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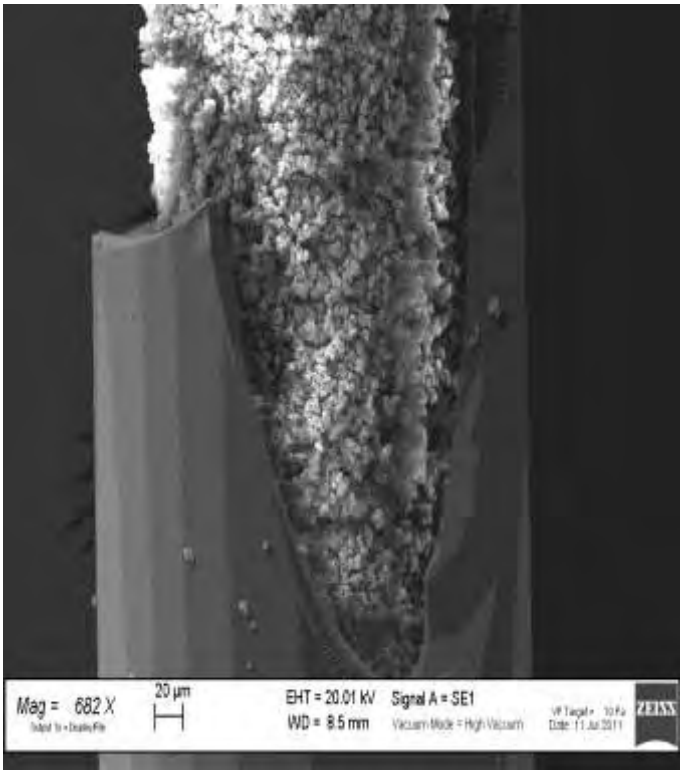
DEPARTMENT OF DRUGS

ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ

ΤΜΗΜΑ ΒΙΟΧΗΜΕΙΑΣ ΚΑΙ ΒΙΟΤΕΧΝΟΛΟΓΙΑΣ

IMMOBILIZED TRYPSIN FOR PROTEOMIC ANALYSIS

ΑΚΙΝΗΤΟΠΟΙΗΜΕΝΗ ΤΡΥΨΙΝΗ ΓΙΑ ΠΡΩΤΕΟΜΙΚΗ ΑΝΑΛΥΣΗ



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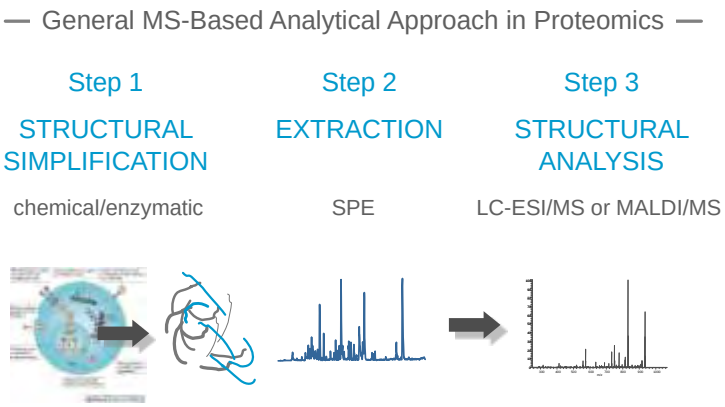
Abstract

The use of monolithic supports for a wide variety of applications has rapidly expanded during the past few years. An immobilized enzyme reactor (IMER) in the form of capillary monolith was developed for a micro-liquid chromatography system. Trypsin was immobilized on three monoliths (GMA-Di GMA, Tri GMA-, NAS) prepared in fused silica capillaries. Enzyme activity behaviour was characterized by the apparent Michaelis constant and the apparent maximum velocity using BAEE as substrate. Bradford assay was also applied for the quantitative determination of the immobilized trypsin on the three monoliths. The results reveal a relation between the amount of the immobilized trypsin and the activity of the enzyme. Also, the influence of various parameters (flow rate and the composition of mobile phases) on enzymatic activity was investigated.

Keywords: Immobilized enzyme reactors; immobilized trypsin; monoliths; Micro-liquid chromatography; HPLC; Kinetic studies

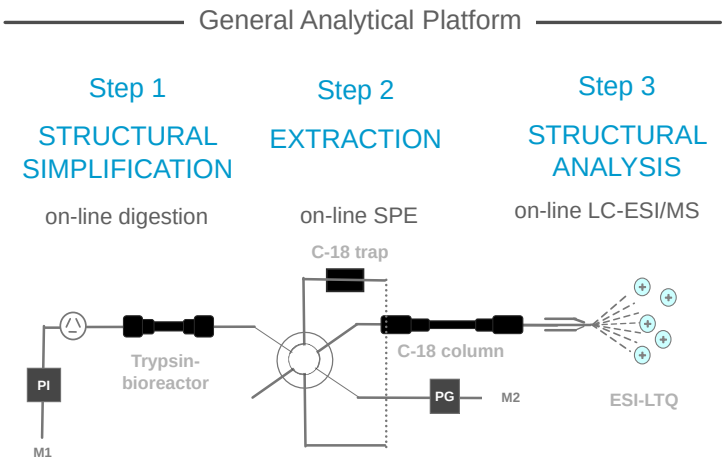
1. Introduction

Proteomics is the large-scale study of proteins devoted to define their structures and understand their functions. A vast number of studies and experiments have been reported to explain the “world of the proteins”. Proteolysis is the key process to simplify the structure of the proteins, as the modern technologies allow determining the exact amino acid sequence of peptides instead of the entire proteins. After a protein is simplified by proteolytic cleavage of its primary chain into smaller peptides, they are identified by Mass Spectrometry, with a preliminary separation step by appropriate chromatographic methods.



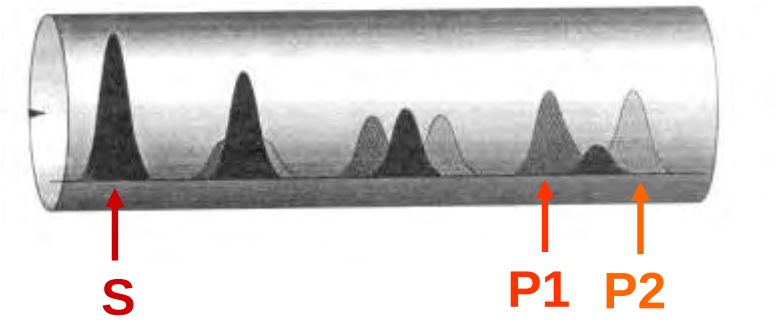
While in the last decade significant improvements have been reached with the technologies for MS and HPLC, with fast and efficient separations by HPLC and UPLC, high resolution mass spectrometers and potent bioinformatics tools for data interpretation, the digestion of the target protein(s) is still the bottleneck of the entire analysis process, requiring long incubation times and presenting often inadequate reproducibility and capability of automation. [3]

It is therefore, indispensable to improve the current digestion, separation and identification technology of proteins with the least drawbacks. In this study, a high-throughput system with low digestion time and high digestion efficiency for protein analysis is proposed.



In order to achieve fast and reproducible protein identification, a solid support to immobilize an hydrolytic enzyme model it was used a solid support to immobilize an hydrolytic enzyme-model (trypsin, the most widely used proteolytic enzyme in proteomics) was used.

A new trend and promising approach to avoid drawbacks such as enzyme auto-digestion, low efficiency, extended incubation time and manual operation, is the use of immobilized enzymes on appropriate solid supports. If this solid support possesses the requirements to be used as chromatographic stationary phase in chromatographic devices (i.e. columns) then the so-called "Immobilized enzymes reactors (IMERs)" are created. [2,7]



These devices allow carrying out the reactions in continuous-flow systems using immobilized catalyzer(s): when injected, the substrate (S) is converted into products (P1 and P2) during its elution through the chromatographic reactor. The main advantages of this format for enzymatic reactions are: enzymes re-usage, high reproducibility of the catalytic process, and high efficiency of the process as in continuous flow systems.

The most used methods for enzyme immobilization are based on covalent binding of the biomolecules on solid supports with different chemistry and morphology (particulate and monolithic organic polymers, particulate and monolithic silica materials). It is important to study the properties of supporting materials and to understand the better immobilization processes in order to preserve the catalytic properties of the immobilized enzyme.

A relatively recent advance in chromatographic materials is the development of monolithic stationary phases. These self-supporting columns do not require frits, while the bimodal pore structure and the high through-pore volumes provide low back pressure and hence increased flow rates relative to conventional columns. Based upon the nature of the material from which they are made, monolithic columns can be classified as organic polymer- or silica-based columns.

Silica monoliths perform best for small molecules, and polymeric monoliths perform best for large molecules. However, although they are prepared in different ways and contain vastly different chemistries, polymer based and silica-based monoliths have some distinct similarities. Organic polymer-based monoliths are classically prepared using a simple molding process carried out within a chromatographic column or capillary. Polymerization of a mixture comprising monomers, initiator, and porogenic solvent affords macroporous materials. [9]

The use of porous polymeric monoliths as supports for the preparation chromatographic enzymatic reactors has become very popular in the last ten years. The preparation of organic polymer is simply

based on the mixing of monomers, initiators, cross-linkers and porogenic solvents in a mold and subsequently exposition of the solution to UV light or heat to initiate polymerization. Despite this advantage, the non-specific adsorption of proteins and peptides on the supporting material is still a challenge when using polymer-based monoliths for the preparation of bioreactors. Therefore, actual efforts are focused on developing new hydrophilic polymer-based monoliths that enable to minimize the non-specific adsorption of biomolecules on IMERs as well as preserving their native conformational state and related biochemical functions. Moreover such hydrophilic matrices would offer a better accessibility of the reaction sites on the monolithic support, hence resulting in higher digestion efficiency.

2. Aim of the work

In this work, we have immobilized trypsin on three monoliths with different chemical and activation properties, in order to synthesize a new bioreactor for on-line digestion of proteins.

The Bradford assay was used to quantify the amount of the immobilized trypsin in each IMER. The IMERs were applied to micro-HPLC-UV where the hydrolysis of a standard substrate N-a-benzoyl-L-arginine-ethyl-ester (BAEE) takes place. This was followed by the separation and quantification of the substrate BAEE, and the product, benzoyl arginine acid (BAA). This model reaction has been used to study in detail the kinetic parameters – (Michaelis constant- Km and the maximum velocity- Vmax) of the enzyme immobilized on the three monoliths. Also, the experimental conditions have been optimized in terms of the flow rate and mobile phase composition to find out the optimal support and experimental conditions for the maximization of the catalytic performances of immobilized trypsin.

The final, experimental purpose was to find out the most useful monolith that can be coupled to a LC-MS system for on-line digestion, separation and structural characterization of proteins in high-throughput integrated systems (Figure 2).

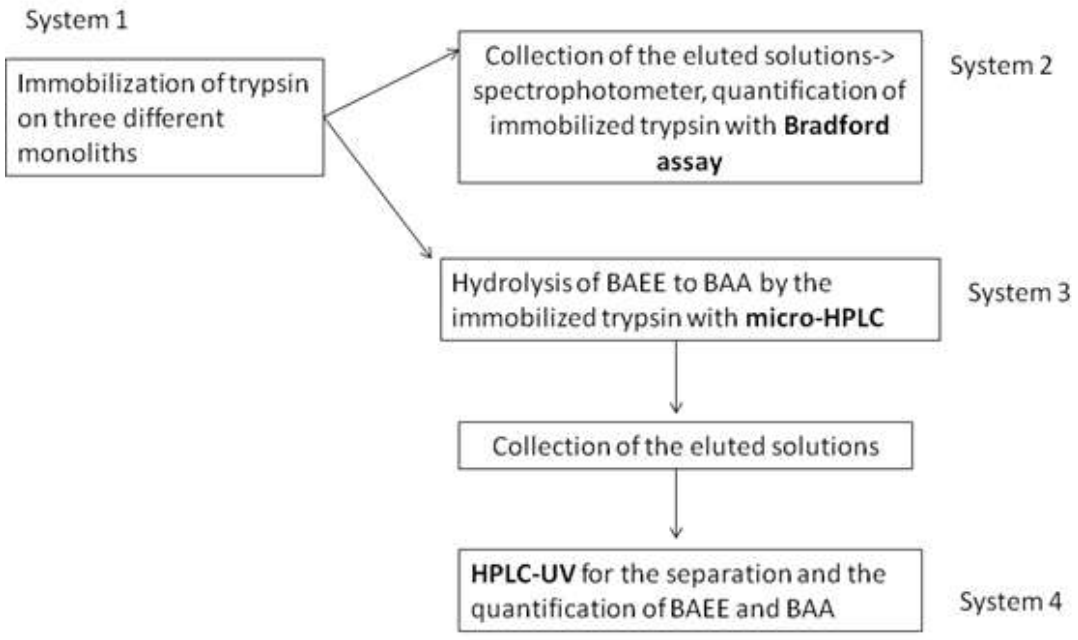
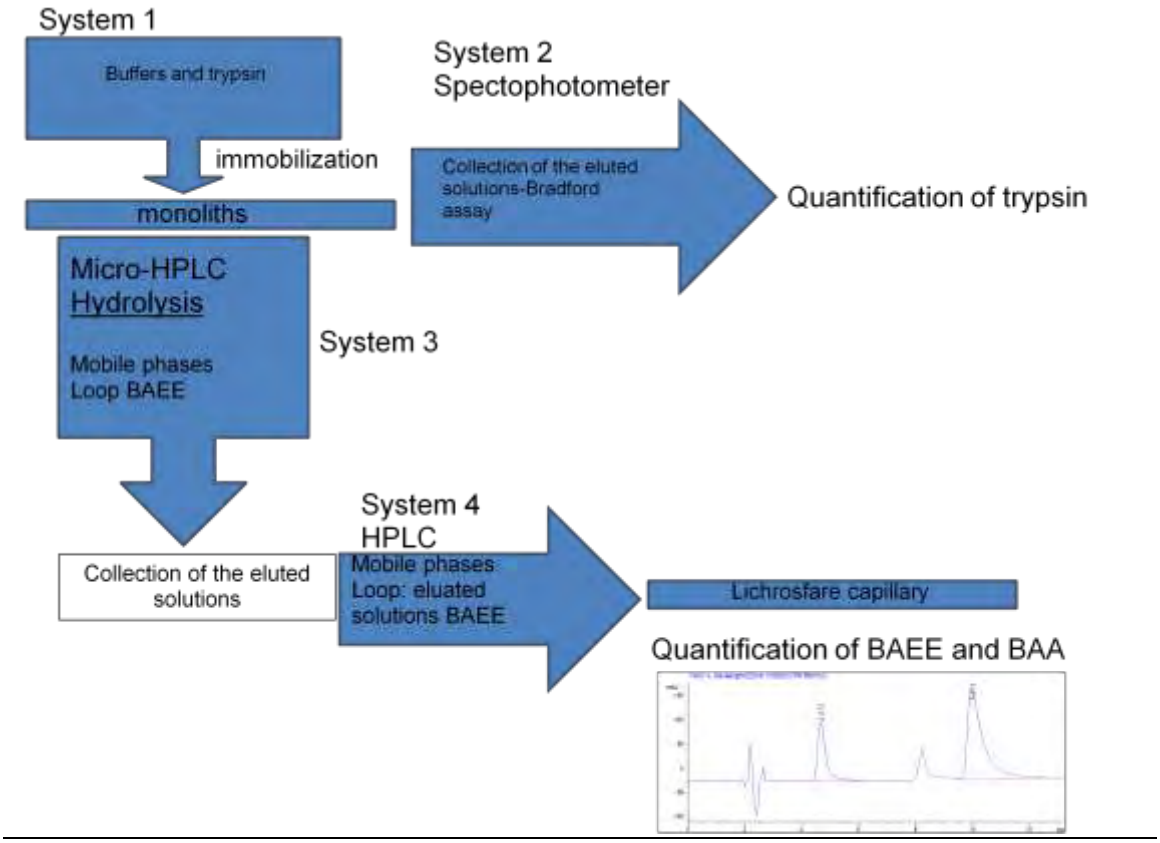


Fig1. Schematic diagram of the chromatographic system for the off-line bioreactors activity determination

3. Experimental

3.1 Instrumentation

At the different stages of the study, four analytical systems were used, namely System1-4 (see scheme below)



System 1: The immobilization of trypsin was performed on the tree monolithic capillaries (M1-3) by pumping the immobilization solutions through a syringe pump (500 µL) at a flow rate of 5 µL/min. The syringe pump was a LTQ-MS Thermo Finnighan.

System 2: The immobilization solutions were collected and analyzed spectrophotometrically to quantify trypsin before and after the immobilization process. The Bradford method was used for the quantification of trypsin. The Spectrophotometer was UV-visible UV-1601, Shimadzu.

System 3: The monoliths with the immobilized trypsin from system 1 were applied to a micro-HPLC to carry out hydrolysis reactions. The micro-HPLC was Phoenix 40 equipped with binary pumps at a range of flow rates, manual injector with loop of 0.2µL, UV-detector spectra 100 controlled by the software Chrom-Card for data analysis.

System 4: The eluted solutions from hydrolysis system (system 3) were collected and were injected on an HPLC-UV for the separation and quantification of the substrate and the product. The instrument was an AGILENT HPLC 1100 Series consisting of manual injector with a loop of 20µL, quaternary pumps at flowrate 1mL/min, degasser, UV-detector and software HP Chem Station (Hewlett pachard) for elaborated results. The chosen column was a Lichrosphere ® (250mm x 4mm I.D.), a Reverse Phase

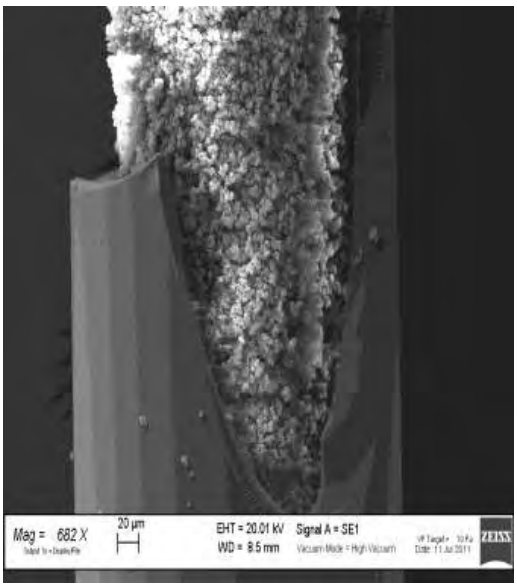
stationary phase containing silica particles derivatized with C18 chains, which confer the characteristic hydrophobicity to the stationary phase(RP-18 column). [8]

3.2 Monoliths preparation and characterization

Three different monolithic columns were prepared by the research group of Prof. Gasparrini, University of Rome (Italy).

1. γ 40_ACR_RPMD_GMA/DiEGMA, 150x0,25 mm ID (M1)
2. γ 40_ACR_RPMD_GMA/TriEGMA, 150x0,25 mm ID (M2)
3. γ 40- PEGDA/NAS 09-06A (400x 0.250 mm, L x I.D.) (M3)

Monoliths were prepared in fused silica capillaries. The characteristic flexibility of the capillary is due to the polyimide material external to the silica. The internal wall of the silica capillary was activated and monolith was linked to it to improve stability [6, 8].



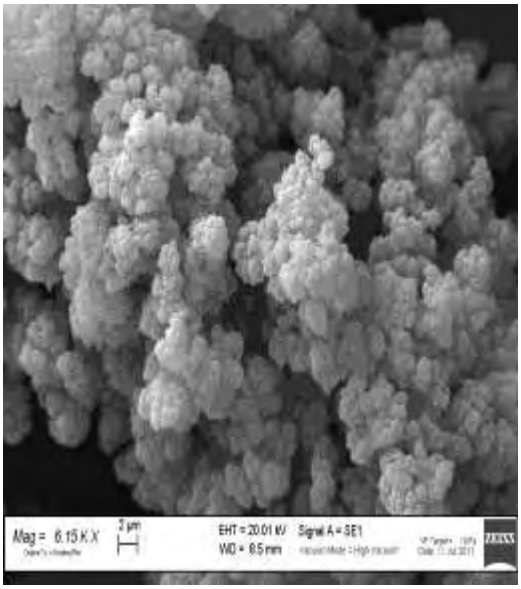
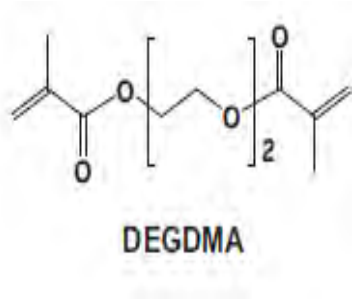
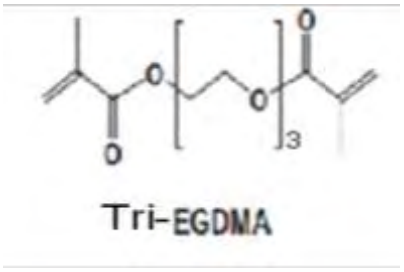


Fig2. Representative scanning electron microscope (SEM) photos, showing the size distribution and the surface morphology

The silica wall with the internal monolith The polymeric structure, with internal pores

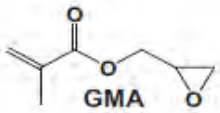
M1, M2: The organic polymer-based monoliths were prepared using diethylene glycol dimethacrylate (DEGDMA) and triethylene glycol dimethacrylate (Tri-EGDMA) as monomers. The functional group is the epoxy group. The epoxy group can be derivatized by aminolysis during coupling with the bound species, in this case lys residues of the trypsin surface. Primary and secondary amino hydrogens can react with the epoxides so the immobilization of trypsin is based on epoxy-amine reactions.





M1: monomer of monolith 1

M2: monomer of monolith 2



M1,M2: functional group

Fig3. Chemical structures of the monomer glycidyl methacrylate (GMA) which is the functional group of M1 and M2, and cross-linkers diethylene glycol dimethacylate (DEGDMA) used for the preparation of M1 and triethylene glycol dimethacylate (Tri-EGDMA) for the preparation of M2.

For M1 and M2, the polymerization was initiated by γ-radiation with a short reaction time at ambient temperature. [8]

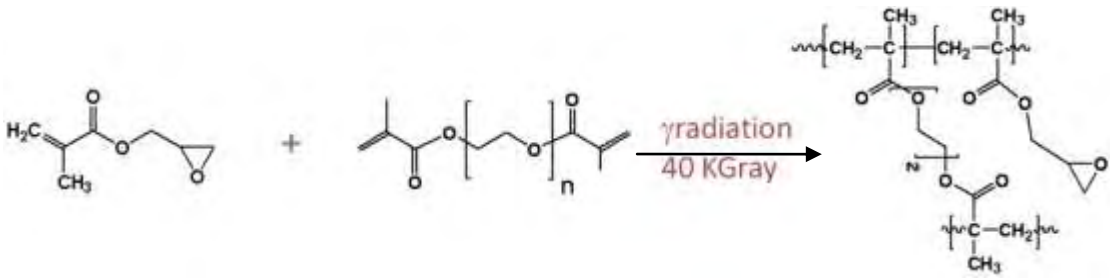
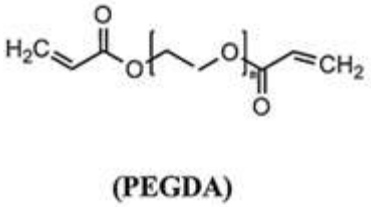
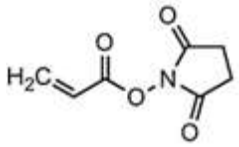


Fig4. This reaction describes the initiation of the polymerization for M1 and M2. The columns were placed inside a Gammacell and irradiated at a temperature of 25°C with a total dose of 40KGray.

M3: The monolithic support was prepared with NAS (an acrylate group and a succimide group) and PEGDA, an acrylate group at each end of the molecule and a PEG chain as a spacer. N-acryloxysuccinimide (NAS) is a high reactive monomer towards the amine group of a protein and it is used to accelerate the enzyme immobilization. [4]



(PEGDA)



(NAS)

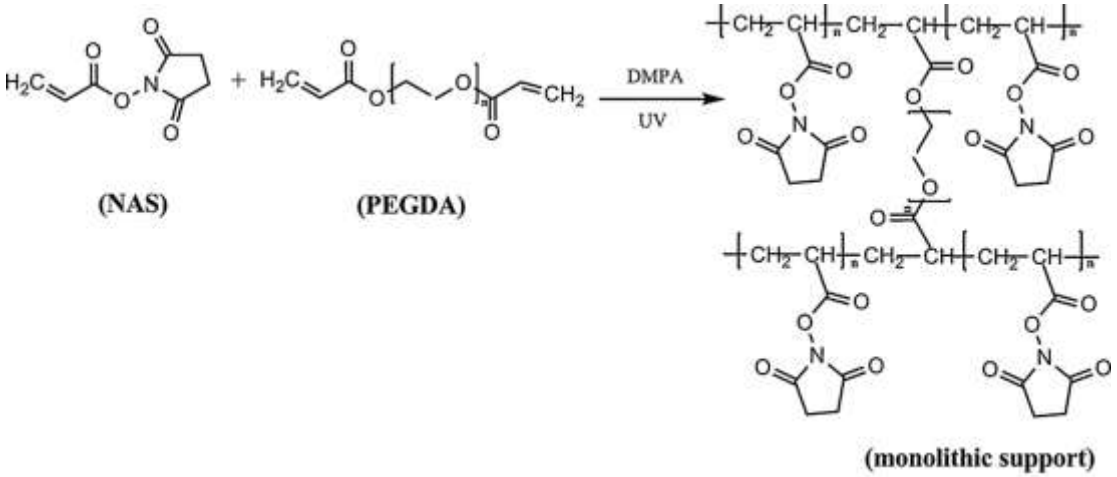
M3: monomer of the monolith 3

M3: functional group of monolith 3

Fig5. Chemical structures of the NAS which is the active monomer for M3 (functional group) and offers high activity to react with the amino group of the enzymes and the PEGDA used as the cross-linker.

For M3 preparation, photopolymerization was applied and dimethoxy-2-phenylacetophenone-DMPA [5] as photoinitiator was needed to introduce the polymerization. Variation in the chemistry of the polymerization mixture was expected to change the polarity of the monolith surface. Different hydrophilicity using more polar monomers was achieved.

The photopolymerization reaction [4]



(NAS) (PEGDA) (monolithic support)

Fig6. The M3 was prepared by the photopolymerization of NAS and PEGDA with CH3OH as the porogen and DMPA as the photoinitiator. NAS is the functional group and the characteristic hydrophilicity is caused by the PEGDA-the cross-linker. The reaction time was 15min.

3.3 SEM photos-Morphological characterization of monolithic supports

Representative scanning electron microscope (SEM) photos of poly (GMA-co-EDMA) and poly (NAS - PEGDA) monoliths are given in the following figures. As seen here, the monolith was well attached onto the inner wall of the fused silica capillary. Also, SEM photos show the size distribution and the surface morphology of the pores.

M1: GMA-DE-GMA

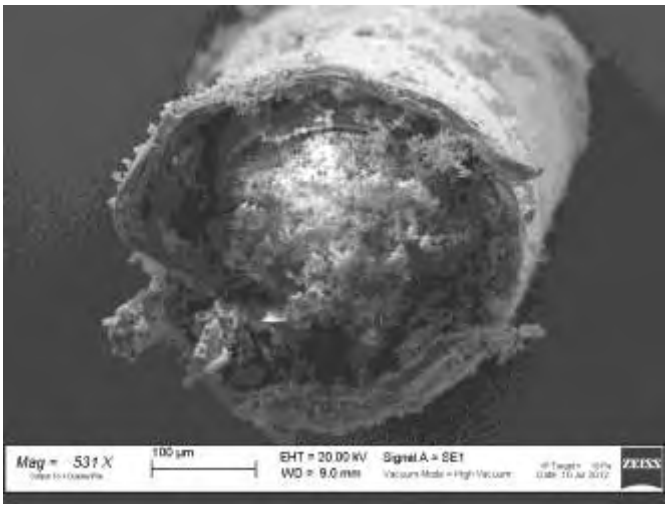


Fig.7. the external part of the capillary, made by silica

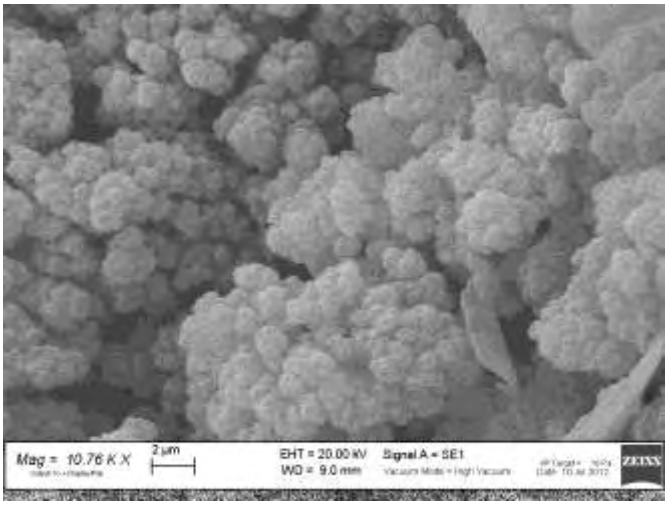


Fig.8. the distribution of the pores

M2: GMA-TE-GDMA

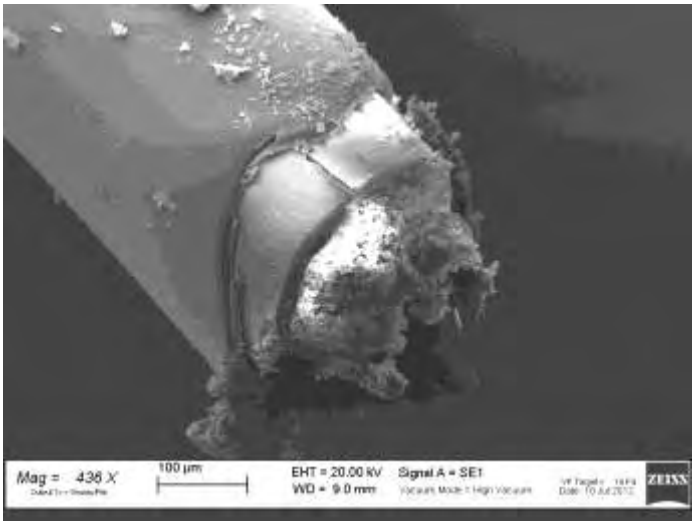


Fig.9. the external part of the capillary, made by silica

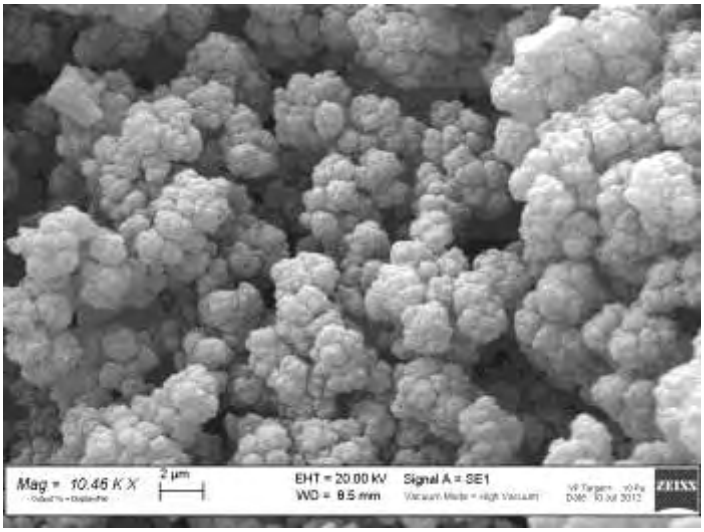


Fig.10. the distribution of the pores

M3: PEGDA-NAS

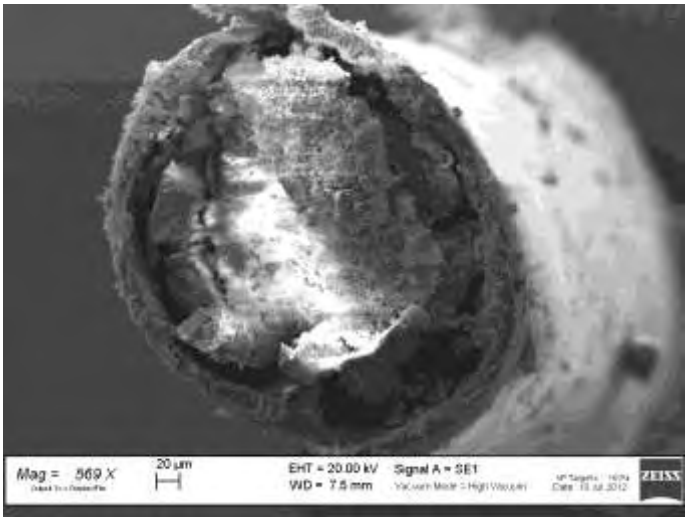


Fig.11. the external part of the capillary, made by silica

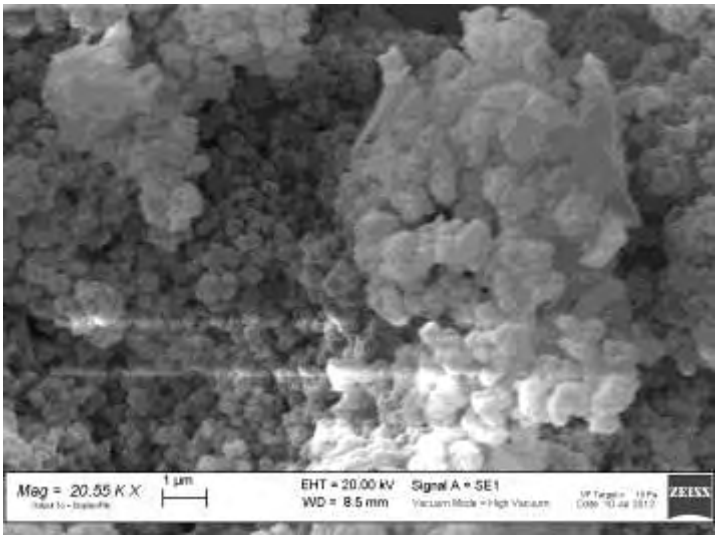


Fig.12. the distribution of the pores

3.4 Modular systems

3.4.1 Immobilization of trypsin (system 1)

M1 and M2: First the monolith was conditioned with deionized water for 30 minutes at a flow rate of 5 µL/min. After that, a 0,5M phosphate buffer pH 9 was flushed for 30 minutes in order to prepare the environment for trypsin immobilization. Then a solution of 1mg/mL trypsin and 50mM Benzamidine (trypsin inhibitor) in the phosphate buffer was passed for 4 hours at a flow rate of 5 µL/min. The inhibitor is necessary to avoid the auto-hydrolysis of trypsin. In order to regenerate the enzyme in its active conditions, after the immobilization, the inhibitor was washed out pumping pure phosphate buffer through the monolith for 20 min at a flow rate of 5 µL/min. To quench all the unreacted epoxide functionalities, 0.5 M phosphate buffer pH 9 with 1 M glycine was passed for 1 hour delivered at a flow rate of 5 µL/min. Finally, the phosphate buffer was pumped for 30 minutes to keep the active conditions of trypsin and to remove all the remained glycine. [8]

M3: The monolith was conditioned with deionized water for 15 minutes at a flow rate of 5 µL/min. The solution for the immobilization consisted of 5 mg/mL trypsin, 0.2 M $NaHCO_3$, 0.5 M NaCl and 50 M Benzamidine pH 8 was passed through the capillary for 4 hours at a flow rate of 5 µL/min. The presence of $NaHCO_3$ and NaCl is required to modify the ionic strength to promote chemical reactivity of both the monolith and trypsin surface. In order to avoid the auto-hydrolisis of trypsin, in this solution, is also included Benzamidine. A solution with 1M Tris-HCl pH 8 was pumped through the monolith for 2 hours at flow rate of 5 µL/min to quench all the unreacted succimide functionalities. The last step was the passage of a solution of 50mM NH_4HCO_3 pH 8 for 2 hours at a flow rate of 5 µL/min for bioreactor conditioning. [4]

3.4.2 Quantification of trypsin- Bradfrord assay (system 2)

The amount of immobilized trypsin on each capillary was determined by the Bradford method. The Bradford method is a colorimetric protein assay based on an absorbance shift of the dye Coomassie Brilliant Blue G-250 in which under acidic conditions the red form of the dye is converted into its blue form to bind to the protein being assayed. During the formation of this complex, two types of bond interaction take place: the red form of Coomassie dye first donates its free electron to the ionizable groups on the protein, which causes a disruption of the protein's native state, consequently exposing its hydrophobic pockets. These pockets on the protein's tertiary structure bind non-covalently to the non-polar region of the dye via van der Waals forces, positioning the positive amine groups in proximity with the negative charge of the dye. The bond is further strengthened by the ionic interaction between the two. The binding of the protein stabilizes the blue form of the Coomassie dye; thus the amount of the complex present in solution is a measure for the protein concentration, and can be estimated by use of an absorbance reading. The (bound) form of the dye has an absorption spectrum maximum historically held to be at 595 nm. The cationic (unbound) forms are green or red. The binding of the dye to the protein stabilizes the blue anionic form. The increase of absorbance at 595 nm is proportional to the amount of bound dye, and thus to the amount (concentration) of protein present in the sample [11].

In all the immobilization protocols used, before and after pumping the solution containing trypsin, an aliquot of each sample was collected in order to quantify the enzyme contained in the solutions. The difference between the amount applied for the immobilization and the amount remained after the immobilization, gives the data on immobilization yield.

Concentration of the solution $_{\text{before the immobilization}}$ – concentration of the eluted solution $_{\text{after the immobilization}}$

For this quantification, the Bradford method was used. Before the quantification, a calibration curve was built by using a standard protein sample of albumin in a range of concentrations from 1 to 4 μg/ml. The equation of this calibration curve is y=0.0435 x + 0.0313, R^2=0.9958. Specifically, the y can be replaced with the determined absorbance of the spectrophotometer thus the x will correspond to the concentration of the trypsin in each sample.

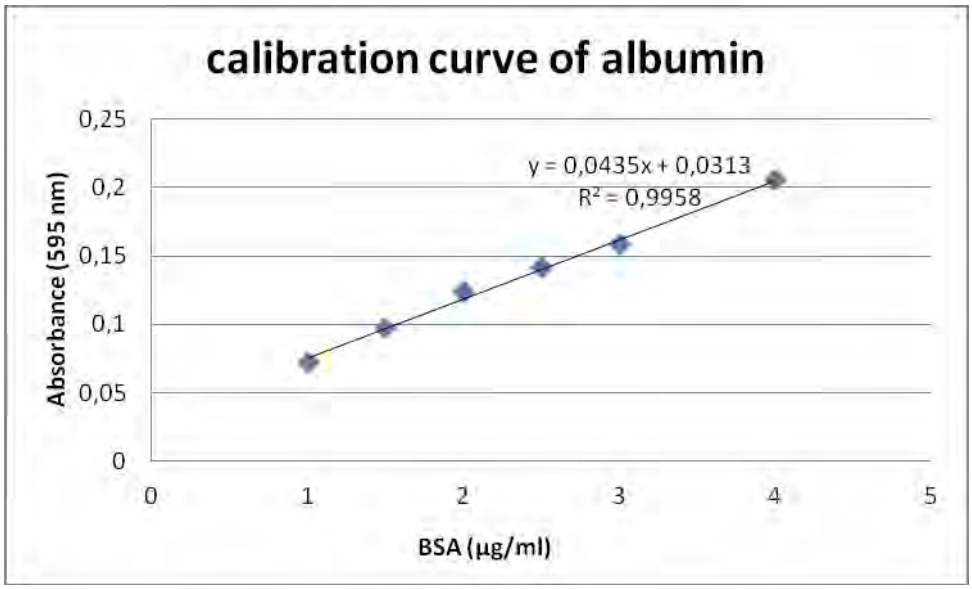


Fig.13. Calibration curve of the standard protein, sample albumin. The equation is y=0.0435x+0.0313 and the R^2=0.9958. This equation allows the determination of trypsin amount in volume 2000μL.

The difference between the concentration of trypsin in the solution before the immobilization and in the eluted solution (after the immobilization) will give the amount of the immobilized enzyme. [12]

All the collected samples of the first two capillaries were diluted from 1mg/ml to 0,1mg/ml into water and the collected samples that correspond to the third monolith were diluted from 5mg/ml to 0,1mg/ml into water.

A blank sample was also prepared with water, Bradford reagent and 5mM Benzamidine. In the blank Benzamidine was also included since it is possible Benzamidine to interact with the Bradford reagent and indicate a different/mistaken absorbance. Three different values of volume (low, middle and high value) of the trypsin-Benzamidine solution were used in the Bradford assay to find the appropriate value of absorbance inside the linearity of the calibration curve of albumin. From the values range of the standard calibration curve (range of the concentration: from 1 to 4 μg/mL), a value corresponding to the highest absorbance-concentration of albumin's calibration curve was finally selected. Therefore, the samples were prepared with 1520 μL water, 80 μL of the trypsin- Benzamidine (5mM) solution and 400 μL Bradford reagent. The blank sample was prepared by adding 1520 μL water, 80 μL of 50 mM Benzamidine solution and 400 μL Bradford reagent. All samples were covered with parafilm to avoid oxidation of the Bradford reagent. The reaction was carried out for 15 min in the dark at ambient temperature. After the incubation, the definition of the absorbance using the spectrophotometer was followed.

3.4.3 Hydrolysis by trypsin (system 3)

The Schwert and Takenaka assay [14] for measuring free activity by using BAEE is the reference method used to express the trypsin activity in terms of active units. BAEE is a non-chromogenic ester that is hydrolysed by trypsin into the corresponding carboxylic acid BA. Both substrate and product have the same UV-Vis absorption profile, except for a narrow wavelength window (253-255 nm) where the product is more absorptive. Due to this similar spectroscopic behaviour, a chromatographic method for the separation of the two chemical species was required.

This reaction is a useful tool for the determination of the affinity between the active site of the immobilized trypsin and the standard substrate, BAEE. The solution with BAEE, substrate can reach the pocket site of the enzyme because of the special construction of the monolith. The dominant mass transfer mechanism for the transport of substrate to the enzymatic active sites within the porous matrix should be diffusion. Therefore, this event permits the hydrolysis reaction.

The conversion of BAEE-substrate to BA-product and ethanol by trypsin:

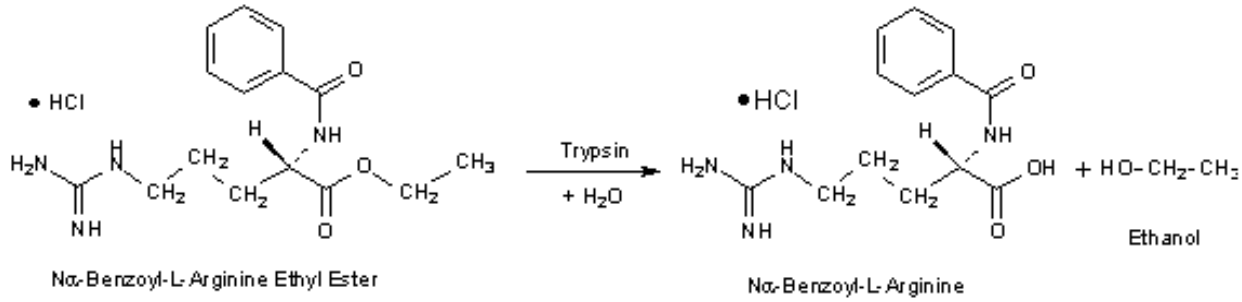


Fig.14 The effect of covalent immobilization of trypsin in the monoliths was assessed using BAEE-BA. BAEE(ester) is the substrate which is hydrolyzed to BA(acid) and ethanol. This reaction shows the conversion, catalyzed by trypsin and added H_2O.

For the on-line hydrolysis experiments, the BAEE samples were prepared in a range of concentrations from 1mM to 600mM coming from two solutions with multiple dilutions.

BAEE is solid, sensitive (kept in 4^{o} C-8^{o} C)

Molecular weight (M.W.) of BAEE: 342.84 gr/moles

Solution 1: the concentration of this solution was 1M. 1.7142 gr of BAEE were weighed and dissolved in 5mL water.

Solution 2: the concentration of this solution was 100mM. 0.34284 gr of BAEE were weighed and dissolved in 10mL water.

Mobile phases:

Molecular Weight (M.W.) of K_2HPO_4=174.2 gr/moles

The concentration of phosphate buffer is 50mM. 0.871 gr of K_2HPO_4 were dissolved in 100mL water. The pH of the buffer is 8 (the pH adjustment was made by the KOH- conjugate base and H_3PO_4-conjugate acid). The prepared solution was filtered and degassed.

The ratios in which the mobile phases were prepared:

Phosphate buffer (pH: 8) / Acetonitrile: 80/20

To find the most appropriate composition of the mobile phases, different ratios of phosphate buffer/acetonitrile were performed to the micro-HPLC.

After the immobilization of trypsin, the monoliths were applied to micro-HPLC (micro High Performance Liquid Chromatography). Aliquots of 10μL BAEE solutions were injected in micro HPLC at a range of flow rates for the monolith 1 (2μL/min, 5μL/min, 15μL/min and 20μL/min). Finally, a flow rate of 5μL/min was used to compare the hydrolysis yield of the three capillaries. As it will be referred (part of the results) the flow rate is a factor that can change the kinetic parameters. In order to obtain comparable results, the value of the flow rate should be constant (5μL/min in this case). The other flow rates were applied to achieve results about the effect of this factor on the kinetic parameters.

The BAEE solutions were injected to the instrument. The micro-HPLC technique allows the hydrolysis of the substrate BAEE to BA by the immobilized enzyme, trypsin. This way, the instrument detects the total quantity of BAA and BAEE which is appeared by a peak. The time that needs the peak to come up, shows the time of the reaction since the peak corresponds to the total quantity of the BAEE and BAA. Accordingly to the detection of BAA-product, as a part of the peak, the end of the reaction is indicated. All the eluted samples were collected in small plastic tubes (eppendorf type). The expected, collected volume is 100μL (with the flow rate 5μL/min and time for the running analysis 20 min).

Fig.15. Chromatograms from BAEE injection on a trypsin Bioreactor, the blue line corresponds to the BAEE injection with the mobile phase 80% phosphate buffer and 20% Acetonitrile, the green line corresponds to the BAEE injection with 100% phosphate buffer as the mobile phase. A single peak can be observed as no chromatographic separation of produced BA and remained BAEE occurs on the bioreactor. Thus, the detection of the product suggests the end of the reaction. The time of the reaction doesn't change with the composition of the mobile phases.

3.4.4 Separation and quantification of hydrolysis products (System 4)

The following step was the separation and the quantification of the BAEE and BAA. The technique used was the HPLC-UV.

All the collected samples by micro-HPLC, were injected offline to HPLC containing amount of the product BA and unreacted substrate BAEE. The flow rate was 1 ml/min and the UV detector was set at a wavelength of 223nm.

The composition of the mobile phases:

A: Water (H_2O) and 0.12% v/v Trifluoroacetic acid (TFA)

B: Acetonitrile (ACN) and 0.1% v/v Trifluoroacetic acid (TFA)

Timetable for the mobile phases:

0-5 min->A: B (20:80 v/v)

5-5:01 min->A: B (30:70 v/v)

5:01-15 min->A: B (30:70 v/v)

Mobile phases are used to adjust the chromatographic separation and retention in liquid chromatography.

The technique used for the quantitative analysis of BAEE and BAA was the HPLC (High Performance Liquid Chromatography) using an AGILENT 1100 Series system. The selected column, Lichrosphare (250mm*4mm I.D. t_{col}=25^{o}C) is a Reverse Phase stationary phase (RP) type column containing silica particles derivatized with Carbon 18(C18) chains, which confer the characteristic hydrophobicity to the stationary phase. This analysis is based on the different polarities of the molecules. The substrate (BAEE) is more hydrophobic molecule as an ester, and the product (BAA) is more hydrophilic molecule being acid. Also, it was referred that the chosen column is quite hydrophobic because of the C18 chains. This fact allowed us to hypothesize that the acid will correspond to the first peak, while the ester will be more retained and will elute as the second peak in the chromatogram.

The injection of a standard solution is proposed to valid the retention times of the analyzed samples.

Retention times

BA- 4.675 min

BAEE- 9.972 min

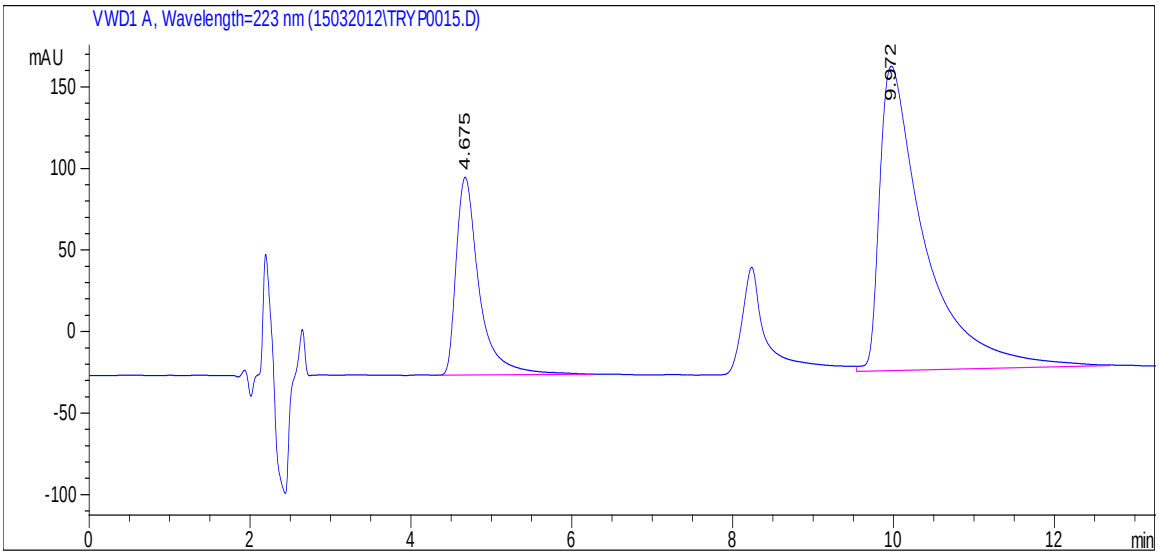


Fig.16. Chromatogram obtained by the injection of the eluted BAEE with concentration 400mM (flow rate: 2µL/min). The double peak at t_0 -2.4 min- represents the solvent (t_0) . The first peak with retention time 4.675 min corresponds to BA, the product, as the most hydrophilic molecule. The next peak is due to the change of the mobile phases. The last peak with retention time 9.972 min corresponds to BAEE, the substrate. The BAEE, being an ester is more hydrophobic molecule so it is retained more to the column than BA.

3.5 Quantitative analysis of BA and BAEE

The HPLC-UV instrument detects the amount of BA and BAEE as peak area. The area is proportional with the injected concentration of the sample.

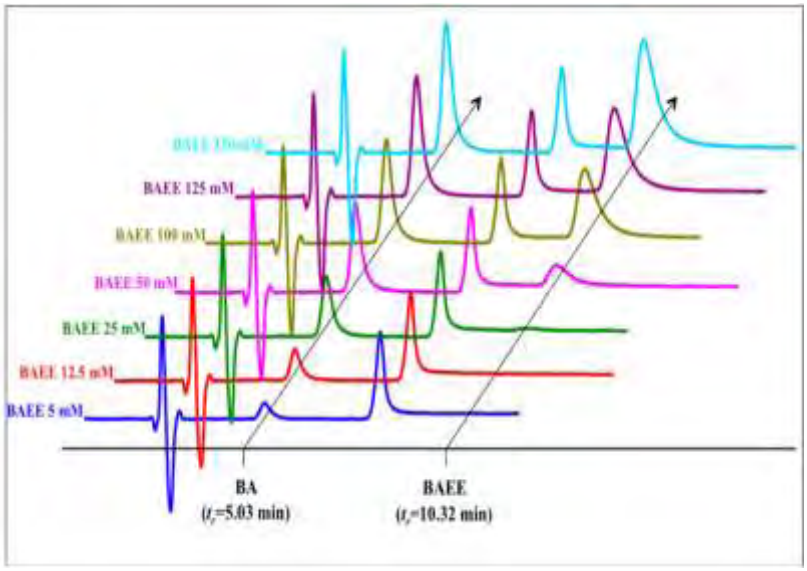


Fig.17. Chromatograms from injections of increasing concentrations of standard BAEE and BA (from 5mM to 150mM)

Two calibration curves were constructed, one for BA and the other for BAEE. This way, the concentration of BA and BAEE that corresponds to a specific area could be easily calculated. For this reason, a range of standard concentrations of BA and BAEE was separately injected to HPLC-UV.

3.5.1 Calibration curve of the substrate-BAEE

Preparation of BAEE samples:

BAEE is solid, sensitive (kept in 4°C-8°C)

Molecular weight (M.W.): 342,82 gr/moles

mg of sample weighed:0,34282 and volume in which dissolved:10mL

Stock-standard solution: 100 mM BAEE in volume: 10mL water-pH=8

Working solutions: 5-900 μM with total volume 10mL (standard solution 100mM & the solution with composition 50mM phosphate buffer and 20% ACN)

The concentration of analyzed samples (BAEE) range was from 5 μM to 900 μM. In all the analysis, the peak area was elaborated by manual integration using the Software HP Chem Station (Hewlett Pachard).

Conc. BAEE (μM)	Area 1	Area 2	Area 3	Average of areas	Standard Deviation (SD)	relative SD (RSD) %
5	51.7	51.1	73.4	58.7	12.7	21.6
30	384.9	381.6	383.2	383.2	1.6	0.4
150	1695.4		1564.1	1629.7	92.9	5.7
300	3340.9	3209.7	3445.8	3332.1	118.3	3.6
500	5622.7	5572.1	5697.4	5630.7	63	1.1
700	7792	7642.5	7977.4	7804	167.8	2.1
900	10560.1	10471.5	10329.2	10515.8	326.3	3.2

Table 1.Data for the construction of BAEE calibration curve

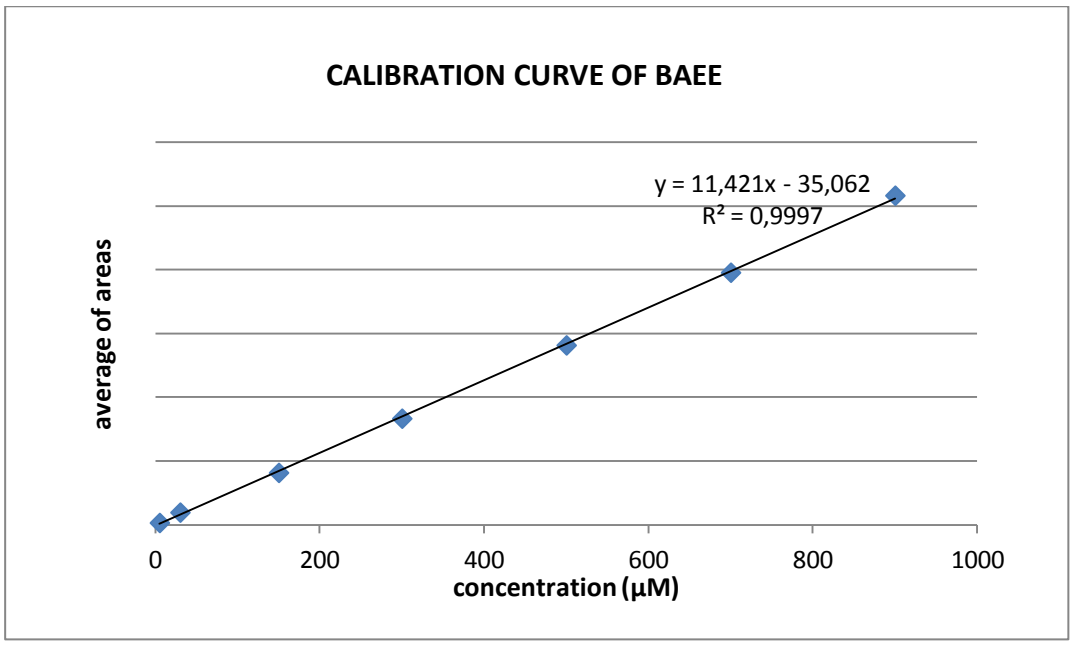


Fig.18. The calibration curve of BAEE allows the calculation of the exact quantification of the products and has been used to estimate the kinetic parameters (Km and Vmax).

3.5.2 Calibration curve of the product-BA

Preparation of BA samples:

The preparation of BA was curated by the Synthesis Lab (University of Pavia), through hydrolysis of the ester BAEE under basic conditions.

Molecular weight (M.W.): 278.307 gr /moles

Standard solution: 0.0278 gr of BA were weighed and were dissolved in volume 10mL of 80% phosphate buffer 50mM pH 8 and 20% ACN. The concentration of this solution was 10mM.

Working solutions: concentrations 5, 150, 300, 450, 600 µM in total volume 10mL (composition 80% of 50mM phosphate buffer pH 8 and 20% ACN)

Conc. BA (µM)	Area 1	Area 2	Area 3	Average of areas	Standard deviation	Relative SD (RSD) %
5	94	82.8	90.3	89	5.7	6.4
150	1762.2	1778.3	1770.8	1770.4	8.1	0.5
300	3057.3	3326.6	3258.2	3214	139.9	4.4
450	5009.8	5007.1	5051.8	5022.9	25.1	0.5
600	6603.5	6720.9	6907.4	6743.9	153.3	2.3

Table 2.Data for the construction of BA calibration curve

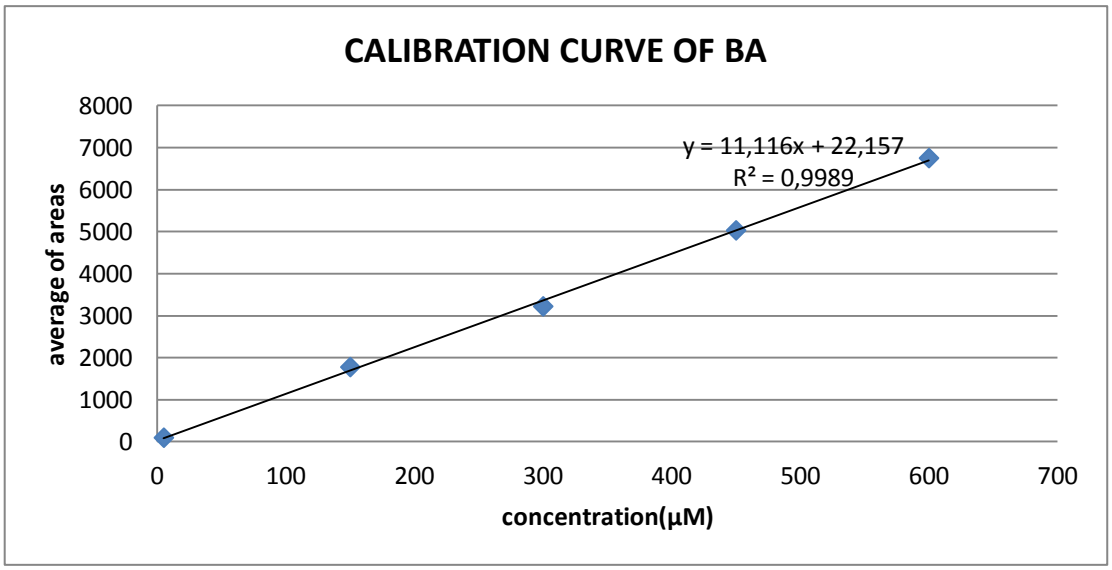


Fig.19. The calibration curve of BA allows the calculation of the exact quantification of the products and,in turns, will be used to estimate the kinetic parameters (Km and Vmax).

4. Results and discussion

In this study, new porous polymer monoliths with modulated hydrophilicity have been prepared using methacrylate chemistry triggered by γ-ray irradiation. The monoliths were physically and chemically characterized, and used for the preparation of novel trypsin-based microreactors which will be used in integrated platforms for protein structural analysis. The amount of immobilized enzyme in each bioreactor was calculated by Bradford assay. By using an off-line method of evaluation of IMERs activities, a comparative kinetic study between various immobilization methods and supports was carried out using BAEE as a standard substrate. The kinetic characterization of the prepared bioreactors indicated the activity of the enzyme in the immobilized form. Indeed, it is well known that both chemical and morphologic characteristics of the chromatographic support can influence the enzymatic activity. Apparent kinetic parameters Km and Vmax, as well as the enzyme specific activity, were estimated in different flow through conditions, and the performances of the IMERs were compared to highlight the influence of the immobilization chemistry and the microsolvation environment within the porous skeleton on the enzymatic performances.

4. 1 Physical characterizations of monoliths

Monolithic supports have been fabricated *in-situ.* The polymerization mixtures have been prepared with the appropriate amount of monomers, cross-linkers and porogenic solvents (**see Table below**). The mixtures have been first degassed using a helium sparger for 5 minutes at ambient temperature. The polymerization reaction has been performed in a Gamma-cell with γ-irradiation dose rate of 2 KGy/h, and a total dose of 40 KGy at room temperature (P= 300-500 psi). After γ-irradiation, the monolithic supports were connected to a micro-HPLC and washed with acetone (100 column volumes). [6,9] Capillary dimensions were:

1. γ 40_ACR_RPMD_GMA/DiEGMA, 150x0,25 mm ID (M1)
2. γ 40_ACR_RPMD_GMA/TriEGMA, 150x0,25 mm ID (M2)
3. γ 40- PEGDA/NAS 09-06A (400x 0.250 mm, L x I.D.) (M3)

upport	Monomer/ crosslinker ratio	Porogen	Monomer/p orogens ratio (v/v)	Total porosit y(HPLC-RP test)	Pores volume*(µL)	Monolith volume *(µL)	Monolith weight* (µg)	µmoles reactive groups/ monolith*	m re gr m
MA/ iEGDMA	GMA/ DiEGDMA 1/3 (v/v)	1-PrOH	30/70	0.75	5.52	1.84	1970	3.38	1
MA/ riEGDMA	GMA/ TriEGDMA 1/3 (v/v)	1-PrOH	30/70	0.73	5.37	1.99	2150	3.69	1
EGDA/ NAS	PEGDA/ NAS 1/3 (v/v)	MeOH	36.4/63.6	0.73	5.37	1.99	2279	3.29	1

Table3. Morphological characterization of the monoliths. Data were generated by the research group of Prof.Gasparrini (University of Rome-Italy).

4.2 Trypsin immobilization and quantification by Bradford Assay

Bradford Assay [11, 12] is a spectrophotometric quantitative method for intact proteins, here used to determine the amount of immobilized trypsin on each monolithic support.

For quantitative measurements, a calibration curve was built using bovine serum albumin as standard protein. The Equation is :

y=0.0435x+0.0313	R^2=0.9958

In this formula, y can be replaced with the measured absorbance and thus x will correspond to the amount of trypsin in the reaction volume (2000µL). This way, the amount of trypsin in volume 80 µL can be easily calculated.

Each enzymatic solution applied to or recovered from the immobilization processes was analyzed as previously described and the amount of immobilized enzyme was calculated. Due to the presence of benzamidine in the enzyme preparations to prevent autolysis phenomena, a blank samples was included in order to account for any unspecific contribution to absorbance.

MONOLITH 1

	Before the immobilization	After the immobilization
a. Absorbance of the blank (H_2O,Bradford,Benzamidine)	0.2382	
b. Absorbance of the solution with trypsin	0.3949	0.3774
c. Difference between b and a	0.3949-0.2382=0.1567	0.3774-0.2382=0.1392
d. Correct absorbance (absorbance of trypsin)	0.1567	0.1392
e. Amount of trypsin	0.8652 mg	0.744 mg
f. amount of the immobilized trypsin	0.8652-0.744=0.1212 mg	

MONOLITH 2

	Before the immobilization	After the immobilization
a. Absorbance of the blank (H_2O,Bradford,Benzamidine)	0.2382	
b. Absorbance of the solution with trypsin	0.3076	0.2997
c. Difference between b and a	0.3076-0.2382=0.0694	0.2997-0.2382=0.0615
d. Correct absorbance (absorbance of trypsin)	0.0694	0.0615
e. Amount of trypsin	0.2628 mg	0.20832 mg
f. amount of the immobilized trypsin	0.2628-0.20832=0.0545 mg	

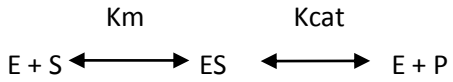
MONOLITH 3

	Before the immobilization	After the immobilization
a. Absorbance of the blank (H_2O,Bradford,Benzamidine)	0.2382	
b. Absorbance of the solution with trypsin	0. 3469	0.3380
c. Difference between b and a	0.3469-0.2382=0.1087	0.2997-0.2382=0.0998
d. Correct absorbance (absorbance of trypsin)	0.1087	0.0998
e. Amount of trypsin	2.6688 mg	2.3622 mg
f. Amount of the immobilized trypsin	2.6688-2.3622=0.3066 mg	

The results obtained clearly demonstrate that the best results in terms of immobilization yield were obtained with Monolith 3, characterized by NAS functionalization. It was Interesting to observe this despite the fact that this support was characterized by the lowest density of reactive groups, which demonstrates the NAS functionality as the most suitable for protein immobilization in terms of effective reactivity and loading capacity.

4.3 Micro-HPLC and HPLC techniques- calculation of kinetic parameters (Km-Vmax)

The next part is reffered to the determination of maximum velocity(Vmax),Michaelis constant (Km), catalytic constant/Michaelis constant (catalytic efficiency- Kcat/Km) and the calculation of specific activity of trypsin.



The first reaction step describes the binding of the substrate to the enzyme (catalyst) and the constant K_m (Michaelis constant) corresponds to the *dissociation constant* of the equilibrium under conditions where the product formation is very slow compared to the dissociation process of the substrate. K_m equals the substrate concentration at half maximal reaction rate $V_{max}/2$(maximum velocity/2). In this case K_m is a good approximation for the dissociation constant and thus describes the *affinity* of the substrate for the enzyme. Its value is independent of the enzyme and substrate concentrations. The K_m constant is expressed into concentration units.

The second reaction step describes the *catalytic rate* or the rate of product formation and referred as the turnover number k_{cat}(catalytic constant). The turnover number of the reaction is the number of the substrate molecules which are converted into product from one molecule of the enzyme per unit time, when the enzyme is totally saturated by the substrate.

The Michaelis-Menten kinetic is valid only under *saturation* conditions, i.e., when the concentration of substrate S is much larger than the enzyme concentration. The maximal reaction rate V_{max} describes a *steady -state equilibrium* of the reaction catalyzed by the enzyme. The steady-state equilibrium is an important concept in biochemistry because many enzyme catalyzed reactions run at saturation and the product is often removed from the reaction site so as to render the reaction irreversible.

Definition of maximum velocity (Vmax): It has been shown experimentally that if the amount of the enzyme is kept constant and the substrate concentration is then gradually increased, the reaction velocity will increase until it reaches a maximum. After this point, increases in substrate concentration will not increase the velocity. It is theorized that when this maximum velocity had been reached, the entire available enzyme has been converted to ES (enzyme-substrate complex). This point on the graph is designated Vmax.

Definition of Michaelis constant (Km): The size of Km tells us several things about a particular enzyme.

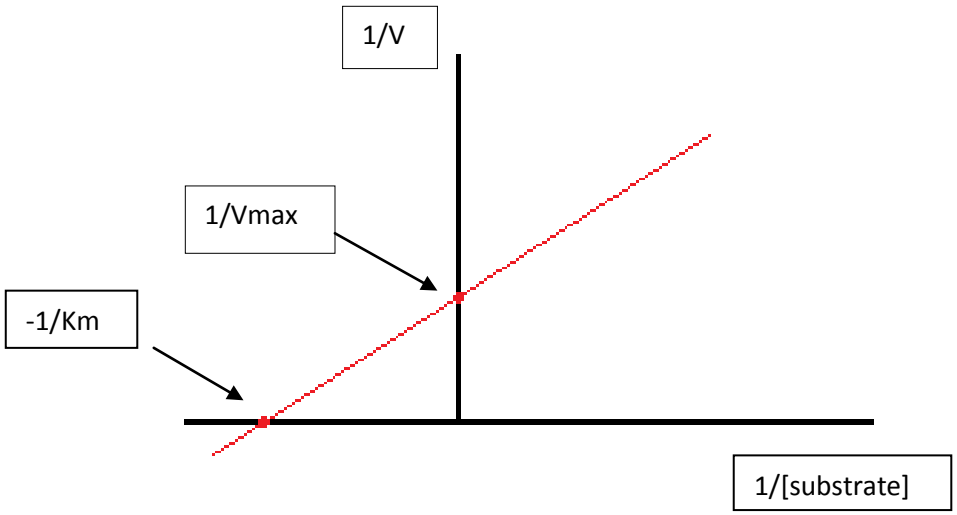
- A small value of Km indicates that the enzyme requires only a small amount of substrate to become saturated. Hence, the maximum velocity is reached at relatively low substrate concentrations.
- A large value of Km indicates the need for high substrate concentrations to achieve maximum reaction velocity.
- The substrate with the lowest value of Km upon which the enzyme acts as a catalyst is frequently assumed to be enzyme's natural substrate, though this is not true for all enzymes.

In order to give an idea about the kinetic parameters of trypsin immobilized on the monolithic micro-column, Lineweaver-Burk plot of monolithic micro-reactor was sketched. Km and Vmax values of the immobilized enzyme were determined using the Lineweaver-Burk slope. If it is supposed that y=mx+q,

the equation (Lineweaver-Burk equation), the replacement of x with 0 will give the Vmax and the replacement of y with 0 will give Km.

X=0 ⟶ y=1/Vmax(on the axon). The above equation will be 1/Vmax=0+q so Vmax=1/q

Y=0 ⟶ mx+q=0 so x=-q/m. But x on the axon will be -1/Km thus Km=m/q



The apparent kinetic parameters of the immobilized enzyme in the conversion of BAEE were estimated by injecting increasing substrate concentrations in the bioreactors at constant experimental conditions. Product and un-reacted substrates from the bioreactors were collected and quantitatively estimated with the developed chromatographic methods (see also paragraphs 3.4.3 and 3.5).

The production of the Michaelis- Menten and Lineweaver-Burk (double reciprocal plot) diagrams allowed to derive the Km and Vmax values for trypsin in each bioreactor. The apparent Km and Vmax were extrapolated by plotting the initial reaction velocity to the injected BAEE concentrations and fitting the experimental points with the Michaelis-Menten equation. The flow rate was kept constant thus the values of Km and Vmax of trypsin can be comparable among the three monoliths.

Monolith 1:

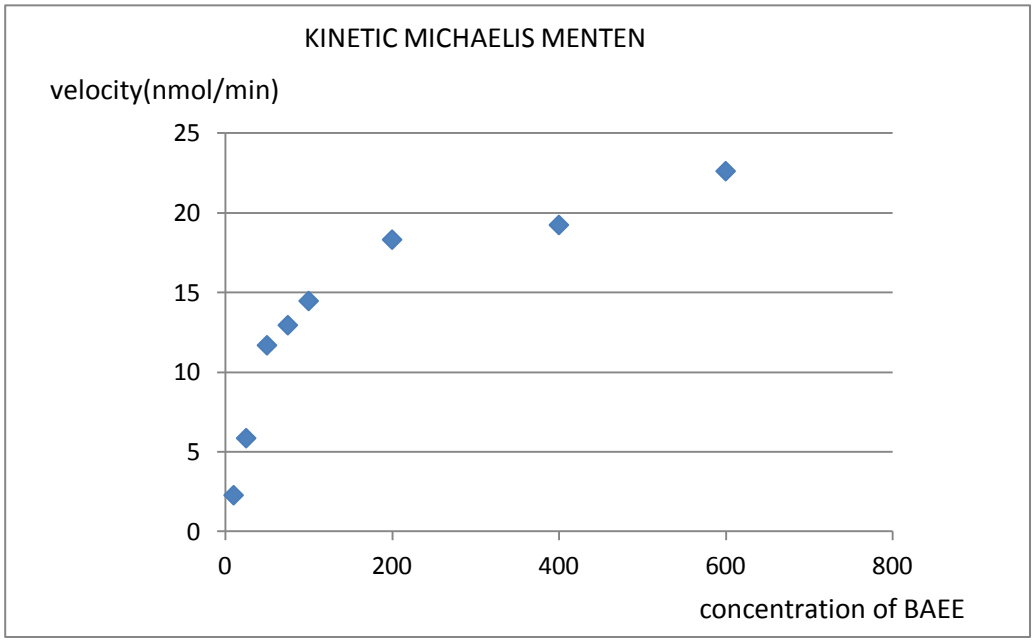


Fig.20. Michaelis- Menten plot for trypsin immobilized on Monolith 1.

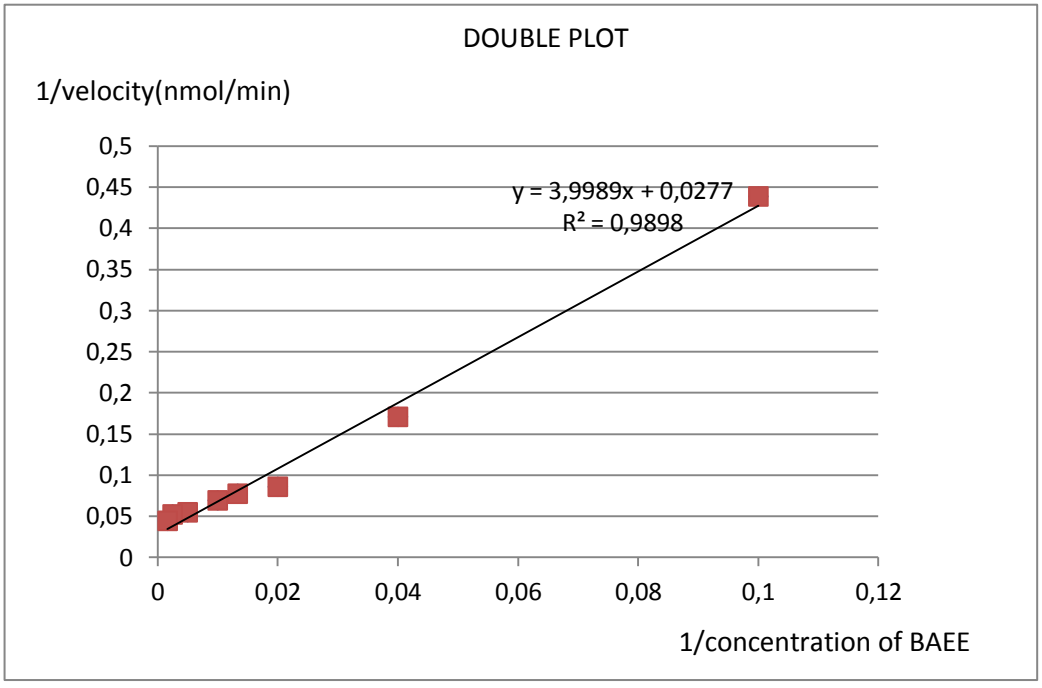


Fig.21. Double reciprocal plot for trypsin immobilized on Monolith 1.

Determination of Km: 142.82 mM
Determination of Vmax: 36.1 nmol /min

Monolith 2:

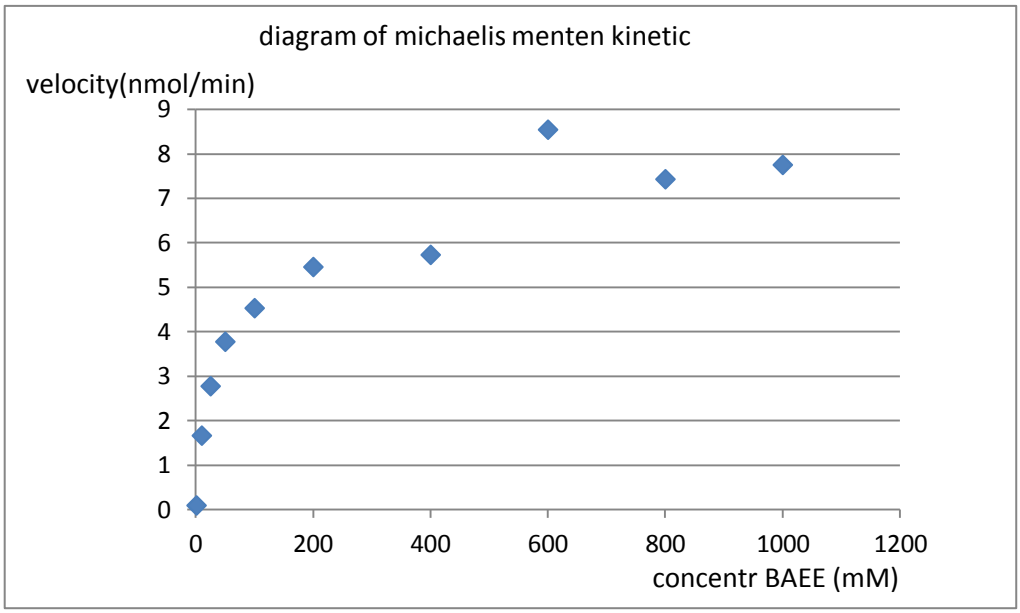


Fig.22. Michaelis- Menten plot for trypsin immobilized on Monolith 2.

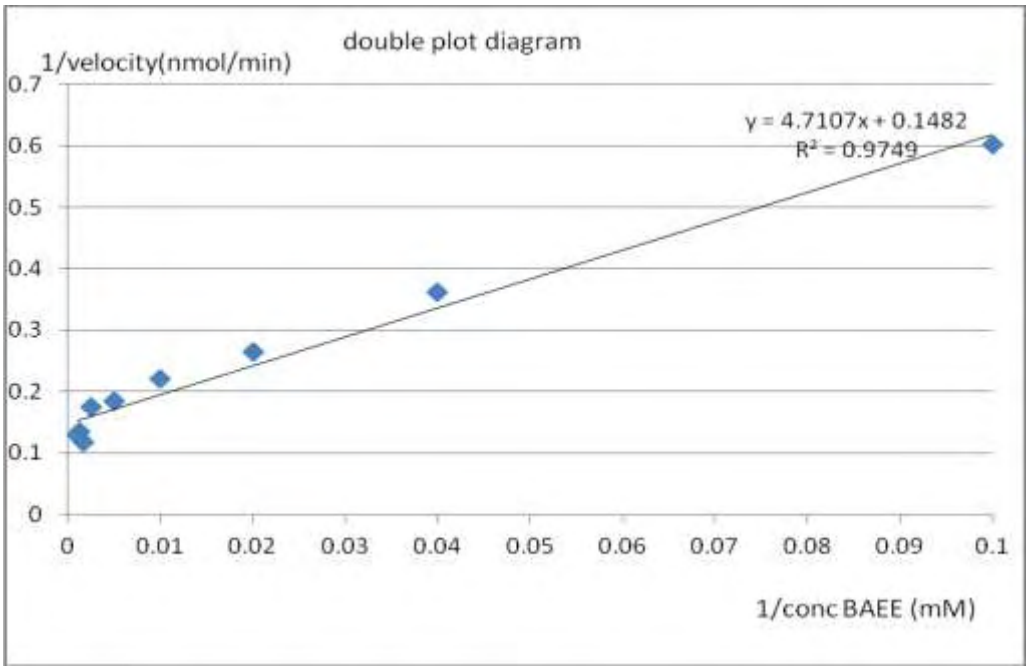


Fig.23. Double reciprocal plot for trypsin immobilized on Monolith 2.

Determination of Km: 31.786 mM
Determination of Vmax: 6.7476 nmol/min

Monolith 3:

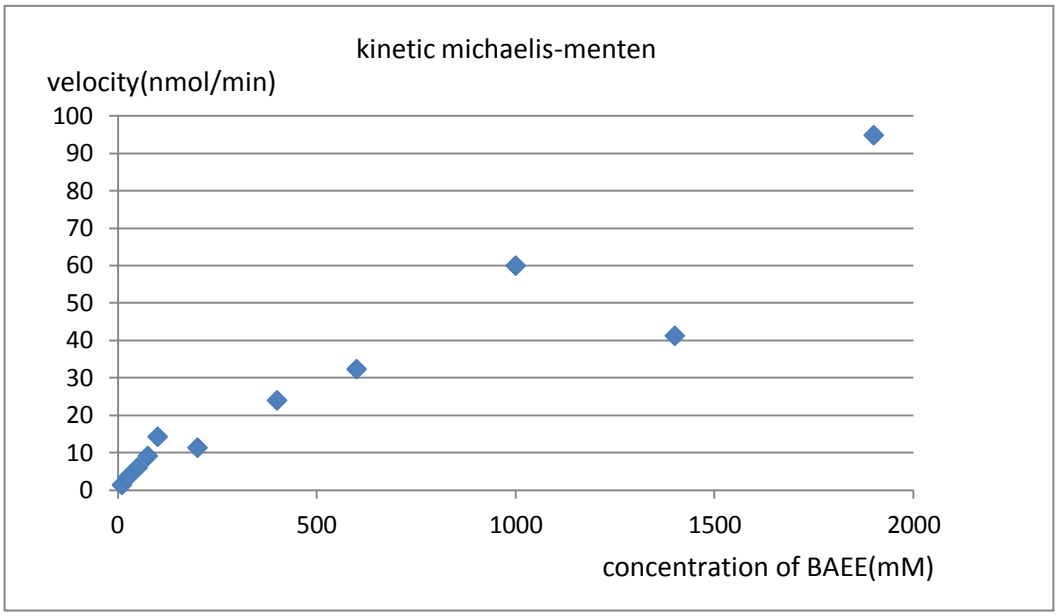


Fig.24. Michaelis- Menten plot for trypsin immobilized on Monolith 3

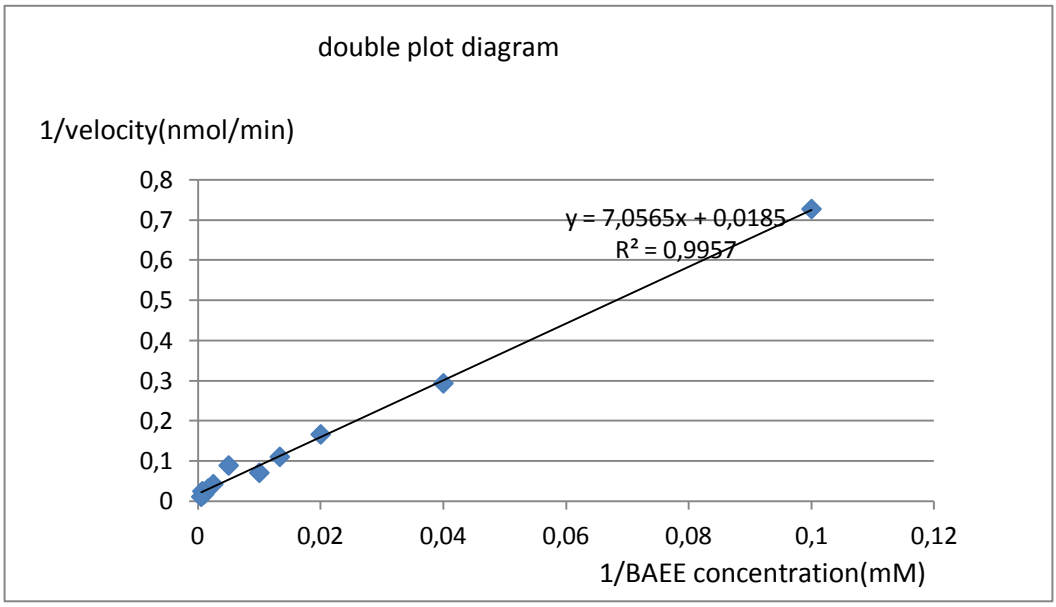


Fig.25. Double reciprocal plot for trypsin immobilized on Monolith 3.

Determination of Km: 381.43 mM

Determination of Vmax: 54.054 nmol /min

After the quantification of immobilized trypsin by the Bradford method and the calculation of the maximum hydrolysis rate (Vmax), the specific activity was also determined. The specific activity is defined as the amount of substrate converted by an enzyme per mg of the enzyme preparation, per unit of time. It is expressed as IU (International Unit), as nmol/min/mg.

This parameter is an index of the catalytic efficiency of the enzyme, and can be representative for the success of an immobilization procedure: when an enzyme is fixed onto a solid support, an unfavourable orientation may occur resulting in partial or total loss of accessibility of the catalytic pocket. This steric hindrance results in a reduced enzyme specific activity: although present (in terms of mg), the enzyme cannot express its catalytic activity.

The three bioreactors' kinetic parameters are here summarized:

Monolithic supports	M1	M2	M3
Equation from Lineweaver-Burk plot	y =1.332x+0.029 R^2=0.991	y = 4.7107x + 0.1482 R^2 = 0.9749	y = 7.0565x + 0.0185 R^2 = 0.9957
Km(mM)	142.82	31.786	381.43
Vmax(nmol/min)	36.1	6.74	54.054
mg of the immobilized enzyme	0.1212	0.0545	0.3066
Specific activity (UI Units/mg)	297.8548	123.6697	176.3014

Table 4.Apparent kinetic parameters and apparent bioreactor specific activity

The value of Km reflects the concentration of the substrate required when initial velocity is half of its maximum value, and a higher Km value indicates a weaker affinity between the substrate and enzyme immobilized on monolith.

In this case, the immobilized trypsin of M3 demonstrated the highest Km, probably due to the fact that in this monolith saturation was not reached. In the bioreactor, it could be arisen that the Km and Vmax values are proportional. As a result, the immobilized trypsin of the M3 will have the highest value of Vmax, as well, and this is in accordance with the extremely higher amount of enzyme bound onto this support.

The immobilized enzyme on the monolith 2 had the lowest Km and Vmax values, which means that the affinity between the substrate and the enzyme was stronger.

As far as the first monolith is considered, the values of the kinetic parameters are in the middle of the second and the third.

The data obtained clearly demonstrate that Vmax parameter is directly proportional to the amount of immobilized enzyme, which means that for a high hydrolytic efficiency of the bioreactor, a high immobilization yield is desirable.

The Km parameter, on the other hand, is influenced by the physico-chemical properties of the solid support carrying the enzyme, but seems also to be strongly dependent on the total amount of active enzyme. Indeed, Monolith 2, characterized by the lowest enzymatic loading, showed the best affinity for the substrate as the result of a higher support hydrophylicity and the lowest enzyme content.

4.3.1 Study of the effect of the flow rate on the kinetic parameters

In a continuous flow reaction system like a bioreactor, the flow rate can determine the contact time between the enzyme and substrate. [3] A series of flow rate values (2µL/min, 5µL/min, 15µL/min, 20µL/min) was selected representing a compromise between reaction time and the chromatographic system constraints (i.e. back pressure of the micro-HPLC). The composition of the mobile phase was 20% Acetonitrile and 80% Phosphate buffer at pH 8.

The first experiment carried out was the calculation of the reaction time at each studied flow rates. This parameter corresponds to the time at which the reaction mixture start to elute from the bioreactor, and was measured following the peak rise at 223 nm. As expected, as the flow rate increases, the reaction time decreases, even if a non linear correlation was observed, indicating that both catalytic and chromatographic factors affect this parameter. The calculated reaction times are reported in the graph below.

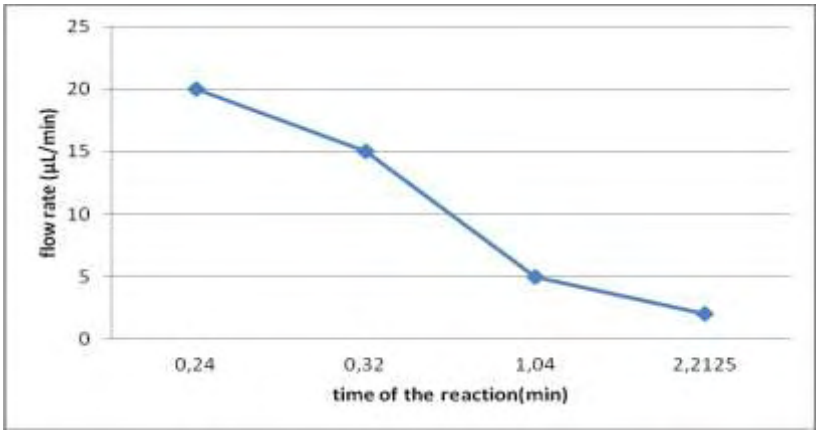


Fig.26. the reduction of the reaction time with increasing the flow rate

The effect of the substrate feed flow rate on the back pressure of monolithic IMERs was also investigated, as this parameter might have a negative effect on the enzyme stability and should be controlled in biochromatographic systems. A solution containing 80% phosphate buffer and 20% Acetonitrile at pH 8 was used as the mobile phase. The back-pressure increased with increasing the flow rate and obeyed the following relationship:

$\Delta P=0.612Q \qquad R^2=0.996$

where ΔP and Q are the back-pressure (kilogram-force per square centimeter (kgf/cm^2)) and the volumetric flow rate of mobile phase (microlitres per minute). [1,3]

Calculation of the back pressure for the above values of the flow rate:

$\Delta P_{2\mu L/min} = 1.224$ kgf/cm^2

$\Delta P_{5\mu L/min} = 3.06$ kgf/cm^2

$\Delta P_{15\mu L/min} = 9.18$ kgf/cm^2

$\Delta P_{20\mu L/min} = 12.24$ kgf/cm^2

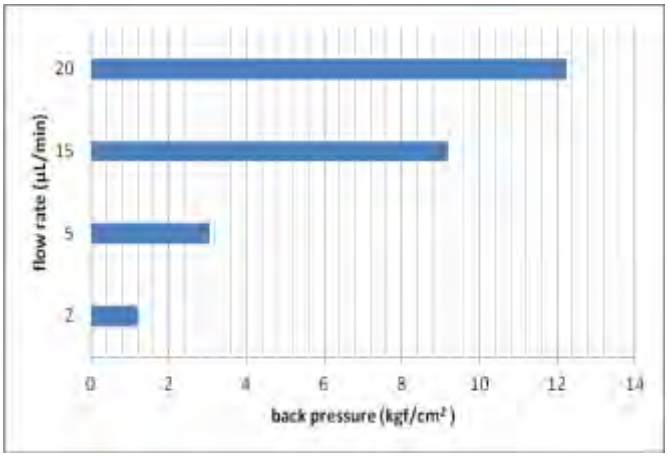


Fig.27. The correlation between flow rate and back pressure

The estimated back pressure for the flow rate 2 and 5 µL/min is negligible, and could be attributed to the high permeability of the monolithic structure. At flow rate higher than 5 µL/min, the system back pressure increases more significantly up to (bar), which was the maximum tolerable by both the monolithic support and the enzyme.

After the reaction time and system back-pressure were assessed, the Km (Michaelis constant) and Vmax (maximum velocity) of immobilized enzyme were determined at each selected flow rate. All the experiments were carried out on M1, which was selected because of its capability to reach substrate saturation, thus providing more reliable kinetic parameters.

Concentration of BAEE injected (mM)	nmols Injected	20µL/min				15µL/min			
		Average area BA	nmols of product	start time (min)	Velocity (nmol/min)	Average area BA	nmols of product	start time (min)	Velocity (nmol/min)
1	0,2	48,30	0,23	0,24	0,98	43,78	0,19	0,33	0,58
10	2	295,20	2,46	0,24	10,23	237,50	1,94	0,32	6,05
25	5	513,40	4,42	0,24	18,41	502,20	4,32	0,32	13,49
50	10	603,00	5,22	0,24	21,77	630,70	5,47	0,32	17,11
75	15	668,90	5,82	0,24	24,24	884,70	7,76	0,33	23,30
100	20	698,70	6,09	0,23	26,46	852,60	7,47	0,32	23,34
200	40	740,40	6,46	0,23	28,09	915,60	8,04	0,32	25,12
400	80	962,60	8,46	0,24	35,25	1117,75	9,86	0,33	29,87
600	120	965,40	8,49	0,24	35,35	1096,60	9,67	0,31	31,48

Concentration of BAEE injected(mM)	nmols injected	5µL/min				2µL/min			
		Average area BAA	nmols of product	Start time	Velocity (nmols/min)	Average area BAA	nmols of product	start time (min)	Velocity (nmols/min)
1	0,2	49,35	0,24	1,12	0,22	34,675	0,11	2,47	0,05
10	2	270,40	2,23	0,98	2,28	241,4	1,97	2,28	0,86
25	5	651,95	5,67	0,97	5,86	536,605	4,63	2,31	2,01
50	10	1327,75	11,74	1,01	11,69	1109,09	9,78	1,92	5,09
75	15	1467,50	13,00	1,00	12,95	1524,095	13,51	2,23	6,07
100	20	1974,30	17,56	1,21	14,47	1876,615	16,68	2,08	8,01
200	40	2045,35	18,20	0,99	18,32	2624,52	23,41	2,13	10,98
400	80	2282,35	20,33	1,06	19,24	2805,9	25,04	2,28	10,99
600	120	2593,65	23,13	1,02	22,62				

Table 5, 6: Analytical data for Monolith 1 at a range of flow rate (2µL/min, 5µL/min, 15µL/min, 20µL/min)

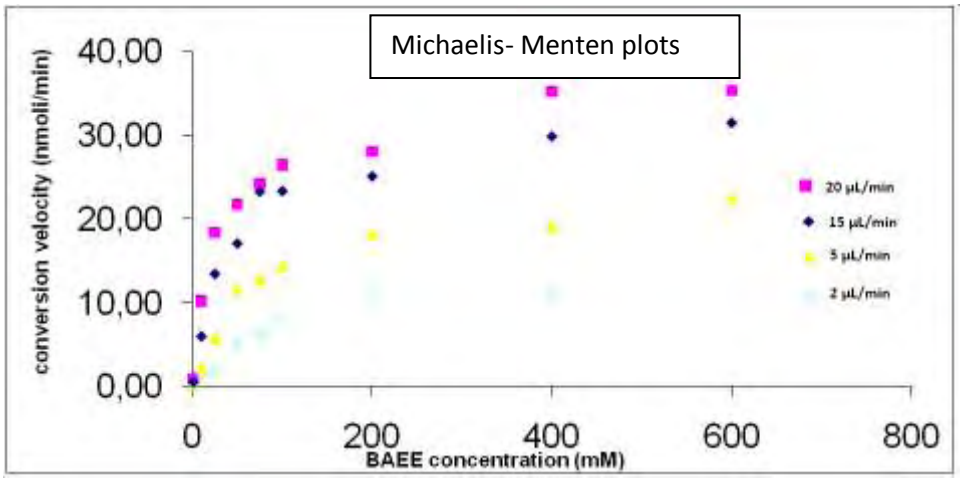


Fig.28. Michaelis – Menten plots for trypsin immobilized on Monolith 1. The parameter changed was the flow rate of micro-HPLC. (2 µL/min, 5 µL/min, 15 µL/min, 20 µL/min)

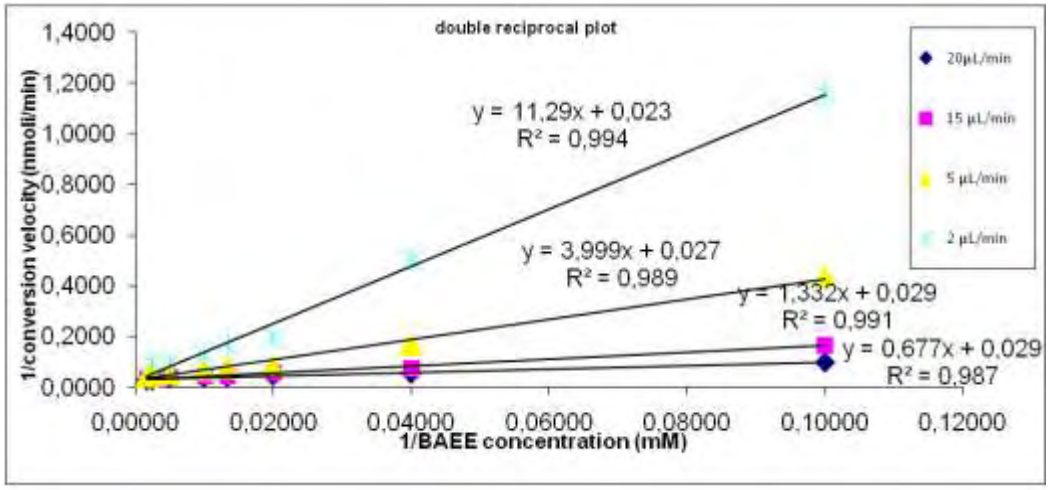


Fig.29. Double plot of the immobilized trypsin with different flow rates to micro-HPLC (2 µL/min, 5 µL/min, 15 µL/min, 20 µL/min)

1/injected concentration (mM)	1/velocity			
	20µL/min	15µL/min	5µL/min	2µL/min
1,00000	1,0218	1,7149	4,5636	22,0201
0,10000	0,0977	0,1652	0,4384	1,1562
0,04000	0,0543	0,0741	0,1706	0,4985
0,02000	0,0459	0,0585	0,0856	0,1964
0,01333	0,0413	0,0429	0,0772	0,1648
0,01000	0,0378	0,0428	0,0691	0,1249
0,00500	0,0356	0,0398	0,0546	0,0911
0,00250	0,0284	0,0335	0,0520	0,0910
0,00167	0,0283	0,0318	0,0442	
Km (mM)	**22,65**	**45,95**	**142,82**	**474,71**
Vmax (nmol/min)	**33,44**	**34,48**	**36,10**	**42,02**

Table7. Analytical data for the construction of double plot diagrams of the flow rate study.

From the data reported in the table we can observe that the highest flow rate gives the lower Km (i.e. better affinity). This effect can be ascribed to the intrinsic monolith's properties. Indeed, the flow rate in monolithic materials is mainly convective instead of diffusive, being the diffusion paths on these stationary phases almost neglectable. This characteristic results in improved mass transfer due to the absence of diffusive limitations and the accessibility of the stationary phase (the catalytic site, in this case) is improved. The positive effect is evident from the Km values: a 20-fold increase in substrate affinity is observed when the flow rate increases from 2 to 20 µl/min (10-fold increase).

Concerning the Vmax, as this parameter indicates the hydrolysis rate, thus the amount of product per minute, the increase in contact time would result in an increase of product obtained. This effect is well evident because the nmol product increase is higher than that of the contact time, as their ratio, Vmax, increases by reducing the flow rate.

4.3.2 Study of the effect of the composition of the mobile phase on kinetic parameters

Another experimental parameter that was explored in this study is the effect of the mobile phase composition on the kinetic parameters of immobilized trypsin. This parameter was studied in order to evaluate the possibility to add or eliminate the organic modifier during hydrolysis, when required to maximize the hydrolysis of a studied substrate.

A small aliquot of organic modifier in the mobile phase might prevent any unspecific binding of substrate/product on the monolith support due to hydrophobic interactions. However, in view of on-line coupling of the bioreactor to an on-line-SPE-LC-MS system for peptide enrichment and analysis, the elution of produced peptides in a non totally aqueous environment could result in unwanted loss of peptides from the trapping column (C-18 derivatized stationary phase), especially for the most hydrophilic ones. [1, 3]

For these reasons, the behavior of immobilized trypsin in buffered media containing different percentages of organic modifier was studied in detail.

The results obtained in a preliminary study (figure below) demonstrate that the reaction time is almost independent by the presence of organic modifier. In absence of acetonitrile, some unspecific binding can be observed by the enlarged peak shape of eluting reaction mixture, even if the entity of this effect is extremely low and could be neglected.

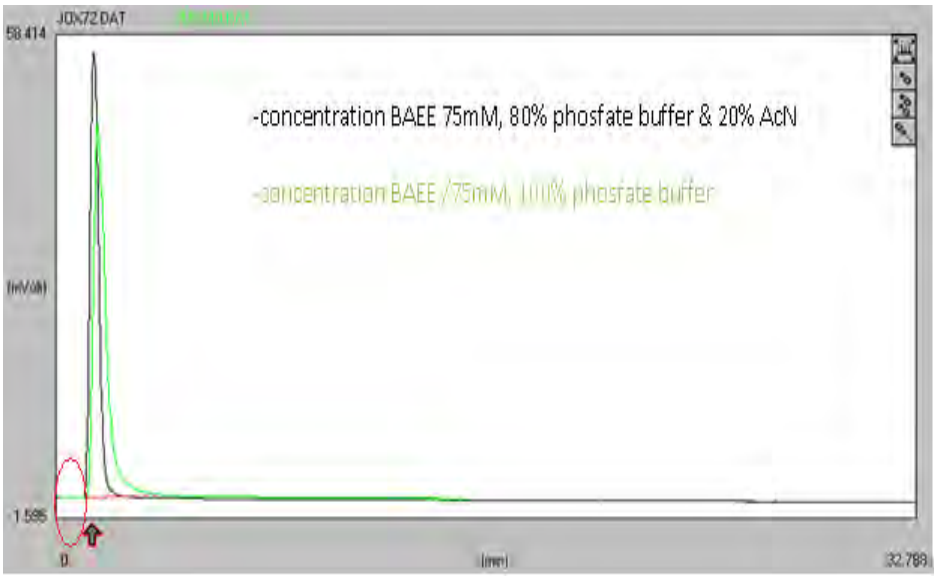


Fig.30. Chromatograms obtained by injection of the same substrate concentration on Monolith 1 in the presence (blue line) and absence (green line) of acetonitrile in the mobile phase.

The next step was a detailed characterization of kinetic parameters of immobilized trypsin in presence of 0, 5, 10 and 20%ACN in the buffered mobile phase.

	kinetic without AcN			Kinetic with 5%AcN		
Conc BAEE mM	Tstart (min)	BAA product (nanomoli)	velocity (nano moli/min)	Tstart (min)	BAA product (nanomoli)	velocity (nano moli/min)
1	1,21	0,00	0,001	1,19	0,21	0,1744
10	1,11	1,73	1,565	1,12	1,85	1,6547
25	1,11	4,86	4,388	1,09	4,16	3,8080
50	1,09	9,17	8,390	1,08	6,94	6,4206
75	1,05	10,83	10,281	1,07	8,05	7,5443
100	1,05	13,72	13,025	1,08	9,54	8,8287
200	1,07	13,83	12,960	1,09	13,37	12,2685
400	1,05	14,71	13,616	1,04	14,88	14,3064
600	1,05	18,71	17,769	1,03	20,04	19,5119

	Kinetic with 10%AcN			Kinetic with 20%AcN		
Conc BAEE mM	Tstart (min)	BAA product (nanomoli)	velocity (nano moli/min)	Tstart (min)	BAA product (nanomoli)	velocity (nano moli/min)
1	1,07	0,49	0,4566	1,12	0,2443	0,2191
10	1,12	1,76	1,5712	0,98	2,2329	2,2808
25	1,08	4,97	4,6064	0,97	5,6653	5,8617
50	1,09	5,32	4,8660	1,01	11,7449	11,6864
75	1,07	8,16	7,6454	1,00	13,0021	12,9503
100	1,08	11,29	10,4513	1,21	17,5613	14,4736
200	1,11	10,55	9,5284	0,99	18,2004	18,3195
400	1,08	13,93	12,8936	1,06	20,3325	19,2360
600	1,07	16,92	15,8591	1,02	23,1330	22,6239

Table 8, 9 the effect of the mobile phases with different percentage of ACN on the kinetic parameters (for the micro-HPLC)

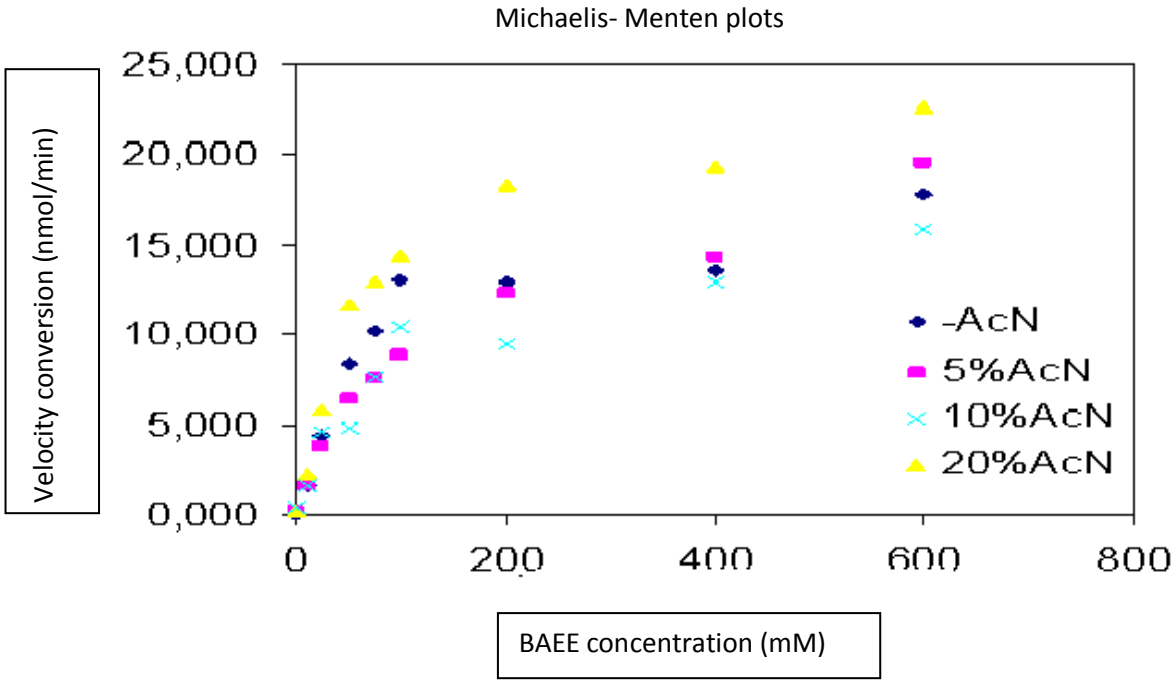


Fig.31. Kinetic Michaelis-Menten diagrams with changing the percentage of Acetonitrile in the mobile phase

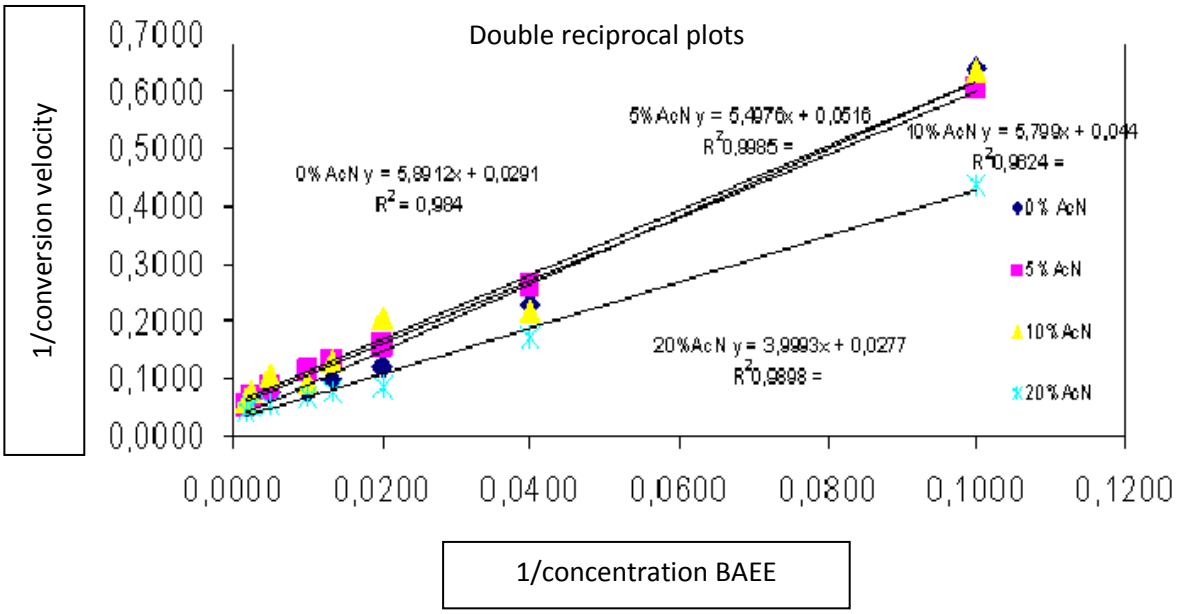


Fig.32. Double plots diagrams with changing the percentage of Acetonitrile in the mobile phase

		1/velocity			
Conc BAEE mM	**1/conc**	**0% AcN**	**5%AcN**	**10%AcN**	**20%AcN**
1	1,0000	1230,9185	5,7346	2,1901	4,5636
10	0,1000	0,6391	0,6043	0,6365	0,4384
25	0,0400	0,2279	0,2626	0,2171	0,1706
50	0,0200	0,1192	0,1557	0,2055	0,0856
75	0,0133	0,0973	0,1326	0,1308	0,0772
100	0,0100	0,0768	0,1133	0,0957	0,0691
200	0,0050	0,0772	0,0815	0,1049	0,0546
400	0,0025	0,0734	0,0699	0,0776	0,0520
600	0,0017	0,0563	0,0513	0,0631	0,0442
Km (m/q)		**202,4**	**106,5**	**131,8**	**144,4**
Vmax (1/q)		**34,4**	**19,4**	**22,7**	**44,1**

Table10. Analytical data for the construction of the Double plot diagram

The obtained data demonstrate that both affinity and hydrolytic activity of immobilized enzyme are influenced by mobile phase composition. A low percentage of ACN (5%) gives the best results in terms of affinity, but the lowest hydrolytic capacity. To gain maximum activity of the bioreactor, 20% ACN in the mobile phase seems to be recommendable. However, if these chromatographic conditions could not be applied (i.e. in case of on-line SPE with RP C-18 materials), 100% buffer might be also used with a loss of enzymatic activity of 22%, only (Vmax is reduced from 44.1 to 34.4 nmol/min) .

4.4 Stability test of bioreactors.

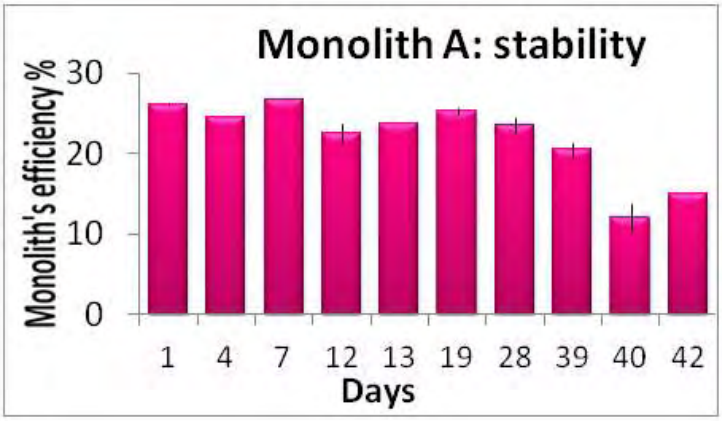
The stability behavior of monolithic IMER was also assessed. The immobilization of trypsin on these type monolith is a novel technique, and the maintenance of the enzyme stability upon immobilization on the monolith is a crucial point for applicative purposes of the synthesized bioreactors.

In order to verify enzyme stability, a test analysis was set. The experimental conditions for this test were kept constants, so that the results were be comparable.

In more detail, the flow rate was 5 μL/min. The selected BAEE concentration was 400mM, which corresponded to saturation conditions of monoliths 1 and 2. For comparative purposes, BAEE concentration of 400mM was also applied to monolith 3 despite the fact of the unsaturated conditions. The mobile phase was a mixture of 20% ACN (Acetonitrile) and 80% Phosphate buffer, and the data were expressed as enzymatic efficiency as follows:

efficiency= area of BA/ (area of BA+ area of BAEE)

Monolith1



Monolith2

Monolith 3

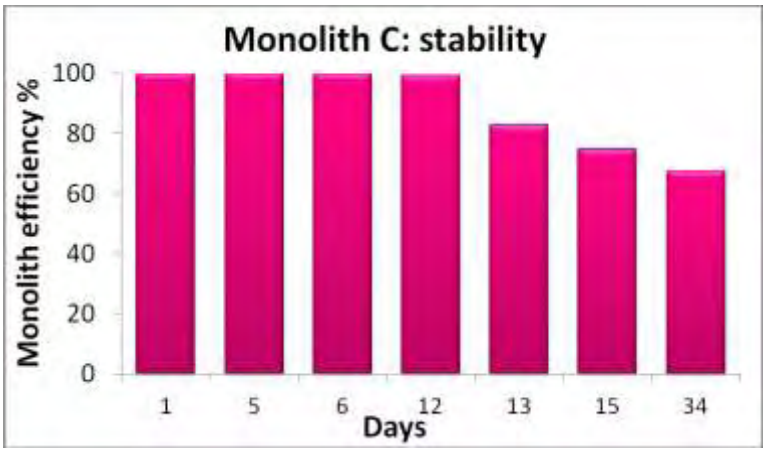


Fig. 33, 34, 35. The efficiency, expressed as the percentage of products area divided with the total areas of the product and the substrate, per days. No significant changes were observed during the days of trypsin on each Monolith. Though, the trypsin on Monolith 3 is active-stable even the pass of 34 days with 80% efficiency.

The results obtained clearly show in all bioreactors an excellent stability during time, in particular monolith 1 and 3, containing the highest enzyme amount immobilized, were stable for more than one month under continuous use.

So we can affirm that this novel type of monolith supports are able to give good conversion kinetics with a good stability of the support.

Conclusions

An immobilized enzyme reactor (IMER) in the form of capillary monolith was developed for a micro-liquid chromatography system. Trypsin was immobilized on three monoliths, prepared in fused silica capillaries. (GMA-Di GMA, Tri GMA-, NAS). A model reaction, using BAEE-as a standard substrate and BA as the product, has been assigned to study in detail the kinetic parameters – (Michaelis constant- Km, and the maximum velocity- Vmax) of the enzyme immobilized on the three monoliths. The activity behavior of trypsin was characterized by the apparent Michaelis constant and the apparent maximum velocity. The kinetic evaluation of immobilized trypsin was used for the comparison of monoliths. Bradford assay was applied for the quantitative determination of the immobilized trypsin on the three monoliths. The immobilized trypsin of M3 demonstrated the highest Km and the highest value of Vmax, as well, and this is in accordance with the extremely higher amount of enzyme bound onto this support. The immobilized enzyme on the monolith 2 had the lowest Km and Vmax values, proposing that the affinity between the substrate and the enzyme was stronger. Monolith 2, characterized by the lowest enzymatic loading, displayed the best affinity for the substrate as the result of a higher support hydrophylicity and the lowest enzyme content.

The data obtained clearly demonstrate that Vmax parameter is directly proportional to the amount of immobilized enzyme, which means that for a high hydrolytic efficiency of the bioreactor, a high immobilization yield is desirable. The Km parameter, on the other hand, is affected by the physico-chemical properties of the solid support carrying the enzyme, but seems also to be strongly dependent on the total amount of active enzyme, probably due to steric effects.

Also, the experimental conditions have been optimized in terms of the flow rate and mobile phase composition to ascertain the optimal support and experimental conditions for the maximization of the catalytic performances of immobilized trypsin. The appropriate flow rate is 5μL/min. As it is observed, Vmax, increases by reducing the flow rate and the highest flow rate gives the lower Km (i.e. better affinity). The effect of the mobile phase composition on the kinetic parameters of immobilized trypsin was studied in order to evaluate the possibility to add or eliminate the organic modifier during hydrolysis, when required to maximize the hydrolysis of a studied substrate. To gain maximum activity of the bioreactor, 20% ACN and 80% phosphate buffer (pH:8) in the mobile phase seems to be recommendable.

The current thesis provides the results for the most useful monolith that could be coupled to a LC-MS system for on-line digestion, separation and structural characterization of proteins in high-throughput integrated systems, for further studies.

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