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# Activity assay based on the immobilized enzyme kallikrein and mass spectrometry

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Deregulated activity and expression of human kallikreins (KLKs) may be involved in various pathologies, so these enzymes are an attractive biological target for identifying molecules that can modulate KLK activity. This identification involves applying fast and efficient screening methods. This work describes an off-line assay with mass spectrometry (MS) detection that uses KLK immobilized on Sepharose-NHS as a micro-column configuration (IMER-KLK-Sepharose-NHS). The mass spectrometry used has an ion trap analyzer and electrospray ionization (EIS). The HPLC-MS method for quantifying KLK activity was developed. The enzymatic assay conditions were optimized, and the IMER-KLK-Sepharose-NHS kinetic parameter ( $K_{Mapp} = 15.48 \pm 3 \mu\text{mol L}^{-1}$ ) was evaluated. Finally, the method was validated by using leupeptin as a reference inhibitor ( $IC_{50} = 0.85 \pm 0.10 \mu\text{mol L}^{-1}$ ). The developed method was able to identify the reference inhibitor and can be an alternative for screening KLK inhibitors.

## KEYWORDS

immobilized enzyme, HPLC-MS, off-line assay, kallikrein enzyme, ligand screening assay

## Introduction

Kallikreins (KLKs) are a group of serine protease enzymes that play an important role in biological fluids and tissues. KLKs can be divided into two groups: plasma kallikrein (KLK 1B) and tissue KLKs (KLK1–15) (Prassas et al., 2015). KLK 1B is exclusively expressed by hepatic cells and is part of the plasma kallikrein-kinin system, which, among other things, participates in the processes of fibrinolysis, blood coagulation, inflammation, cancer, and pathologies related to the cardiovascular system (Schmaier and Mccrae, 2007; Costa-Neto et al., 2008). Under physiological conditions, plasma kallikrein serves as a cardioprotective enzyme. However, its increased plasma concentration or hyperactivity perpetuates cardiovascular disease (CVD) (Kolte and Shariat-Madar, 2016). Tissue KLKs comprise human tissue kallikrein (KLK1) and 14 kallikrein-related peptidases (KLK2–15)

(Prassas et al., 2015) that share genetic and protein structural similarities. Amino acid identity among human tissue KLKs ranges from 40 to 80% (Yousef and Diamandis, 2001).

Tissue KLKs are present in several tissues of the human organism, and their unregulated expression may be associated with various diseases (Costa-Neto et al., 2008). For example, in patients with Alzheimer's and Parkinson's disease, KLK6 has decreased expression in brain tissue (Diamandis et al., 2000; Bayani and Diamandis, 2012). The human kallikrein-related peptidases 5 (hKLK5), 6 (hKLK), 7 (hKLK7), 8 (hKLK8), and 14 (hKLK14) are also involved in inflammatory skin disorders (Di Paolo, Diamandis and Prassas, 2020) with abnormal desquamation and inflammation, such as atopic dermatitis (AD), psoriasis, and the rare disease Netherton syndrome (NS) (Zani et al., 2022). In addition, KLKs are dysregulated in most solid tumors, especially in hormone-dependent tumors (Kryza et al., 2016). KLK3 also known as prostate specific antigen (PSA) is used as a biomarker for prostate cancer because this enzyme is overexpressed in these types of cancers and KLK3 presents dysregulated expression in breast tumors (Yu et al., 1995). KLK2 is overexpressed in prostate tumors (Darson et al., 1997). KLK7 overexpression in colon cancer tissues is involved in cancer cell proliferation (Walker et al., 2014).

Given that some enzymes underlie pathologies that affect humanity, searching for molecules that can inhibit them is a relevant topic when searching for a treatment or cure for these pathologies (Copeland, 2013). In this scenario, KLKs are important targets when it comes to developing drugs that can inhibit their activities or restore their expression (Kolte and Shariat-Madar, 2016; Xie et al., 2020). This is particularly relevant in the context of anticancer studies because KLKs have been shown to modulate cancer cell multiplication (Shang et al., 2014; Walker et al., 2014). The PROSTVAC vaccine, developed for prostate cancer treatment, is an example of a therapeutic resource that targets KLK3 and multiple T cell co-stimulatory molecules (TRICOM) (Madan et al., 2009).

Developing tools that allow enzyme inhibitors to be discovered and developed is therefore essential. In this sense, enzyme immobilization is an important tool for screening ligands that can potentially inhibit enzymes. Assays with immobilized enzymes offer advantages over the use of enzymes in solution—they allow enzymes to be better handled and reused, and they increase enzyme stability under varying conditions (Brena, González-Pombo and Batista-Viera, 2013). Additionally, enzyme immobilization allows enzymatic activity modulation to be monitored in the presence of libraries of molecules (synthetic or natural) and complex matrixes, like crude extracts (Singh et al., 2014). Moreover, enzyme immobilization enables high-throughput screening (HTS) of ligands from such matrixes (De Simone et al., 2019). Indeed, different enzyme immobilization protocols (Rodrigues et al., 2021) and ligand screening assays, ranging from online (flow)

to offline assays, have been developed (Wubshet et al., 2019; Cieśla and Moaddel, 2016; Zhuo et al., 2016; Cheng and Chen, 2018; de Moraes, Cardoso and Cass, 2019; Q. Chen et al., 2021; Trindade Ximenes et al., 2021; de Oliveira et al., 2022).

In online assays, ligands are analyzed and screened simultaneously (Zhuo et al., 2016). These assays basically consist of an immobilized enzyme reactor (IMER) coupled to an automated system, such as high-performance liquid chromatography (HPLC) or capillary electrophoresis (Cheng and Chen, 2018; G. Y. Chen et al., 2021), which can be coupled with different detection techniques, including ultraviolet (UV) (da Silva et al., 2013), fluorescence (Hai et al., 2011), electrochemical detection (Sun, Gao and Jin, 2006), and mass spectrometry (MS) (Vilela and Cardoso, 2017).

HPLC-MS is considered the most powerful analytical tool for analyzing polar compounds, including peptides, phenols, polymers, and biomolecules (Loos, Van Schepdael and Cabooter, 2016). HPLC-MS combines separation by HPLC with the broad analysis capability of MS. The advantages of HPLC-MS include the need for a small amount of sample, high resolution power, high molecular specificity, and high detection sensitivity (Beccaria and Cabooter, 2020). The ionization mode is crucial for the performance of HPLC-MS method depending on the analytes targets, between others the electrospray ionization source (ESI) is the main ionization mode analysis for thermally labile, nonvolatile, and polar compounds. (Beccaria and Cabooter, 2020). Specifically, ESI ionization source and ion trap mass analyzer (IT) has been successfully used in IMER coupled with HPLC-MS. For example, Beta secretase1 and acetylcholinesterase (AChE) were co-immobilized and used to dual-ligand screening (Vilela, Narciso dos Reis, and Cardoso 2021) AChE-IMER (Vanzolini et al., 2013) used to screening coumarin derivatives and butyrylcholinesterase (BChE)-IMER and AChE-IMER used in on-flow mass spectrometry dual enzyme assay (Seidl et al., 2019).

On the other hand, off-line assays entail independent screening and analysis steps. However, the detection techniques mentioned above can be used in offline assays, as well (Zhuo et al., 2016; Zhang et al., 2018).

Numerous supports, such as magnetic particles (Wang et al., 2018; Q. Chen et al., 2021), silica gel microspheres (Shi et al., 2015), and Sepharose (Zofair et al., 2020), can be used to construct bioreactors that can be applied in off-line assays. Previously, we immobilized the KLK enzyme on Sepharose-NHS solid support and employed it in ligand screening assays during which we used a 96-well flat-bottom microplate to measure fluorescence (Carvalho et al., 2021). The immobilized KLK showed excellent operational and storage stability and proved an efficient tool for identifying KLK inhibitors (Carvalho et al., 2021).

Bearing the advantages of enzyme immobilization and the growing search for KLK inhibitors in mind, we present an off-line

assay based on KLK immobilized on Sepharose-NHS as a micro-column configuration and MS detection. We will show that this bioreactor, IMER-KLK-Sepharose-NHS, exhibits excellent stability, can be reused, is a useful tool in off-line ligand screening, and can be combined with an HPLC-MS method.

## Materials and methods

### Chemical and reagents

Porcine pancreas kallikrein (KLK, 250 units), *N*-hydroxysuccinimidyl-Sepharose<sup>®</sup> 4 Fast Flow, CBZ-Phe-Arg-7-amido-4-methylcoumarin hydrochloride (Z-Phe-Arg-AMC), leupeptin hemisulfate salt (Acetyl-Leu-Leu-Arg-al), and 7-amino-4-methylcoumarin (AMC) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Buffer components and other chemicals were acquired from Sigma-Aldrich, Merck (Darmstadt, Germany), Synth (São Paulo, Brazil), or Acros (Geel, Belgium). The water that was used in all the preparations was obtained from a MILLI-Q<sup>®</sup> system (Millipore<sup>®</sup>, São Paulo, Brazil). All the chemicals and solvents used here were analytical or HPLC grade and were employed without any further purification.

### Instrumentation and system configuration for analyses

HPLC-MS analyses were carried out on a NexeraXR high-performance liquid chromatography system (Shimadzu, Kyoto, Japan) equipped with three LC 20ADXR pumps, an SIL-20A automatic injector, a DGU-20A degasser, a 10-port two-position high-pressure switching valve (Valco Instruments Co. Inc., Houston, United States), and a CBM-20A system controller. The HPLC system was coupled to ion trap mass spectrometer, model AmaZon speed dual ion funnel, QIT technology (IT-MS) (Bruker Daltonics, Bremen, Germany) equipped with an electrospray source (ESI). The system was controlled by Bruker Compass Hystar software (version 4.5, Bruker Daltonics Inc., Billerica, United States). Data were acquired and analyzed by using the Compass DataAnalysis software (version 4.3, Bruker Daltonics Inc., Billerica, United States).

### Preparation of IMER-KLK-sepharose-NHS micro-column

KLK from porcine pancreas (100  $\mu\text{g ml}^{-1}$ ) was covalently immobilized on *N*-hydroxysuccinimidyl-Sepharose<sup>®</sup> 4 Fast Flow (Sepharose-NHS, solid support) as previously described (Carvalho et al., 2021). After immobilization, 80 mg of the immobilized enzyme was placed in a micro-column device

(Mobitec, Göttingen, Germany), to give IMER-KLK-Sepharose-NHS. The micro-column was washed with 50  $\text{mmol L}^{-1}$  Tris-HCl buffer (pH 8.0), and stored in the same buffer solution at 4 C. Control columns containing 80 mg of Sepharose-NHS (without enzyme) were prepared as described above.

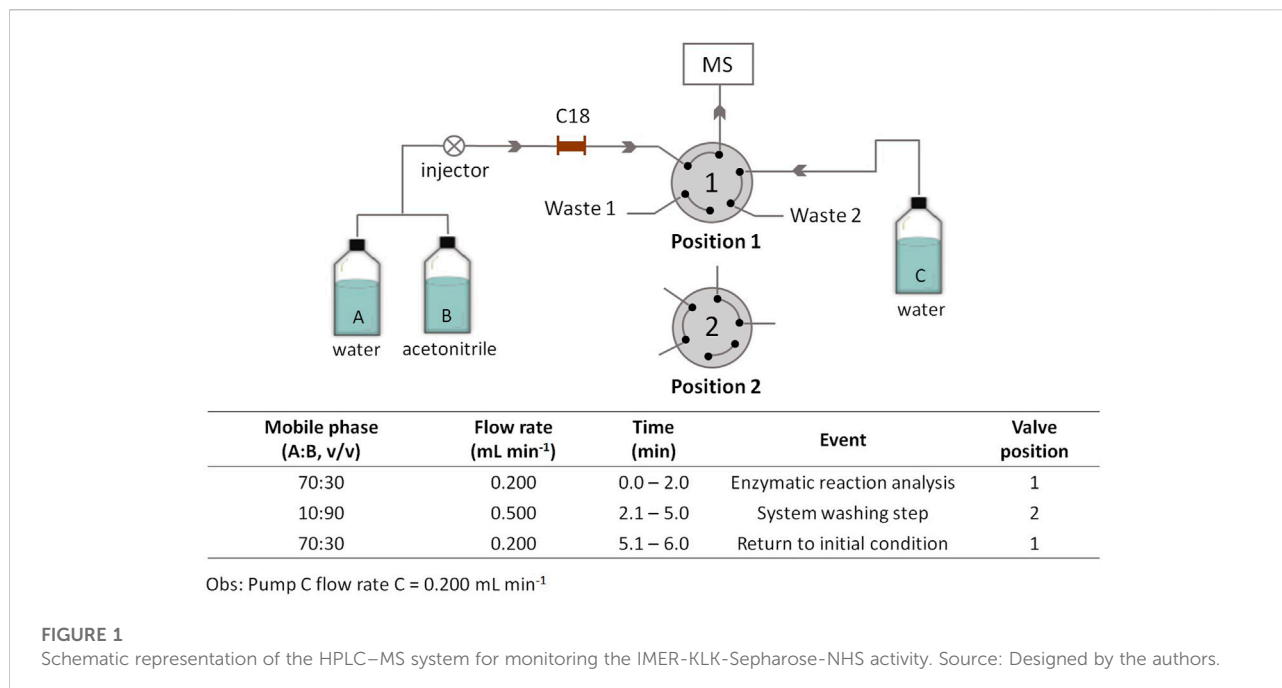
### IMER-KLK-sepharose-NHS micro-column off-line assay

Aliquots of 120  $\mu\text{l}$  of 10  $\text{mmol L}^{-1}$  ammonium acetate solution (pH 8.0), and 50  $\mu\text{l}$  of 200  $\mu\text{mol L}^{-1}$  Z-Phe-Arg-AMC substrate were added to the micro-column and incubated under gentle agitation at room temperature for 5 min. After incubation, the reaction medium was filtered and collected. Then, 20- $\mu\text{L}$  aliquots of the reaction mixture (filtered) were transferred to vials, diluted with 80  $\mu\text{l}$  of 10  $\text{mmol L}^{-1}$  ammonium acetate solution (pH 8.0), and analyzed by HPLC-MS. At the end of each reaction, the IMER-KLK-Sepharose-NHS micro-column was washed with 5 ml of Milli-Q water (three times), followed by 5 ml of 5  $\text{mmol L}^{-1}$  ammonium acetate solution (pH 8.0) three times. At the end of each reaction, the immobilized KLK micro-column was washed with 5 ml of Milli-Q water (three times), followed by 5 ml of 5  $\text{mmol L}^{-1}$  ammonium acetate solution pH 8.0 (for 3 times). Blank samples were prepared under the same reaction conditions by using control micro-columns (without enzyme). All the experiments were performed in triplicate.

The optimization of IMER-KLK-Sepharose-NHS off-line assay conditions included the effect of the reaction time and ammonium acetate solution concentration on the enzymatic activity. The effect of ammonium acetate solution concentration was evaluated at concentrations ranging from 2 to 15  $\text{mmol L}^{-1}$  (pH 8.0). For this, the immobilized KLK micro-column was previously washed with 5 ml of each concentration of ammonium acetate pH 8.0 (three times) and left under conditioning for 10 min at each concentration. The reaction time was evaluated for incubation times ranging from 5 to 40 min. The enzymatic reactions were carried out under the same conditions described above, and Z-Phe-Arg-AMC was employed as substrate. The reproducibility of the IMER-KLK-Sepharose-NHS activity assay was verified by carrying out five consecutive reaction cycles.

### Analysis of the IMER-KLK-sepharose-NHS activity by HPLC-MS

HPLC analyses were carried out by using an Ascentis<sup>®</sup> Express C18 micron guard cartridge (5 mm  $\times$  2.1 mm  $\times$  2.7  $\mu\text{m}$ , Supelco, Bellefonte, PA, United States) connected to a 10-port/two-position valve in the HPLC-MS system (Figure 1); the mobile phase consisted of water (solvent A) and acetonitrile (solvent B). Initially, with the valve in position 1, the enzymatic



reaction products were eluted from the column with an initial mobile phase composed of 30% solvent B at a flow rate of 0.2 ml min<sup>-1</sup> for analysis in the mass spectrometer. After 2.0 min, the valve was switched to position 2; solvent B was increased to 90% at a flow rate of 0.5 ml min<sup>-1</sup>, which was maintained until 5 min, to wash the column and to remove excess substrate. At 5.1 min, the valve was switched to position 1 again, and the mobile phase was returned to the initial condition. The total run time was 6 min. Pump C (solvent water) at a flow rate of 0.2 ml min<sup>-1</sup> was used to deliver the mobile phase to the MS during the column washing step. All the analyses were performed at 21 C (controlled room temperature), and the injection volume was 5 µl. The ESI ionization parameters were as follows: positive ionization mode, capillary voltage = 4500 V, end plate voltage = 550 V, drying gas flow = 7 L min<sup>-1</sup>, drying temperature = 270 C, and nebulizer pressure = 27 psi. The product of the enzymatic reaction, AMC, was monitored at  $m/z$  176 [M + H]<sup>+</sup>.

## Method qualification

Method qualification was performed on the basis of literature criteria (FDA 2011). Method linearity was evaluated by constructing a standard curve; AMC at the following concentrations was used: 50, 70, 100, 200, 300, 400, 500, 600, 800, and 1,000 nmol L<sup>-1</sup>. For this purpose, from a standard 10 mmol L<sup>-1</sup> AMC solution (in DMSO), the AMC solutions at concentrations ranging from 50 to 1,000 nmol L<sup>-1</sup> were prepared in 10 mmol L<sup>-1</sup> ammonium acetate solution. For the analytical curve, the solutions were prepared in triplicate from the concentrated stock solutions, and 5-µL aliquots

were injected into the HPLC-MS system as described above. The analytical curve was constructed by linear regression of the plot of the chromatogram area of the extracted ions with  $m/z$  176 [M + H]<sup>+</sup> as a function of the concentration. Method selectivity was evaluated by buffer analysis under the same conditions described above, but without adding AMC. The limit of quantification and the limit of detection were evaluated from the injection of AMC solutions at concentrations of 50, 30, 10, 5, and 2.5 nmol L<sup>-1</sup>. The -limit of quantification was defined as the lowest concentration that produced a signal-to-noise value higher than ten times that response obtained by analyzing the blank (buffer without AMC), while the limit of detection was defined as the lowest concentration of an analyte in a sample that can be consistently detected with a stated probability and could be reliably differentiated from the baseline. When based on signal-to-noise establishing the minimum concentration at which the analyte can reliably be detected. Typically, acceptable signal-to-noise ratios are 2:1 or 3:1. (FDA 2011). The intra- and inter-day precision and accuracy were evaluated by analyzing samples at three different concentration levels (50, 500, and 1,000 nmol L<sup>-1</sup>); each concentration level was prepared and analyzed in quintuplicate.

## IMER-KLK-sepharose-NHS kinetic constant ( $K_{Mapp}$ )

The IMER-KLK-Sepharose-NHS kinetic constant ( $K_{Mapp}$ ) was determined by using different Z-Phe-Arg-AMC concentrations (4–1,200 µmol L<sup>-1</sup>) in 10 mmol L<sup>-1</sup> ammonium acetate solution, pH 8.0. After incubation for 5 min, the reaction medium was filtered and collected, and 20-µL aliquots of the

solution were transferred to vials, diluted with 80  $\mu\text{l}$  of 10 mmol  $\text{L}^{-1}$  ammonium acetate solution (pH 8.0), and analyzed by HPLC-MS as describe above. The enzymatic activity was monitored by quantifying the AMC that was formed ( $m/z$  176  $[\text{M} + \text{H}]^+$ ). Data were fitted by using nonlinear regression into a Michaelis-Menten plot, and  $K_{\text{Mapp}}$  values were obtained with the GraphPad Prism 8.0 software.

## Inhibition studies

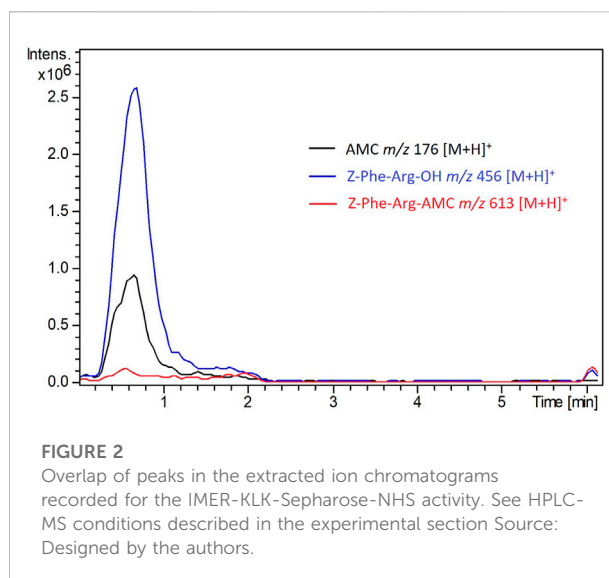
Inhibition studies were carried out by using leupeptin as standard inhibitor. To determine their half maximum inhibitory concentration ( $\text{IC}_{50}$ ), 110- $\mu\text{L}$  aliquots of 10 mmol  $\text{L}^{-1}$  ammonium acetate solution (pH 8.0), 10  $\mu\text{l}$  of leupeptin (1–2,500  $\mu\text{mol L}^{-1}$ ), and 50  $\mu\text{l}$  of the substrate Z-Phe-Arg-AMC at 200  $\mu\text{mol L}^{-1}$  were added to the micro-column and incubated under gentle agitation at room temperature for 5 min. After incubation, the reaction medium was filtered and collected, and 20- $\mu\text{l}$  aliquots of the solution were transferred to vials, diluted with 80  $\mu\text{l}$  of 10 mmol  $\text{L}^{-1}$  ammonium acetate solution (pH 8.0), and analyzed by HPLC-MS as describe above. The enzymatic activity was monitored by quantifying the AMC that was formed ( $m/z$  176  $[\text{M} + \text{H}]^+$ ). The activities in the presence ( $A_i$ ) and absence ( $A_0$ ) of the inhibitor were compared, and the percentage of inhibition was calculated by the expression:  $\%I = 100 - [(A_i/A_0) \times 100]$ . The inhibition curve was constructed by plotting the percentage inhibition *versus* the corresponding leupeptin concentration, and the  $\text{IC}_{50}$  value was determined by plot sigmoidal curve build by log [leupeptin] *versus* % inhibition with the GraphPad Prism 5.0 software.

## Results and discussion

Assays based on immobilized enzyme coupled to HPLC-MS systems have emerged as a promising alternative to colorimetric screening assays in microplates. Here, we developed an off-line assay with MS detection by using KLK immobilized on Sepharose-NHS as a micro-column configuration. We prepared the immobilized enzyme by a batch approach. After we covalently immobilized KLK onto Sepharose-NHS, we packed it into micro-column devices, to obtain IMER-KLK-Sepharose-NHS.

### HPLC-MS analysis method development

We performed chromatographic analysis in the isocratic elution mode with a mobile phase composed of water/ acetonitrile; a C18 micron guard cartridge (5 mm  $\times$  2.1 mm  $\times$  2.7  $\mu\text{m}$ ) was used. To eliminate the substrate excess and to avoid possible problems caused by ionization suppression and/or



contamination in the MS ionization source, we added a clean-up step, as described in Figure 1.

Figure 2 shows the chromatogram corresponding to the analysis of the IMER-KLK-Sepharose-NHS enzymatic reaction by the proposed method. Under the employed conditions, we eliminated the interference caused by excess substrate, but the products AMC and Z-Phe-Arg-OH co-eluted. In this case, there was no need for high-resolution chromatographic separation even though the analytes co-eluted because we later identified them according to their respective  $m/z$  ratio. Therefore, this method allowed us to monitor the KLK activity by fast analysis, which lasted only 6 min. In routine analyses, we evaluated the IMER-Sepharose-NHS enzymatic activity by monitoring the ion with  $m/z$  176  $[\text{M} + \text{H}]^+$ , which refers to the AMC product.

### Method qualification

We evaluated method linearity by constructing a calibration curve for the AMC product. The curve showed a linear response and  $R^2 = 0.99$  ( $n = 3$ ) for AMC concentrations ranging from 50 to 1,000  $\text{nmol L}^{-1}$  ( $y = 5.07 \times 10^7 x + 8.85 \times 10^5$ ); the relative standard deviation (RSD) values were below 15% for the triplicates. When we injected only the buffer under the same assay conditions, no signal due to AMC emerged, which demonstrated method selectivity. The limits of detection and quantification for AMC were 5 and 30  $\text{nmol L}^{-1}$ , respectively. The intra- and inter-day precision values were expressed in RSD and ranged from 5 to 15%. The intra-day accuracy of the method ranged from 85 to 110%. The inter-day accuracy lay between 87 and 101%. Thus, the developed HPLC-MS method can be applied to monitor the immobilized KLK activity.

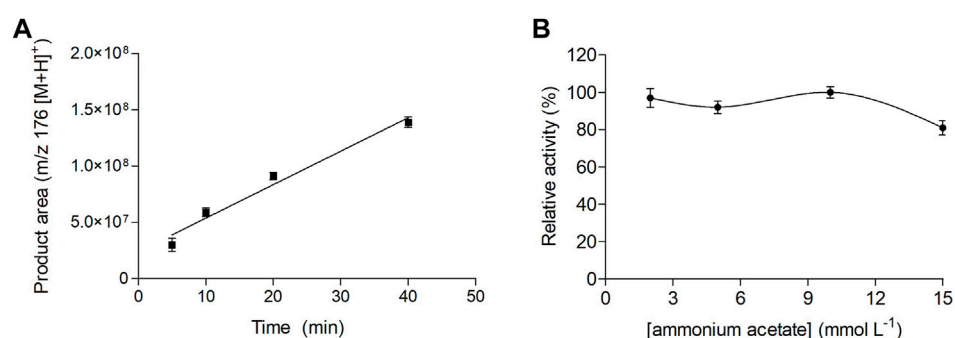


FIGURE 3

Effect of reaction time (A) and ammonium acetate solution concentration (B) on the IMER-KLK-Sepharose-NHS activity. Source: Designed by the authors.

## IMER-KLK-sepharose-NHS off-line assay conditions

We evaluated the reaction conditions for the IMER-KLK-Sepharose-NHS activity assay, namely reaction time, ammonium acetate solution concentration, and immobilized KLK micro-column washing steps. We evaluated the IMER-KLK-Sepharose-NHS activity at different incubation times (Figure 3A). We obtained a linear response for AMC formation as a function of reaction time, which showed that the proposed assay model can be used to monitor the IMER-KLK-Sepharose-NHS activity. Among the analyzed conditions, incubation for 5 min was sufficient to measure the enzymatic activity, so we chose this time for further analyses.

Figure 3B shows how the ammonium acetate solution concentration, pH 8.0, affected the immobilized KLK activity. The immobilized enzyme remained active between 2 and 10 mmol L<sup>-1</sup> ammonium acetate. In contrast, IMER-KLK-Sepharose-NHS showed lower activity at higher ammonium acetate concentration (15 mmol L<sup>-1</sup>).

Hence, the optimized assay conditions for the IMER-KLK-Sepharose-NHS activity off-line assay were incubation time of 5 min and 10 mmol L<sup>-1</sup> ammonium acetate solution (pH 8.0).

Another important parameter to evaluate is the clean-up step of the immobilized KLK micro-column, to ensure that it can be reused and that results are reproducible. KLK immobilized on Sepharose-NHS had previously been shown to have excellent reusability after simple washing steps with washing buffer (10 mmol L<sup>-1</sup> Tris-HCl, pH 8.0) (Carvalho et al., 2021). Here, we adjusted the clean-up step to the conditions used during MS detection, and we replaced the washing buffer with 5 mmol L<sup>-1</sup> ammonium acetate (pH 8.0). The final clean-up conditions of the IMER-KLK-Sepharose-NHS were 5 ml of Milli-Q water followed by 5 ml of 5 mmol L<sup>-1</sup> ammonium acetate solution (pH 8.0). The washing procedure proved adequate for cleaning the immobilized KLK micro-column. In routine analyses, we repeated this washing procedure three times.

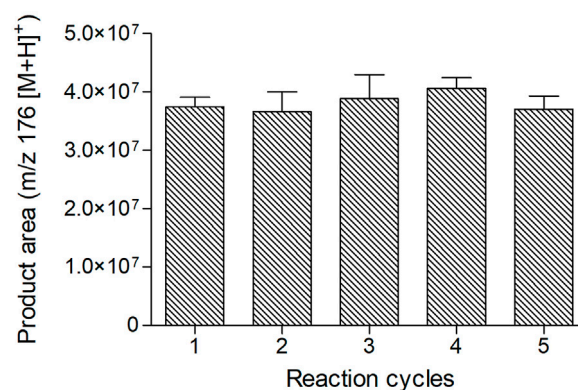


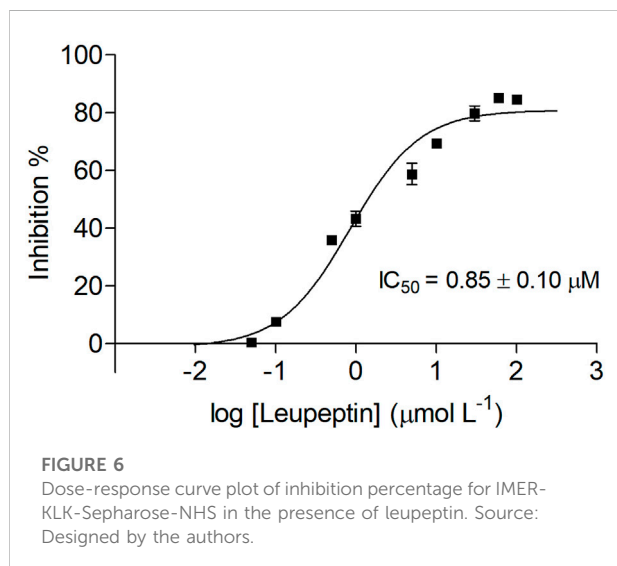
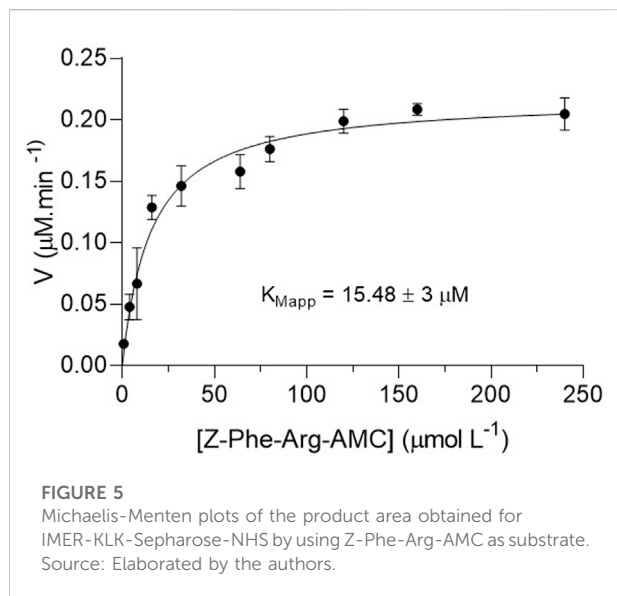
FIGURE 4

IMER-KLK-Sepharose-NHS reusability. Source: Elaborated by the authors.

In addition, we tested IMER-KLK-Sepharose-NHS in repeated reaction cycles (Figure 4), which showed excellent reproducibility in five consecutive reactions (CV = 4.3%,  $n = 5$ ) and indicated that the proposed model assay was suitable for monitoring the IMER-KLK-Sepharose-NHS activity. The immobilized enzyme showed good stability and remained active throughout the assay.

## IMER-KLK-sepharose-NHS kinetic constant ( $K_{Mapp}$ )

We determined the kinetic parameter  $K_{Mapp}$  for Z-Phe-Arg-AMC hydrolysis by IMER-KLK-Sepharose-NHS under the conditions established for the enzymatic reaction and for the HPLC-MS analysis method. The  $K_{Mapp}$  value was  $15.48 \pm 3 \mu\text{mol L}^{-1}$  (Figure 5), corroborating the value previously obtained by the fluorescence assay on microplates of  $10.3 \pm 0.9 \mu\text{mol L}^{-1}$



(Carvalho et al., 2021). Thus, we selected  $40 \mu\text{mol L}^{-1}$  substrate (corresponding to more than twice the  $K_{Mapp}$ ) for the inhibition studies.

## Inhibition studies

As a proof of concept, we used the peptide leupeptin as a reference inhibitor to validate the off-line assay employing IMER-KLK-Sepharose-NHS as a tool for screening ligands. To determine the inhibitory potency, we evaluated the IMER-KLK-Sepharose-NHS activity in the presence of increasing leupeptin concentrations and obtained  $IC_{50}$  of  $0.85 \pm 0.10 \mu\text{mol L}^{-1}$  (Figure 6), which is in the same order of magnitude as the

value obtained by the microplate assay and fluorescence detection  $IC_{50}$  of  $0.13 \pm 0.01 \mu\text{mol L}^{-1}$  and to  $IC_{50}$  obtained for the free enzyme in solution ( $IC_{50} = 1.62 \pm 0.18 \mu\text{mol L}^{-1}$  (Carvalho et al., 2021)). The  $IC_{50}$  values vary when the test conditions are modified, so it is a relative comparison parameter (Holdgate, Meek and Grimley, 2018). In general, IMER-KLK-Sepharose-NHS was able to identify the reference inhibitor and proved to be effective in determining the  $IC_{50}$  parameter.

## Conclusion

KLK immobilized on Sepharose-NHS as a micro-column configuration is a useful approach for measuring KLK activity; exhibits satisfactory stability; allows the enzyme to be reused; and can be combined with an HPLC-MS method off-line. We determined the KLK activity by quantifying the AMC product through the proposed HPLC-MS method. The developed assay was also able to identify the known KLK inhibitor leupeptin, demonstrating that it can be used for screening inhibitors. Thus, the off-line assay with MS detection reported here represents a good alternative to the fluorescence microplate assays.

## Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

## Author contributions

DC: methodology, validation, formal analysis, investigation, writing—original draft, BL: formal analysis, validation. CR: writing—original draft formal analysis. EA: software, writing—review GS: writing—review. JK: writing—review. CC: conceptualization, funding acquisition, project administration, resources, visualization, supervision, writing—review and editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/frans.2022.1018115/full#supplementary-material>

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