcoholic organic solvent [24,25]. Furthermore, it was reported that the yields of phenolic acids from fruits and plant extraction increased with increasing amounts of methanol in the solvent [26] and the methanolic extracts obtained were directly used for total phenolic content analysis.

Herein, it were performed in an automatic mode kinetic studies of the mushroom tyrosinase-mediated oxidative coupling using caffeic acid (phenolic acid) as substrate. The influence of methanol on enzyme activity, similar caffeic acid solutions were prepared in the stock solution by suitable dilutions in the [bmim][BF4]/buffer mixtures. All such solutions were shielded from light throughout use.

A bromocresol green sodium salt dye (Fluka) solution was used to determine the dilution factors and concentrations of the reactants in the flow cell by measuring absorbance at 614 nm. A series of dye dilutions were prepared to ensure that the absorbance was linearly proportional to the concentration within the range investigated.

### 2.2. Flow manifold and instrumentation

System components were arranged as shown schematically in Fig. 1. The analytical SIA flow system comprised a Gilson Minipuls 3 (Villiers-le Bel, France) peristaltic pump, equipped with a 1.30 mm i.d. Gilson PVC pumping tube. This pump was connected to the central channel of a ten-port electrically controlled selection valve (Fig. 1, SV) (Valco, Vici C25-3180D, Houston, USA). Omnitfit polytetrafluoroethylene (PTFE) tubing (0.8 mm i.d.) together with Gilson end-fittings and connectors were used to assemble the different manifold components. The holding coil (HC) and reaction coil (RC) were respectively 3.0 and 0.5 m length, both of which had a figure-eight-shaped configuration.

A Heλios Gamma UV visible spectrophotometer equipped with a thermostatic cell holder and flow-through Hellma cell (80 µL, ref. 178.710QS) were used as detection system and the wavelength set at 311 nm. The cell holder was connected to a GFL Thermoiled 5001 thermostatically controlled bath.

The SIA flow system was controlled by means of a microcomputer equipped with an interface card (Advantech Corp., PCL 711B, San Jose, CA). Software was developed in QuickBasic 4.5 (Microsoft) and permitted operation of the peristaltic pump and multi-position selection valve, enabling the run-time definition of all analytical parameters such as flow rate, flow direction, sample volume, reagents volume and valve positioning as well as data acquisition and processing. Analytical signals were also recorded on a strip chart recorder (Linseis, L 250 E).

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Fig. 1. SIA manifold for performing the biocatalytic oxidation of the caffeic acid in [bmim][BF4]/buffer and methanol/buffer mixtures as reaction media and for evaluating the inhibitory effect of trans-cinnamic acid and 3,4-dihydroxybenzoic acid. C: Carrier, Britton and Welford universal buffer pH 6.5; PP: peristaltic pump; HC: holding coil; SV: selection valve; S: caffeic acid solution or caffeic acid-inhibitor mixture; E: enzyme solution; RC: reaction coil (50 cm); D: spectrophotometric detector; and W: waste.
A Perkin-Elmer, Lambda 45 UV–VIS spectrophotometer was used to perform the batch determinations on the oxidation of the caffeic acid in ionic liquid by the tyrosinase.

2.3. Sequential injection procedure

The analytical cycle established for kinetic evaluation of caffeic acid oxidation in the SIA system is summarised in Table 1. Initially, the substrate and enzyme were sequentially aspirated to the holding coil (Steps a and b). Following flow reversal, the reaction zone was propelled through the reaction coil into the detector cell by the carrier solution and during a pre-established stop period, the depletion of the caffeic acid was monitored with signal acquisition (Fig. 2). The cycle was concluded by cleaning the flow cell with the carrier solution (Step e).

2.3.1. Initial rate measurement

The stacked zones were directed to the flow cell (Step c) and a stopped-flow period of 60 s was implemented (Step d) with absorbance readings being continuously taken (4 readings/s). On completion of the stop period, the pump was reactivated and the contents of the flow cell washed out, thus being ready for the next analytical cycle. Within this stopped-flow period, a fixed data collection interval was chosen (20–60 s) during which the slope of the absorbance readings being continuously taken (4 readings/s). On completion of the stop period, the pump was reactivated and the contents of the flow cell washed out, thus being ready for the next analytical cycle.

Reaction rates (determined in ΔA/s units) were then calculated according to zero-order kinetics by linear least-squares regression of the data obtained over the respective period. This relationship was considered linear after visual inspection of the graphs (number of points >50) and when the correlation coefficient was greater than 0.995.

The apparent Michaelis–Menten constant (K_{app}^m) and maximum reaction rate (V_{app}^m) were obtained by nonlinear regression to a plot of the initial rate values (V_0 − ΔA/s) against [S] by using the GraphPad Prism 4 program for Windows. When caffeic acid depletion was measured, the V_0 values were determined at short reaction times from quadruplicate measurements at each [S].

3. Results and discussion

The development of the automatic system for kinetic studies with ILs was tested using the oxidation of caffeic acid, a poorly water-soluble substrate, by the tyrosinase (Fig. 3). To this end, the water miscible ionic liquid ([bmim][BF_4]) was used as a nonaqueous component of the reaction system to help solubilise the caffeic acid.

Several batch methods have been used to measure tyrosinase activity, including radiometric (measuring ^3H released from radiolabeled dopa) [28], electrometric, amperometric [29], voltammetric [30] and spectrophotometric methods. Radiometric methods are discontinuous, laborious and time consuming in nature. Continuous electrometric methods (based on the measurement of oxygen consumption [31], proton production or uptake [32]) have also been reported but are not widely used. More recently, amperometric and voltammetric methods have also been proposed as an alternative to spectrophotometric methods, permitting significant improvements in analysis time. Spectrophotometric methods combine features such as convenience, sensitivity, low cost and the ability to continuously follow the course of the reaction to be studied. Some of these methods measure the formation of o-quinones or their products as they evolve to form aminochromes [22,33,34]. Other methods based on reaction of the corresponding o-quinones with coupling reagents such as ascorbic acid [35] and NADH [36] measure their disappearance through oxidation by the quinones, and other nucleophilic reagents that trap the o-quinones and generate chromophoric adducts [37–39]. However, some problems were reported such as instability of the o-quinones formed and interference from absorption of the intermediates or products [40] which restrict the applicability of methods based on this principle. On this basis, it is preferable to follow the course of the reaction by measuring depletion of the substrate at its λ_{max} [25].

At the outset, a 10-min incubation period of caffeic acid with tyrosinase in the IL-containing aqueous system was tested under a batch mode. This produced characteristic changes in the UV–vis spectrum (Fig. 4) which did not occur in mixtures without enzyme or caffeic acid. Two distinct isosbestic points appeared at 267 and 339 nm, while absorbance declined at 311 with an increase over the 400–450 nm range (due to formation of the characteristic quinones [41]) after enzyme addition. Therefore, in the present work, the

![Fig. 3](image-url) Oxidation of caffeic acid to o-quinone catalyzed by mushroom tyrosinase using molecular oxygen.
monitoring wavelength selected to carry out the biocatalytic oxidation of the caffeic acid in the SIA system was set at 311 nm.

3.1. Design and optimization of the SIA method

The optimum working conditions of the SIA system for evaluating the enzyme kinetics and inhibition in IL-containing aqueous systems were studied by the univariate approach with the influence of several parameters on its working characteristics being evaluated. The studied range of parameters as well as final conditions selected for kinetic evaluation of caffeic acid oxidation by tyrosinase are summarised in Table 2.

To determine the concentration of each reagent in the detector cell, dye injection experiments were conducted to determine the dilution coefficients of the reactants [42], using the protocol outlined in Table 1. Therefore, all concentrations presented in this work refer to the concentrations in the flow cell. These experiments also showed a complete overlap of the corresponding zones of the substrate caffeic acid and enzyme tyrosinase solutions in the flow cell detector, which illustrated a complete mixture of both reagents [43].

3.1.1. Protocol sequence

The solutions involved in this reaction were drawn into the HC in the following order: caffeic acid substrate in the different [bmim][BF₄]/buffer mixtures and then enzyme. The enzyme should be the last reagent to be aspirated into the HC to minimize its dilution. Furthermore, the caffeic acid plug in the [bmim][BF₄]/buffer mixtures should be kept as small as possible to facilitate an effective mixing of the aspirated aliquots due to their viscosity. A 0.5 m figure-eight-shaped reaction coil connected the valve to the detector. Consequently, the final cycle involved the aspiration of 10 μL of substrate in the [bmim][BF₄]/buffer mixture and 20 μL of enzyme since this sequence guaranteed repeatable and single peak-shape analytical signals.

3.1.2. Enzyme concentration

This parameter was studied between 11 and 217 U mL⁻¹ by maintaining the same experimental conditions described above. The enzymatic reaction rate increased proportionally with the amount of enzyme up to 54 U mL⁻¹ and from this concentration it exhibited a steady-state profile. For the highest tested enzyme concentration (217 U mL⁻¹), the absorbance/time relationship was not linear over the 60 s signal-monitoring period. Similar results were found for the other caffeic acid solutions tested. Bearing in mind that zero-order conditions can only be achieved as long as the catalyst is limiting and that the reaction rate must be proportional to enzyme concentration to evaluate the Michaelis–Menten kinetics [44], a 54 U mL⁻¹ enzyme concentration was chosen.

3.1.3. pH and reaction temperature

The influence of pH of the carrier buffer solution on reaction rate development was investigated within the 5.5 and 7.5 range as the optimum pH of mushroom tyrosinase is between 6 and 7 (Sigma, Product Information). Herein, one should account for some buffering effect of the ionic liquid used from both the cation and anion [13]. Concentrations of caffeic acid ranged between 0.026 and 0.158 mM while enzyme concentration was 54 U mL⁻¹ and the temperature was set at 25 °C. An increase of reaction rate up to pH

Preliminary studies were performed to assess if the volume of solution sent to the detector (Table 1, Step c) influenced the reaction rate. Hence, 10 μL of caffeic acid standard (0.311 mM) and 20 μL of enzyme solution (109 U mL⁻¹) were sequentially aspirated into the HC containing carrier buffer at a pH of 6.5. These stacked zones were sent over different time intervals through the flow cell before the flow was halted, to get the corresponding sections of the reaction zone to different volumes and dilutions. In each of these experiments, the content of the flow cell was allowed settle for 20 s after reaching the detector and before data was considered for slope calculation over 40 s. The results obtained for intervals between 15 and 22 s are depicted in Fig. 5. For further studies, this interval was fixed at 18 s (corresponding to a displacement volume of 600 μL) since it yielded the steepest slope rates.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
<th>Tested values</th>
<th>Selected value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time interval for propelling HC to detector (s)</td>
<td>15–22</td>
<td>15, 16, 17, 18, 19, 20, 21, 22</td>
<td>18</td>
</tr>
<tr>
<td>Enzyme concentration (U mL⁻¹)</td>
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<td>11, 27, 54, 82, 109, 163, 217</td>
<td>54</td>
</tr>
<tr>
<td>pH of carrier buffer</td>
<td>5.5–7.5</td>
<td>5.5, 6.0, 6.5, 7.0, 7.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>25–50</td>
<td>25, 30, 40, 50</td>
<td>30</td>
</tr>
<tr>
<td>Time period for signal acquisition (s)</td>
<td>0–120</td>
<td>–</td>
<td>20–60</td>
</tr>
</tbody>
</table>

Table 2

Range of values used in the study of system parameters and selected values for its operation.
From Table 3 that caffeic acid showed a higher affinity with tyrosinase in the reaction medium of the 1:1 [bmim][BF4]/buffer mixture ratio than that obtained at the higher concentration of IL reaction media, as indicated by the respective \( K_{M}^{\text{app}} \) values of 0.13, 0.24 and 0.29 mM for [bmim][BF4]/buffer mixture ratios of 1:1, 1.5:1 and 2:1, respectively. On the other hand, catalytic efficiency (namely \( V_{\text{app}}^{\text{max}} / K_{M}^{\text{app}} \) ratio) was also used as an indication of the ability of the enzyme to convert substrate into the corresponding final products under a given condition. Once again, the catalytic efficiency of tyrosinase in the reaction media of the 1:1 [bmim][BF4]/buffer mixture ratio was higher than that obtained in high IL content media. The corresponding \( V_{\text{app}}^{\text{max}} \) were kept fairly constant.

These observations illustrate the activity of tyrosinase towards caffeic acid in this water-miscible IL but also its impairment in the presence of increasing concentrations of [bmim][BF4]. Several properties of ionic liquids including polarity, hydrogen-bond basicity and anion nucleophilicity have shown strong influences on the activity and stability of enzymes [45]. Herein (particularly for tyrosinase), the polarity of the B–F bond in BF4– could partly account for the decrease in its activity although with tyrosinase being a copper-containing enzyme [46], the possibility of the fluoride ion binding to the copper ion at the enzyme’s active site cannot be ruled out [13]. Sgalla et al. demonstrated that inhibition of the reaction involving horseradish peroxidase with water insoluble phenolic substrates in [bmim][BF4]/H2O mixtures was due to the binding of fluoride anions released from the tetrafluoroborate anion to the heme iron [47]. Moreover, it was reported that a large portion of the substrate binding energy involves interactions of the polar groups on the side chain, far from the reaction site on the phenolic or diphenolic nucleus, with these polar interactions being particularly responsible for tyrosinase catalysis [48]. In this sense, the water-miscible ionic liquid used could interfere with those polar interactions. On the other hand, due to the high hydrophilicity of the IL, it is possible that this solvent could enter the aqueous microenvironment by surrounding the enzyme molecules and would therefore undergo some direct interactions with the enzyme to inactivate it. This in agreement with the fact that the presence of a bulk of water (hydration shell) is critical for enzymatic activity [49].

In summary, the overall findings show that maximal efficiency for tyrosinase catalysis of caffeic acid oxidation was demonstrated in the medium containing the 1:1 [bmim][BF4]/buffer mixture ratio, followed by those of the medium containing the 1.5:1 [bmim][BF4]/buffer mixture ratio and that of the 2:1 [bmim][BF4]/buffer mixture ratio. In fact, the observed decline in enzyme activity in aqueous solution with increase in IL content was in agreement with the literature [13,47,50]. Yang et al. [13] investigated the activity of mushroom tyrosinase towards the substrate 4-methylcatechol in IL ([bmim][BF4] or [bmim][MeSO4])—containing aqueous systems. As in our case, \( K_{M}^{\text{app}} \) values increased with IL content. Corresponding \( V_{\text{app}}^{\text{max}} \) values showed a slight increase until IL content was below 8% (v/v). Hinckley et al. [50] also reported a decrease in laccase activity on syringaldazine oxidation with increasing concentrations of the 4-methyl-N-butylpyridinium tetrafluoroborate IL but in this case, due to a simultaneous increase in \( K_{M}^{\text{app}} \) and decrease in \( V_{\text{app}}^{\text{max}} \) values.
A decrease in catalytic efficiency with an effective decrease in activity in the methanolic media was evaluated. This assay showed on this basis, using the same manifold and same reaction conditions organic solvents when performing kinetic biocatalysis is suggested. Moreover, the use of ILs instead of the toxic and hazardous conventional above-cited ionic liquid-containing aqueous systems. Additionally, evaluation of the kinetic behaviour using methanol as reaction medium was performed and was aimed at a comparative study on the catalytic activity of the tyrosinase in the above-cited ionic liquid-containing aqueous systems. Moreover, enzyme activity in the methanolic media was evaluated. This assay showed a decrease in catalytic efficiency with an effective decrease in when compared under the same conditions, in the reaction medium containing the 1:1 [bmim][BF_4]/buffer mixture ratio (Table 3). As known, enzyme structure and catalytic activity depend on the direct or indirect role of water in all interactions, including hydrogen bonding, ionic, hydrophobic and van der Waals interactions [54]. On this basis, the organic solvent used could distort the native protein structure and somewhat destroy enzymatic activity to a greater extent than the IL assayed. These experiments strengthened the use of the ILs as reaction media for tyrosinase biocatalysis evaluation instead of conventional organic solvents.

3.3. Effect of inhibitors on the diphenolase activity of mushroom tyrosinase in ionic liquid–aqueous systems

Inhibition studies could provide information on the mechanistic properties of enzyme-catalyzed biochemical processes. Indeed, to date, no kinetic studies of inhibitors on enzymatic catalysis using IL as reaction media have been reported.

Cinnamic acid and benzoic acid derivatives have been reported as inhibitors of mushroom tyrosinase using L-3,4-dihydroxyphenylalanine as substrate, in phosphate buffer medium [55,56]. Herein, it was intended to test the tyrosinase inhibitory ability of such compounds using caffeic acid as substrate in the IL-containing aqueous system reaction medium. Thereafter, by taking trans-cinnamic acid and 3,4-dihydroxybenzoic acid as the effectors, the impact of these compounds on the activity of mushroom tyrosinase for the biocatalytic oxidation of caffeic acid was probed. The inhibitors were dissolved in the 1:1 [bmim][BF_4]/buffer mixture ratio and assays without caffeic acid were performed to disregard any interference at the wavelength of the determination. Therefore, several inhibitor concentrations were initially mixed with different concentrations of substrate caffeic acid solution. Resulting solutions were then aspirated through the available inlets of the selection valve (Table 1, Step a) and the analytical cycle proceeded as described.

Activities measured with the caffeic acid and inhibitors mixture were considered as a relative percentage of the tyrosinase activity obtained without the inhibitors, which correspond to 0% inhibition. Thus, tyrosinase inhibition efficiency was expressed as the percentage inhibition of the biocatalytic action of the tyrosinase in the caffeic acid. Results obtained for the compounds tested are presented in Fig. 7.

Both inhibitors presented higher inhibition efficiency with the increase of their concentration (up to 0.395 mM) for each caffeic acid concentration tested. However, the behaviour of such inhibitors was demonstrated to be quite different. Regarding the trans-cinnamic acid as inhibitor, the results obtained pointed to a concomitant increase in inhibition efficiency with increase in caffeic acid concentration. In fact, this statement could expose an uncompetitive inhibition behaviour, which works best when substrate concentration is high [44]. Herein, the trans-cinnamic acid only exhibited its inhibitory effect for caffeic acid concentrations equal to or higher than 0.105 mM. Plotting 1/V_0 versus 1/[I] gives a family of parallel straight lines with the same slopes. Accompanying the increase of the inhibitor concentration, the values of both K_{app} and V_{app} decreased, but the ratio K_{app} / V_{app} remained unchanged. The obtained inhibition constant was 0.23 mM.

In the case of the 3,4-dihydroxybenzoic acid, a distinct response with increase in substrate was observed. However, the obtained Lineweaver–Burk plots did not result in a typical type of inhibition. In fact we believe that if we could have tested higher concentrations of inhibitor, which was impaired by its low solubility, would have resulted in a competitive behaviour.

In addition, the effect of such inhibitors for a fixed caffeic acid concentration (0.105 mM) was studied. With increasing concentrations of inhibitors (up to 0.789 mM) the remaining enzyme activity steadily decreased. The IC_{25}, corresponding to the concentration at which 25% inhibition efficiency was verified, was...
determined to be 0.514 and 0.560 mM for trans-cinnamic acid and 3,4-dihydroxybenzoic acid, respectively.

4. Conclusions

The obtained results substantiate the usefulness and reliability of the developed automatic procedure as a robust alternative to carry out kinetic studies of enzymatic reactions with poorly water-soluble substrates and evaluate the effect of potential inhibitors in ionic liquid/buffer mixtures as reaction media. Consumption of ionic liquid and reagents are all reduced to a minimum as is subsequent waste production. The proposed method permits analyses of various mixtures, including ionic liquid/buffer mixtures as reaction media. Consumption of organic solvent as reaction media for biocatalysis evaluation. However, the method does have a lower degree of automation, thus introducing eventual errors associated with operator intervention.

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