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Development of purification strategies for SCOMT and MBCOMT proteins by affinity chromatography

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Resumo alargado

A catecol-*O*-metiltransferase (COMT, CE 2.1.1.6) consiste numa enzima metiltransferase dependente de magnésio que catalisa a metilação de substratos catecóis utilizando S-adenosyl-$_L$-metionina (SAM) com dador do grupo metil, originando dois produtos *O*-metilados. Esta enzima encontra-se densamente expressa ao longo do córtex pré-frontal e do sistema límbico. Nos seres humanos, a COMT existe sob a forma de duas isoformas, uma solúvel (SCOMT) localizada no citoplasma e que tem como principal função a eliminação de catecóis biologicamente ativos e, outra associada a membranas plasmáticas (MBCOMT) que desempenha um papel importante no metabolismo *in vivo* das catecolaminas. Ambas as isoformas são codificadas pelo mesmo gene, localizado no cromossoma 22, a partir de dois promotores, P1 (promotor transcrito a 1.5 kb relativamente à isoforma MBCOMT) e, P2 (promotor transcrito a 1.3 kb em relação à isoforma SCOMT). Ambas são expressas na maioria dos tecidos humanos, exceto no cérebro, onde a MBCOMT é a isoforma dominante.
Em geral, a função fisiológica da COMT é a eliminação de catecóis biologicamente ativos ou tóxicos, funcionando como uma barreira desintoxicante entre o sangue e vários tecidos. Especificamente, a COMT encontra-se envolvida na inativação dos neurotransmissores no sistema nervoso central e regulação dos sistemas dopaminérgicos e noradrenérgicos.
A COMT ao longo dos últimos anos tornou-se um alvo de estudos de diversas patologias, tais como a doença de Parkinson (PD). A PD caracteriza-se pela degeneração neuronal dopaminérgica na *substância nigra*, induzindo a uma diminuição dos níveis do transmissor da dopamina no cérebro. A degradação dos níveis de dopamina iniciados pela COMT conceberam estratégias de terapia. As atuais terapias passam pela administração de levodopa (precursor da dopamina) conjuntamente com inibidores da COMT e da monoamina oxidase (MAO).
Portanto, existe um grande interesse científico no desenvolvimento de estratégias de purificação para estudos estruturais de ambas as isoformas, de modo a se desenhar moléculas capazes de inibir a COMT.

Assim, o principal objetivo deste trabalho consistiu no desenvolvimento de um processo cromatográfico sustentável de forma a obter quantidades significativas da proteína SCOMT_6His na sua forma ativa e pura para estudos cinéticos e estruturais. Deste modo, pela primeira vez, duas estratégias cromatográficas foram propostas para a recuperação de SCOMT_6His a partir de lisados *P. pastoris* usando o monólito Agmatina depois de uma pré-purificação com a coluna de Q-Sepharose ou diretamente depois de um processo de filtração.
Estudos realizados pelo nosso grupo de investigação demonstraram que a aplicação da Q-Sepharose como permutador aniónico revelou ser eficiente na recuperação de ambas as isoformas. No entanto, neste trabalho houve a necessidade de se adaptar o processo cromatográfico. Assim, tornou-se conveniente analisar a aplicação de vários gradientes com o objetivo de melhorar o processo de purificação da SCOMT_6His. Os resultados obtidos

mostraram que a retenção da proteína SCOMT_6His é alcançada com a aplicação de um aumento linear do gradiente de cloreto de sódio (NaCl) de 0 a 100 mM NaCl seguido de outro gradiente linear de 100 a 310 mM NaCl enquanto, a sua eluição é atingida com um gradiente por passos a alta concentração (450 mM NaCl).
Em termos de recuperação, a coluna Q-Sepharose não demonstrou uma elevada seletividade para o isolamento SCOMT_6His, pois verificou-se uma perda significativa na atividade específica da proteína de interesse em relação à amostra de lisado.
Para melhorar o grau de pureza da proteína alvo obtida pelo ensaio da Q-Sepharose, vários suportes monolíticos foram testados (CDI, Histamina e Agmatina). Os suportes monolíticos têm sido aplicados com sucesso para a purificação de DNA plasmídico, RNA e proteínas, no entanto, estes suportes nunca foram utilizados na purificação da proteína SCOMT_6His. Deste modo, o estudo destes três suportes teve como finalidade avaliar o comportamento cromatográfico da amostra pré-purificada a fim de se explorar diferentes estratégias de eluição por aumento e diminuição de concentrações de cloreto de sódio e sulfato de amónio ($(NH_4)_2SO_4$). De acordo com os resultados obtidos, as estratégias de eluição da proteína SCOMT_6His adotada para monólitos CDI e Histamina foram baseados na manipulação das condições hidrofóbicas e iónicas, no entanto, verificou-se que a proteína alvo não ficou retida nestes monólitos, sendo eluída no flowthrough em conjunto com outras proteínas contaminantes. Por sua vez, a retenção da SCOMT_6His foi conseguida no monólito Agmatina e, a sua eluição foi possível com um gradiente linear crescente de NaCl na fase móvel. Por fim, com o objetivo de se isolar e purificar a SCOMT_6His no monólito Agmatina, houve a necessidade de se injetar diretamente a amostra de lisado de P. p*astoris* no suporte, pois com esta estratégia aumentou-se a quantidade de proteína injetada na matriz originando menores perdas no rendimento do passo cromatográfico.
Em conclusão, a comparação destas abordagens demonstram as limitações existentes no delineamento das estratégias de purificação desta proteína. No entanto, os estudos realizados nos monólitos apoiam claramente que estes têm vantagens sobre os métodos anteriores publicados, devido à sua simplicidade no processo purificação.

Palavras-chave

Parkinson, COMT solúvel, Purificação, Cromatografia de Interação Aniónica, Cromatografia de Afinidade, Suportes monolíticos.

Abstract

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) was first described in 1958. It is a S-adenosyl-$_L$-methionine (SAM) dependent methyltransferase which catalyses the methylation of catechol substrates (catecholamines, catecholestrogens). This protein plays an important role in the brain, since participates in the metabolism of the neurotransmitter dopamine, being involved in neurodegenerative diseases such as Parkinson's disease. Biosynthesis and purification methods have allowed the crystallization of the soluble COMT (SCOMT) from rats and the analysis of the kinetic properties of the enzyme in detail. In this study, the main goal was to develop appropriate strategies for purification of SCOMT_6His by using initially the Q-sepharose column to clarify the sample and then monolithic supports such as CarbonylDiImidazole (CDI, Histamine and Agmatine monoliths. Firstly, recombinant SCOMT_6His production was performed using *Pichia pastoris* X33 cells containing the expression construct pICZα A-hSCOMT_His6. Subsequently, a suitable cell lysis stage employing glass beads was performed, and the lysate was recovered and directly injected onto the Q-sepharose support for clarification and reduction of the homologous proteins from *P. pastoris* lysates. Results shown that for a complete adsorption of SCOMT_6His onto the anionic resin it was necessary a linear salt gradient (0 mM to 310 mM NaCl in 10 mM Tris-HCl, pH 7.8). Subsequently, for SCOMT_6His elution it was performed a stepwise salt gradient of 450 mM and 1 M of NaCl in 10 mM Tris-HCl, pH 7.8. By analysis of several eluted peaks with SDS–PAGE gel and western blot, it can be observed that SCOMT_6His was eluted essentially at one fraction with high NaCl concentration. Also activity levels and SCOMT_6His recovery rates were evaluated after Q-Sepharose chromatography. Thereafter, the pre-purified sample was injected in three monolithic supports (CDI, Histamine and Agmatine) in order to explore different elution strategies by increasing and decreasing of sodium chloride and ammonium sulphate concentrations. According to the conducted studies, it was found that the SCOMT_6His protein was not retained in CDI and Histamine monoliths under hydrophobic and ionic elution conditions. However, after the equilibrium of the Agmatine monolith with 10 mM Tris–HCl buffer at pH 7.8 at 1 mL/min, the SCOMT_6His was retained, being eluted with 1.5 M NaCl in 10 mM Tris-HCl at pH 7.8. Finally, the direct injection of filtrate lysate sample was also tested in the Agmatine monolith by increasing the NaCl concentration in the elution strategy in order to isolate and purify the SCOMT_6His.

Keywords

Parkinson, Soluble COMT, Purification, Anionic Interaction Chromatography, Affinity Chromatography, Monolithic supports.

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List of Acronyms

3,5-DNC	3,5-dinitrocatechol
3-OMD	3-*O*-methyl-levodopa
AADC	Peripheral aromatic $_{L}$-amino acid decarboxylase
AC	Affinity Chromatography
AdoMet	S-Adenosyl-$_{L}$-methionine
B.choshinensis	*Brevibacillus choshinensis*
BSA	Bovine serum albumin
CDI	CarbonylDiImidazole
CEA	Anionic exchange chromatography
CEC	Cationic exchange chromatography
COMT	Catechol-*O*-metytransferase
CV	Column volume
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
HCl	Hydrogen chloride
HIC	Hydrophobic Interaction Chromatography
His	Histidine aminoacid
HPLC	High performance liquid chromatography
IEC	Ion Exchange Chromatography
IMAC	Immobilized metal-affinity chromatography
KDa	kilodaltons
Km	Michaelis- Menten constant
L-Dopa	Levodopa
MAO	Monoamine oxidase
MBCOMT	Membrane bound catechol-*O*-methyltransferase
Met	Methionine
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
MP's	Membrane proteins
Lys	Lysine
OD_{600}	Optical density at 600nm
OPC	Opicapone
PD	Parkinson disease
PI	Isoelectric point
P. pastoris	*Pichia pastoris*
Trp	Tryptophan

Pro	Proline
SAH	S-adenosyl-$_{L}$-homocysteine
SAM	S-adenosyl-$_{L}$-methionine
SCOMT	Soluble catechol-*O*-methyltransferase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Tris	Tris(hydroxymethyl)aminomethane
V_{max}	Maximum velocity
Val	Valine

Chapter I

Introduction

1.1 The enzyme catechol-*O*-methyltransferase

Catechol-*O*-methyltransferase (COMT, E.C.2.1.1.6.) was first characterized by Axelrod and Tomchick in 1958 [1]. Both investigators showed that the enzyme is responsible for the 3-*O*-methylation of catecholamines [2]. This enzyme was firstly discovered in rat liver extracts, and since then, it has been found in plants, yeast, fungi, invertebrates and vertebrates [3, 5]. In mammals, COMT is widely distributed throughout the organs of the body and their higher activity levels have been found in liver, kidney and gut wall. This enzyme is densely expressed throughout both the prefrontal cortex and the limbic system. It is noteworthy that liver is the most important site for the metabolism of circulating catechol containing molecules [1, 4, 5].

COMT is a monomeric magnesium-dependent enzyme that catalyses the methylation of catechol substrates using S-adenosyl-$_{L}$-methionine (SAM) as a methyl donor, taking as reaction products, *O*-methylated catechol and S-adenosyl-$_{L}$-homocysteine (SAH) (Figure I) [3, 5, 6, 11, 16, 24].

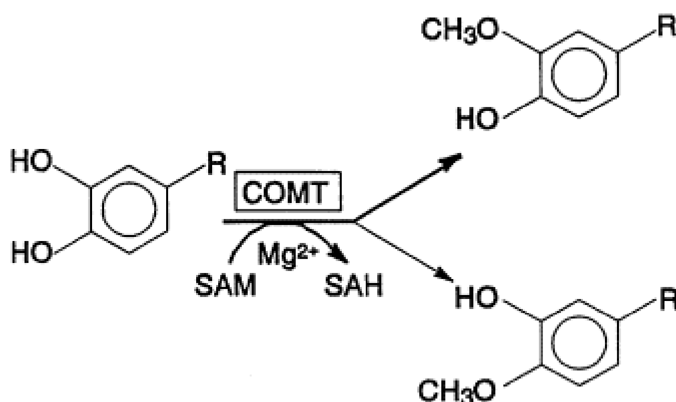

Figure I - A typical reaction catalysed by COMT. SAM: S-adenosyl-$_{L}$-methionine; SAH: S-adenosyl-$_{L}$-homocysteine; Mg^{2+}: Magnesium (adapted from [4]).

Regarding its three-dimensional structure, the protein is composed of a seven stranded β-sheet, wedged between two sets of α-helices as seen in Figure II [8]. The active site of COMT consists of the S-adenosyl-$_L$-methionine (AdoMet) binding domain and few amino acids such as Lysine and Proline that are important for the binding of the substrate, water, and Mg^{2+}. It is noteworthy that the binding motif of the AdoMet site is similar to the Rossman fold [8, 9]. In addition, Mg^{2+} ions are essential for COMT activity, since they are coordinated to both of the catecholic hydroxyls, to a water molecule and to three amino acid residues in the catalytic site of COMT [2].

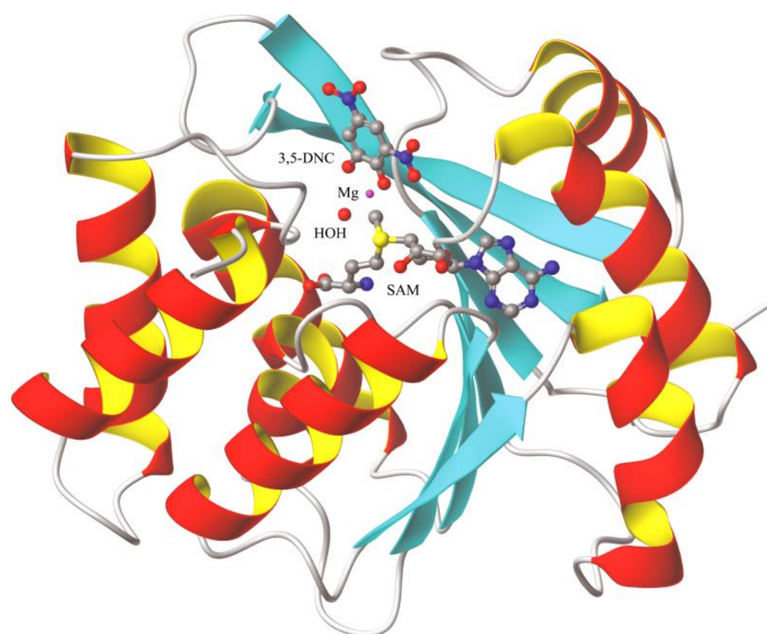

Figure II - Representation of the three-dimensional structure of COMT. The S-adenosyl-$_L$-methionine co-substrate (SAM), the 3,5-dinitrocatechol (3,5-DNC), the magnesium ion (Mg^{2+}), and coordinated water molecules are depicted (adapted from [8]).

For example, amino acid residues such as Lys144 (Lysine 144) accept a proton of the hydroxyl group and residues such as Trp38 (Tryptophan 38), Trp143 (Tryptophan 143) and Pro174 (Proline 174) form hydrophobic walls that define COMT selectivity for the substrate [8].

1.1.1 Functions of COMT

Over the recent years COMT has become a target in pharmacological studies due to its interference in normal brain function and possible involvement in some human disorders such as Parkinson disease (PD), Alzheimer disease and schizophrenia due to lack of dopamine [3, 4, 8, 10, 29].

In general, COMT physiological function consists in the inactivation of biologically active or toxic catechols. Specifically, COMT it is involved in the inactivation of the neurotransmitters in the central nervous system and regulation dopaminergic and noradrenergic systems. This protein catalyses the transfer of a methyl group to catecholamines and degrades dopamine, norepinephrine and epinephrine [5, 12]. Beyond these specifications, this protein has important role in the inactivation of catecholamines, metabolism of catecholestrogens and catecholic drugs, such as L-dopa and carbidopa [2]. Therefore, COMT has been implicated in several human diseases such as cardiovascular diseases, estrogen induced cancers and neurologic disorders [2, 3].

1.1.2 Isoforms: SCOMT and MBCOMT

In humans, COMT presents two molecular forms, a soluble (SCOMT) and a membrane-bound (MBCOMT) [1, 2, 6].

SCOMT is a nonglycosylated protein containing 221 amino acid residues and a molecular weight of 24.7 kDa [1, 2, 10, 11]. This soluble form presents most abundance in the cytoplasm and has principal function as elimination of biologically active or toxic catechols [5, 11, 28]. Relatively to MBCOMT, it is an integral membrane protein and is found mainly associated with the rough endoplasmic reticulum membrane [2, 10, 11, 13, 28].This isoform has an additional peptide in its amino terminal of 50 amino acid residues and a molecular weight of 30 kDa [2, 5, 28]. This extra peptide contains a stretch of 21 hydrophobic amino acid residues that constitute the membrane anchor region [5, 6, 10]. This enzyme plays an important role of metabolism of catecholamines *in vivo* since it has 100-fold higher affinity for catecholamine substrates that soluble isoform [6, 13].

Finally, it is important to note that both forms of COMT are coded by a single gene that is located on chromosome 22 and is composed of six exons [4, 8]. The expression of the COMT gene is controlled by two distinct promoters located in exon 3 [9].

1.1.3 Genetic Polymorphisms

COMT presents two polymorphic forms, a thermolabile low activity form contains Met-108 (158 in MB-COMT) and a thermostable high activity form contains Val-108 (158 in MB-COMT) [2]. Moreover, the COMT polymorphism has been shown to account for individual variability in the response to pharmacological manipulations that alter dopamine [20].

In human tissues, COMT activity is distributed to three levels, low ($COMT^{LL}$), intermediate ($COMT^{LH}$), and high ($COMT^{HH}$) [9]. The most studied genetic polymorphism consists in the substitution of amino acid valine (Val) by methionine (Met) (Val158Met). This functional polymorphism is caused by transition of guanine to adenine at codon 158 of the MBCOMT and is associated for example to Parkinson's disease [9, 14, 15, 21]. The Met108/158 variant is associated with low enzymatic activity and decreased thermal stability, while the Val108/158 is associated with high activity [14, 15, 20, 21]. However, there are other polymorphisms in the COMT gene, such as Ala22Ser (G/T), His12His (C/T), Leu86Leu (C/G and C/T) and Ala52Thr (G/A) in which COMT activity is not affected by mutation [14, 15].

1.1.4 Stability of COMT

The clinical use of therapeutic proteins has increased over the years due to scientific developments and continued growth of biotechnology and biopharmaceutical industries [8]. Therefore, this strategy has enabled the treatment of a wide range threatening diseases [29]. However, the aggregation and misfolding continues to be a problem in experimental approaches using soluble proteins. The tendency to form aggregates induces a decrease in biological activity and reduces the efficiency of separation techniques [30]. In this context, various purification techniques have been developed in order to increase the activity rate and consequently its performance [29, 30]. In order to avoid the activity losses of therapeutic proteins, there are several parameters to analyse such as pH and ionic strength. The ionic strength should be adjusted to the minimum that allows a homogeneous solubilisation and to a maximum which avoid the dissociation of protein structures [8, 29].

The protein COMT is highly unstable and loses rapidly its activity during isolation and storage [2, 9]. The temperature is one of the factors that lower activity. Other factor is pH, essential to ensure maintenance of catalytic activity and specifically for COMT optimum pH is achieved between 7.5 and 8.0 [27].
The enzyme contains few cysteine residues in its primary structure and a reasonable reason for COMT poor stability is the oxidation of thiol (-SH free groups of cysteine) and the consequent formation of intra- or intermolecular disulphide bridges [31]. According to the literature the cofactor SAM and magnesium chloride ($MgCl_2$) reduce cysteine oxidation, preventing COMT inactivation, since these residues are essential to catalytic activity [5, 31]. In addition, several types of stabilizers have a stabilizing effect on proteins protecting them against loss of activity and thermal denaturation such as sugars, divalent metals, glycerol and some amino acids (glycine and proline) [32].

1.1.5 COMT inhibitors

The study and development of COMT inhibitors began in 1975 by authoring of Guldberg Marsden [14] and led to an improvement in the treatment of Parkinson's disease [3, 6, 11, 25].

There are three generations of inhibitors. In the first generation are included derivatives of pyrogallol and catechols, such as gallic acid, caffeic acid, U-0521, 2-hydroxyoestrogens, or flavonoids like quercetin or rutin [9]. Other noncatecholic compounds were also identified such as ascorbic acid, tropolones, and derivatives of 8-hydroxyquinolines and 3-hydroxylated pyrones and pyridines (Figure III) [3, 4, 9]. Typically, inhibitors such as tropolone and pyrogallol present low efficacy *in vivo* and are toxic.

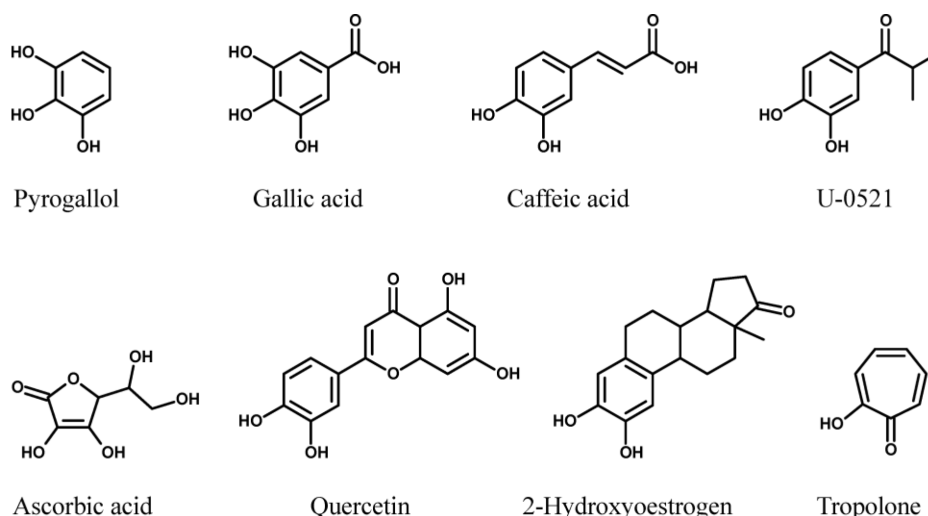

Figure III - Representation of some structures of first generation COMT inhibitors (adapted from [3]).

The second generation of COMT inhibitors includes nitrocatechols as entacapone (OR-611), nitecapone (OR-462) and tolcapone (Ro 40-7592) (Figure IV) [3, 4, 9] have been extensively studied in the context of PD. [6, 8, 16, 22, 25]. These COMT inhibitors have beneficial effects in increasing the half-life of levodopa (L-Dopa), a drug used as substitute of dopamine (Figure V) [3, 4, 20].

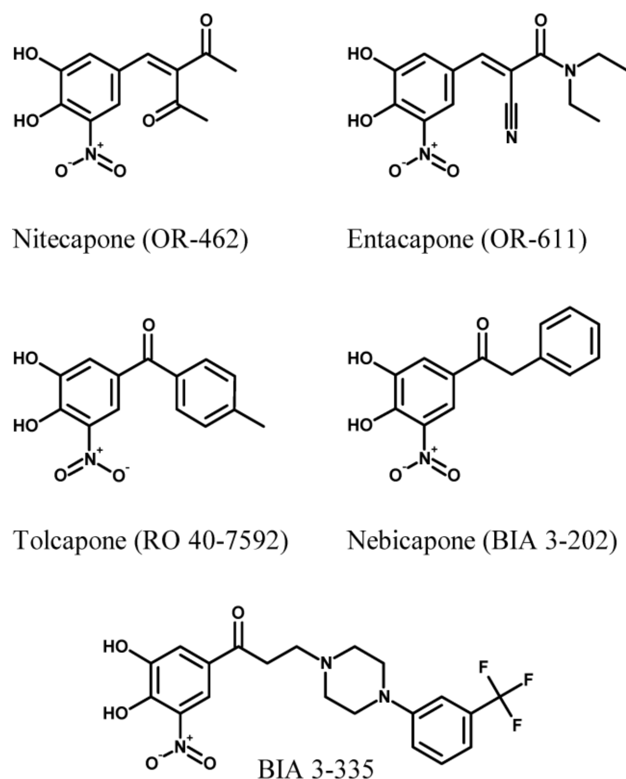

Figure IV - Representation of some structures of second generation COMT inhibitors (adapted from [3]).

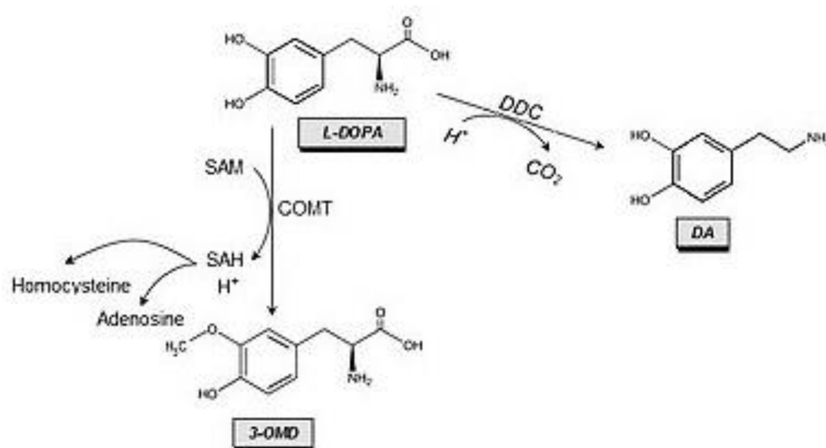

Figure V - Representation of metabolic pathway of L-Dopa (adapted from [1]). L-Dopa: Levodopa; DDC: Dopa decarboxylase; DA: Dopamine; COMT: Catecol-O-metiltransferase; SAM: S-adenosyl-$_L$-methionine; SAH: S-adenosyl-$_L$-homocysteine 3-OMD: 3-O-metil-Levodopa.

Finally, the third generation COMT inhibitor is opicapone (OPC). OPC is a hydrophilic 1,2,4-oxadiazole analogue with a pyridine N-oxide residue at position 3 providing high COMT inhibitory potency and avoiding cell toxicity [17, 18,19]. Another advantage of this inhibitor on the other molecules is the dose be taken 24 hours in 24 hours.

1.1.6 COMT inhibition in Parkinson`s disease

PD is the most common chronic neurodegenerative disease that affects movement behaviour. PD is characterised by dopaminergic neuronal degeneration in the *substantia nigra* and consecutively by striatal dopamine loss with the accumulation of the protein α-synuclein [4, 17]. This happens because COMT initiates the degradation of brain synaptic dopamine levels by introducing a methyl-group from SAM and then later, methylated dopamine is further degraded by monoamine oxidase (MAO) [18, 21].

The treatment of this disease consists in the dopamine replacement therapy with levodopa together with an inhibitor of aromatic amino acid decarboxylase and a COMT inhibitor [5, 6, 9]. The therapeutic effect of levodopa depends on its biotransformation to dopamine in the brain. However, levodopa undergoes rapid and extensive metabolization by peripheral aromatic $_{L}$-amino acid decarboxylase (AADC) and COMT [19]. Therefore levodopa is usually co-administered with an AADC inhibitor (carbidopa or benserazide) which increases levodopa bioavailability, but still approximately 90% of a levodopa dose is converted by COMT to 3-*O*-methyl-levodopa (3-OMD) which competes with levodopa at the level of the blood-brain barrier for transport. Thus, an additional strategy to further inhibit peripheral levodopa metabolism and increase the delivery of levodopa to the brain is the administration of a COMT inhibitor [4, 18-20]. The two inhibitors used for this purpose are tolcapone and entacapone [21]. Actually, it is marketed the opicapone [19].

1.2 Properties of COMT isoforms

Genetic and pharmacological manipulation of COMT activity has demonstrated positive effects in human studies [3]. These studies provide validation for COMT inhibition as a promising avenue for treatment of cognitive deficits in schizophrenia and PD, although no distinctions have yet been made with regards to selective MBCOMT or SCOMT inhibition [21, 22]. As referred above, MBCOMT has much lower capacity and K_m value than the soluble form but a higher affinity to catecholamines [6, 13]. Thus, at high substrate concentrations, the SCOMT activity increases. On the other hand, when concentrations of substrate are low, MBCOMT is the predominant isoform. Furthermore, there are other differences between both isoforms, such as isoelectric point (pI) values, which is 5.2 for SCOMT and 6.2 for MBCOMT [11].

1.2.1 Biosynthesis of SCOMT and MBCOMT

In last two decades, the yeast *Pichia pastoris* (*P. pastoris*) has been used frequently a expression system for recombinant protein production [6, 10]. *P. pastoris* is a single-cell microorganism and by this way, it is easily manipulated and cultivated [23, 26]. The advantages of this system include growth up to high cell densities quantity on defined minimal medium, high expression level of heterologous proteins and efficient secretion of extracellular proteins. Among *P. pastoris*, more remarkable features are the promoter derived from the alcohol oxidase I (AOX 1). This yeast has two alcohol oxidase genes, AOX 1 and AOX 2 of which AOX 1 is much more strongly transcribed than AOX 2, making it a great advantage when induction is effected by methanol. [6, 26, 33].

For expression of the soluble COMT (SCOMT), different expression systems have been used such as transfected mammalian cells [16], insect cells [16] (via mammalian and baculovirus vectors), plant cells (via a potyvirus) [23] and prokaryotic cells, such as *Escherichia coli (E. coli)* [25] that is a Gram-negative bacterium. Usually the major biorecombinant resource of SCOMT, for specific biopharmaceutical and neurological trials, is *E. coli* such as E. coli SG 13009 and BL21 [7, 24, 25, 33]. It should be noted that the optimization of environmental conditions such as temperature, pH, inducer concentration and stabilizers concentration are essential for the production of recombinant proteins to ensure a good quality of the target product [24].

In relation to MBCOMT recombinant protein, several expression systems have been explored to produce high amounts of this enzyme. It´s expression was successfully reached using prokaryotic hosts such as *E. coli* BL21 and SG 13009 strains [6, 33]. Another system used for expression of recombinant protein is *Brevibacillus choshinensis* that is a gram-positive microorganism. This system is well suited for secretory production of heterologous proteins with high efficiency as it produces a small amount of extracellular proteases [63]. Beyond aforementioned systems there are others that are also used for recombinant expression such as, Sf9 insect cells, transfected human embryonic kidney fibroblast cell lines, human HeLa, and hamster BHK cells. However, in some systems is impossible to express this recombinant protein, since its hydrophobic sequence can be toxic to host [6]. Another system used of MBCOMT recombinant protein is *P. pastoris*. This recombinant production, *P. pastoris* has been described as an attractive host for the production of correctly folded and inserted membrane proteins [6].

1.3 Chromatography

Chromatography is a method mainly used in separating components of a sample, whose aim is not only removal of unwanted contaminants, but also the concentration of the desired protein and the transfer to an environment where it is stable and in a form ready for the intended application [34].

Nowadays, chromatographic processes can be differentiated in two types, the analytical and preparative chromatography. Regarding analytical chromatography the principal aim is the rapid detection of specific components through the direct signal acquisition in order to calculate its concentration by a calibration curve [35]. On the other hand, in preparative chromatography it is required the injection of large quantities of sample in the column in order to obtain a given amount of pure product with high recuperation yield [35].
Currently, many chromatographic techniques are available, such as ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), affinity chromatography (AC), reverse phase chromatography (RPC) and gel filtration chromatography (GFC). These different types of chromatography are studied according to different types of interactions involved between solutes in the mobile phase and the stationary phase [8, 28, 36-38]. Therefore, an insoluble matrix is used as stationary phase and is packed into a column and the mobile phase is pumped through the system [36, 39]. Column chromatography is the most common physical configuration, in which the stationary phase is packed into a tube, a column, through which the mobile phase, the eluent, is pumped, such as demonstrated in Figure VI [34, 35].

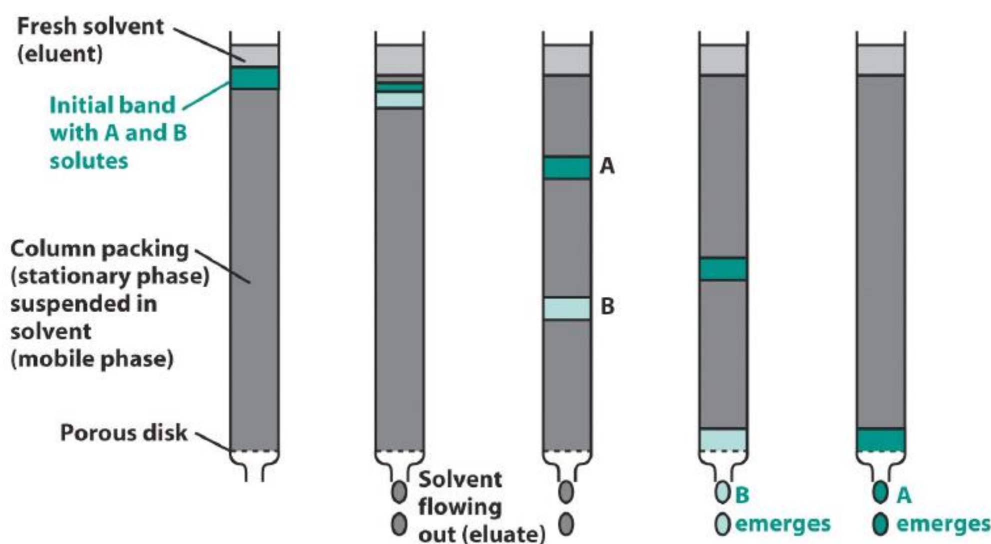

Figure VI - Representation of a typical column apply in chromatography (adapted from [38]).

Generally, a typical chromatographic procedure presents five major stages, equilibrium phase, sample injection, washing of non-retained species, elution of molecules adsorbed in matrix and regeneration (Figure VII) [39]. Briefly, the equilibrium phase aims to maintain optimal conditions at the mobile phase that allow the binding of the target biomolecule to the stationary phase [39]. The sample injection consists in the insertion of a certain quantity of a complex mixture, which contains the target protein, into a stationary phase [38, 39]. The sample is transported by mobile phase and will be distributed between the mobile phase and stationary phase according to its affinity. In the washing stage, impurities that not interact within stationary phase are removed from the column using the same buffer of the column equilibrium [36, 38]. The elution of the retained species is achieved using a buffer that decline the strength of interactions established between the matrix and the target biomolecule. Thus, the proteins strongly adsorbed move more slowly through the column than the weakly bound biomolecules [38]. Lastly, the regeneration step is a very important cleaning process that maintains the binding capacity, selectivity and lifetime of the chromatographic support. The cleaning procedure depends on matrix type but in general are used highly acid or basic solutions, low or high-salt concentrated solutions or organic solvents [38].

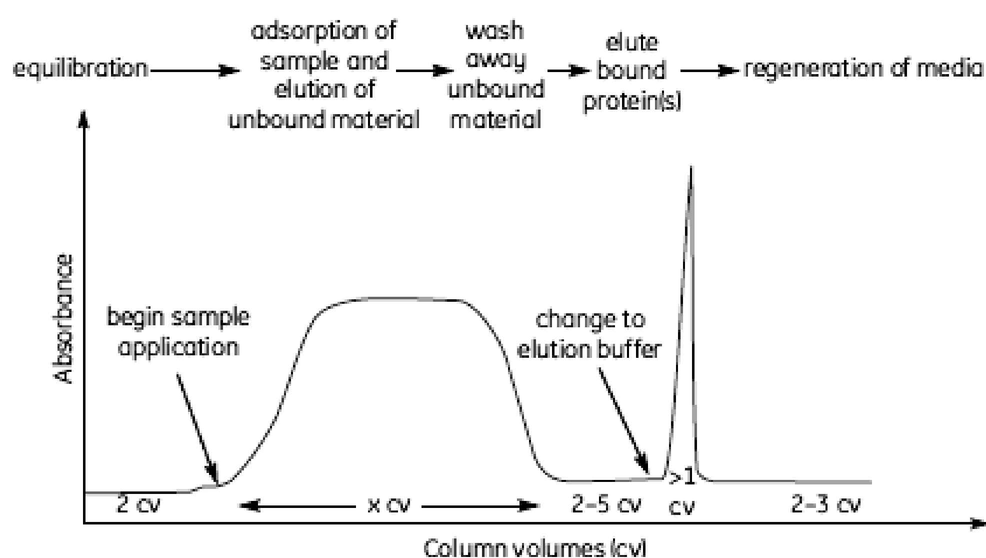

Figure VII - Representation of the phases of chromatographic procedure (adapted from [39]).

1.3.1 Chromatographic Matrix

A chromatographic matrix should be insoluble in the buffer, hydrophilic, easily activated and coupled to a ligand in order to explore different interactions, depending of the ligand nature. It should also have large pores accessible to the protein, have a large surface area to increase

the binding capacity, and be physically and chemically stable to withstand the conditions during derivatization and sterilization [39].

A variety of materials have been used as matrices. These include inorganic materials such as glass, silica, and hydroxyapatite [40]; synthetic organic polymers such as polyacrylamide, polystyrene and polysaccharides [38]. The most commonly used supports are matrices derived from synthetic organic polymers with based agarose, such a Sepharose (Figure VIII) [38, 39]. It should be noted that the agarose is widely used for adsorption chromatography because of its reasonable rigidity, stability, and the ease of surface modification to couple functional groups [39, 40]. In addition, an advantage to use agarose or monoliths is that these supports are fairly easy to prepare in a variety of shapes and sizes [40].

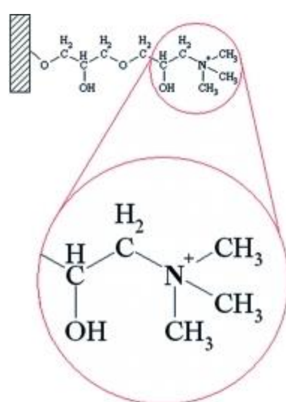

Figure VIII - Structure of Sepharose (adapted from [38]).

1.3.2 Chromatographic methods for SCOMT and MBCOMT purification

The development of techniques and methods for the separation and purification of proteins has been essential for many of the recent advancements in biotechnology research. The choice of a suitable purification technique depends on location, characteristics and desired purity of the target protein [34]. The purification procedure presents high efficiency how lower the number of steps. Thus, it is possible to obtain high yields and the suitable quality and purity [34, 35].

Nowadays, various chromatographic procedures can be applied in purification of proteins. Thus, the chromatography has become one of the preferential techniques for the SCOMT purification due to its high resolving power [16, 42, 43, 45].

1.3.2.1 Hydrophobic interaction chromatography

The HIC is a powerful separation technique where protein purification is based in hydrophobic interactions between hydrophobic ligands immobilized on matrix and non-polar regions on proteins surface. This technique is generally apply immediately after a salt precipitation step [41 - 43]. The adsorption increases with high salt concentration in the mobile phase and the elution is achieved by decreasing the salt concentration of the eluent [37, 41, 42]. The main factors affecting protein chromatographic behavior in HIC are protein hydrophobicity, their surface hydrophobicity distribution and molecular size [41, 43].
This purification process has been extensively applied in purification of SCOMT. This isoform was totally retained on several hydrophobic matrixes and by decreasing the ammonium sulphate concentration, the SCOMT was isolated with a basal loss of specific activity [42]. For instance, butyl-Sepharose resin with an optimized elution gradient revealed the best performance relative to the purity ratio. In addition, the less hydrophobic adsorbent (epoxy-Sepharose) is considered as a last resort due to the high ammonium sulphate concentration needed, which will compromise the protein activity [42].
Regarding to MBCOMT, the purification process is more difficult than SCOMT, because it is highly desirable transfer the protein for a more hydrophobic environment when it is working with a membrane protein. This can be achieved by treating the sample with detergents [5, 13, 37]. Detergents are amphipathic molecules, consisting of a polar head group and a hydrophobic chain that solubilizes membrane proteins by creating a mimic of the natural lipid bilayer environment. For this purpose, the type of detergent can be ionic, non-ionic, zwiterionic or bile acid salts [13]. In this way, it is essential to adjust the detergent type and concentration to the characteristics of target biomolecule in order to avoid irreversible structural loss [37]. Another study to purify the MBCOMT from crude *Brevibacillus choshinensis* cell lysates was conducted by comparing different hydrophobic ligands such as octyl, butyl and epoxy [13, 37]. In case of octyl and butyl ligands, the MBCOMT adsorption was performed at moderate salt concentrations and the elution was promoted by using 1 % Triton X-100. On the other hand, higher salt concentrations were used for protein adsorption in the case of epoxy ligand and its elution was promoted also by using 0.8 % Triton X-100 [37].

1.3.2.2 Ion exchange chromatography

The IEC is one technique of the most chromatographic methods applied in the purification of soluble proteins and membrane protein [49]. The IEC allows the separation of biomolecules with high degree of resolution according to differences in their surface charge at specific pH value [38, 49]. The main advantages of this technique is the capacity to purify biomolecules with positive or negative charge [39, 49].

One of chromatographic techniques that have gained great importance in isolating or purifying the SCOMT was IEC [11]. This chromatography process seeking is not only removal of unwanted contaminants, but to promote the concentration of the desired protein [30].
Actually, there are already some purification processes for MBCOMT using Resource Q column as chromatographic support. This strategy involved the MBCOMT solubilisation with Triton X-100 and its purification was also performed using Triton X-100 [5]. So, MBCOMT adsorption can be promoted with application of 0.5 % Triton X-100 in 10 mM Tris HCl. The elution of isoform was performed by an increase in ionic strength [5, 11].

1.3.2.3 Affinity chromatography

The AC is a high-resolution technique that separating proteins based in highly specific biological interactions between the protein and an affinity ligand, providing elevated selectivity [16, 39]. These methods require often the addition of an affinity tag to protein during vector construction step, which facilitates target protein binding to chromatographic matrix [16]. Consequently, affinity tag removal is necessary after the purification step, which usually implies a significant reduction in process yield and irreversible activity losses [8].
This strategy was successfully described for the isolation of biologically active SCOMT. The elution was performed an increasing NaCl stepwise gradient.

1.3.2.4 Immobilized metal-affinity chromatography

Immobilized metal-affinity chromatography (IMAC) is an affinity technique of chromatographic separation based in affinity between the immobilized metal ions on a solid matrix and the biomolecule in solution [33, 44, 45]. This affinity results of reversible linkages formed between metal ion (the most frequently used are Cu (II), Ni (II), Zn (II), Co (II) and Fe (III)) and electron donor groups located on the surface of the proteins, mainly histidine residues, often introduced into a target protein as a N- or C- terminal peptide ‘tag’ [33, 46, 47]. In general, the biomolecules are retained in IMAC using equilibrium buffer without imidazole or at low concentrations between 1 to 10 mM and the elution is usually achieved by increasing the imidazole concentration [33]. Usually the buffers containing NaCl in order to reduce nonspecific electrostatic interactions. Generally, in IMAC, through the competition with nickel ions, the imidazole is responsible for eluting the proteins and when present at low concentrations in the binding buffer, it may prevent the binding of host proteins with exposed histidines, allowing the removal of contaminants in the flowthrough during the injection of the sample [48]. This methodology is extremely efficient and selective for the direct capture of hexahistidine tagged SCOMT from recombinant *P. pastoris* lysates [33]. The adsorption was carried out with low concentration of imidazole (5 mM imidazole) while their elution was

achieved with increase stepwise concentration of imidazole (300 mM imidazole). The best strategy allowed recovering SCOMT at 300 mM imidazole in a highly purified fraction with a purification fold of 81 and a bioactivity recovery of 57.35% [33].

1.3.3 Ionic Exchange Chromatography

IEC is one of the most frequently used techniques for purification of proteins, peptides, nucleic acids and other charged biomolecules, since it is obtained high resolution and separations with high loading capacity [11, 38, 34, 39] .

In general, in IEC, the charged sample is first loaded into the column at low ionic strength interacting with oppositely charged ligands [32, 38]. After, low affinity proteins are quickly eluted to the column, while the high affinity proteins are retained for further elution. The elution is usually performed by increasing the salt concentration or suitable modification of pH [31, 32]. For this purpose the increase of ionic strength in the eluent affects the retention, since the high salt concentration favour the competition by the solutes, resulting in the elution of the components of lower affinity. Finally, the high ionic strength wash removes any ionically bound proteins before re-equilibration (Figure IX) [32, 38, 39].

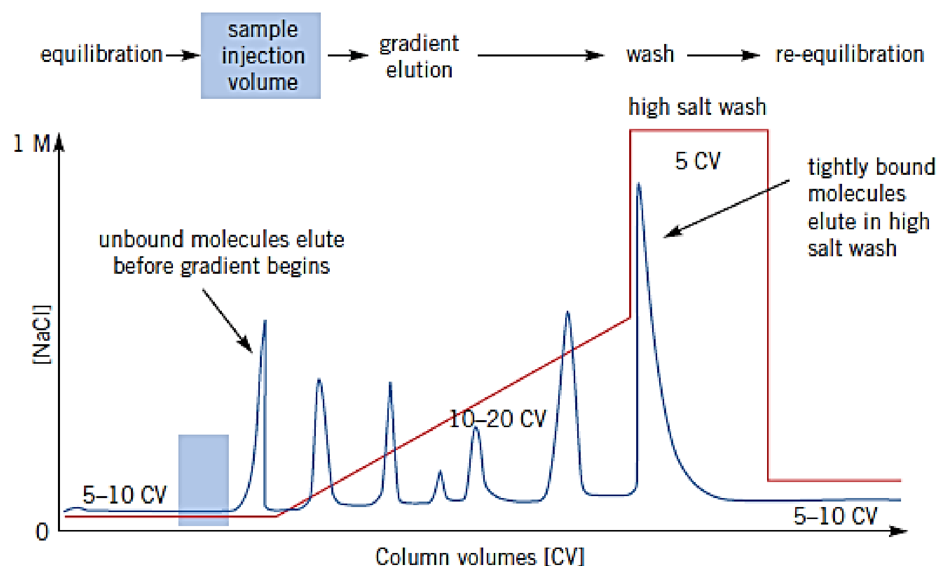

Figure IX - A typical profile from ion exchange chromatography (adapted from [32]).

Whereas, the pH of the elution buffer determines the molecule ionization state. A protein that has no net charge at a pH equivalent to its isoelectric point (pI) will not interact with a charged medium. However, at a pH above its isoelectric point, the protein is negatively charged, and will bind to a positively charged column by anion exchange chromatography

(AEC). On the other hand, at pH below its *pI*, the protein is positively charged, and will bind to a negatively charged medium by cation exchange chromatography (CEC) (Figure X) [32, 38, 39].

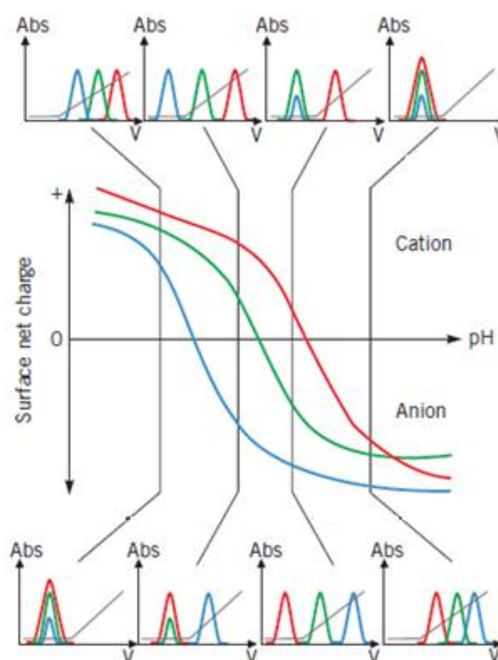

Figure X - Effect of pH on protein binding and elution patterns in Ionic Exchange Chromatography (adapted from [32]).

This chromatographic procedure has revealed to be quite efficient to separate both soluble proteins and membrane proteins (MPs) due to numerous advantages such as purifications in large scale, relatively low cost and use of any neutral detergents, which reduces the risk of MPs instability during the chromatographic step [31, 32, 39].

Overall, IEC requires a stationary phase, usually composed by hydrated insoluble polymers such as cellulose or Sephadex, to which is coupled an ion exchanger group, that can be cationic or anionic. Thus, this type of chromatography can be classified into CEC or CEA [38]. For instance, in AEC, the stationary phase carries positively charged functional groups that are capable of binding anions (e.g. ionized carboxylic acids). The mobile phase usually contains a buffer to maintain stable pH and varying the salt concentration to control the retention of sample ions (counter ions) [38]. In relation to these ion exchangers can be classified as strong and weak exchangers based on the difference between the functional groups (Table I). The ion exchangers are strong due the fact that they are completely ionized at a specific pH, while the weak exchangers have a degree of ionization dependent of pH. An example of a weak anion exchanger is usually diethylaminoethyl (DEAE) [32, 35, 38].

Table I - Types of ion exchangers (adapted from [32]).

Anion exchangers		Functional group
Quaternary ammonium (Q)	strong	$-O-CH_2N^+(CH_3)_3$
Diethylaminoethyl (DEAE)*	weak	$-O-CH_2CH_2N^+H(CH_2CH_3)_2$
Diethylaminopropyl (ANX)*	weak	$-O-CH_2CHOHCH_2N^+H(CH_2CH_3)_2$
Cation exchangers		**Functional group**
Sulfopropyl (SP)	strong	$-O-CH_2CHOHCH_2OCH_2CH_2CH_2SO_3^-$
Methyl sulfonate (S)	strong	$-O-CH_2CHOHCH_2OCH_2CHOHCH_2SO_3^-$
Carboxymethyl (CM)	weak	$-O-CH_2COO^-$

1.3.4 Monolithic supports

Currently, the chromatographic technology is a widely used method in the purification of biomolecules, where one of its main objectives is to develop rapid and efficient separations with high binding capacity, as well as, apply specific ligands to improve the selectivity for the target molecule [50, 51, 53]. An example of this technology is the monolithic chromatography. Monoliths are a special type of chromatographic column, considered the material of choice for the purification and analysis of proteins, plasmid DNA and viruses [51, 52, 53, 54, 56, 58].

The monolithic supports have been increasingly used due to its advantages over traditional matrices, such as, high porosity, high binding capacity for extremely large molecules and convective mass transport [51, 52, 54, 59]. The fact that the monoliths present channels with high porosity makes the binding capacity increased. In fact, pore dimension is correlated with the exclusion limit, which defines the size range of molecules that can enter or be excluded from the pore. In reality, the pore size of a matrix is inversely correlated to its surface area, which in turn directly affects the amount of immobilized ligand [40, 50, 51, 58]. Therefore, another advantage of monolithic columns is low absolute surface area, but a high adsorption due to their channel structure, allowing a high dynamic binding capacity [40, 51]. The separation of biomolecules is another advantage and dominantly happens by convective mass transport that allows high flow velocity, in this way, all the mobile phase is forced to flowthrough to the channels via convection, causing high throughput purifications [40, 50, 57, 58]. Lastly, the chromatographic columns need to be short, supporting higher flow rates, without sacrificing resolution or the bioactivity of the target molecule [40, 52].

1.3.4.1 CarbonylDiImidazole Monolith

The CarbonylDiImidazole (CDI) monolithic support allows the convective mass transport of the macromolecules, offering a great binding capacity due to their single structure with a highly interconnected network of a large diameter channels [50]. This support, when activated, can be used in ligand immobilization by means of a nucleophilic substitution, resulting in a stable amide linkage, as seen in Figure XI [57].

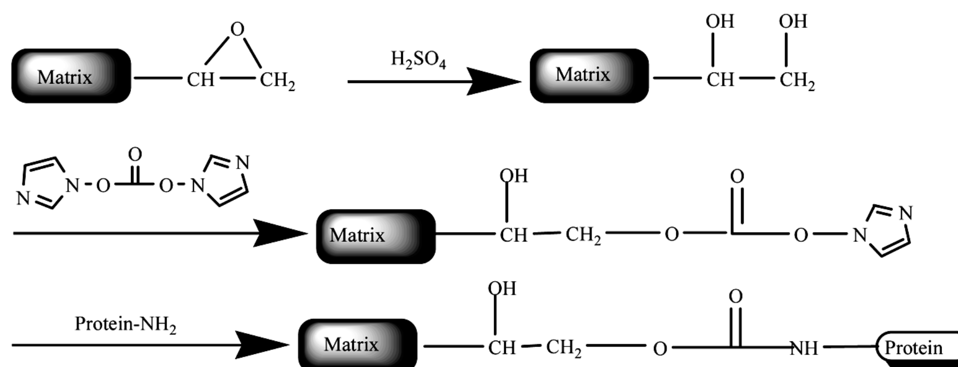

Figure XI - Immobilization of ligands by CarbonylDiImidazole monolithic support (adapted from [57]).

However, the CDI disc without modification has been successfully used in the separation of plasmid DNA (pDNA) isoforms due to the imidazole functional groups by using a decreasing stepwise ammonium sulphate ($(NH_4)_2SO_4$) gradient [61]. Should be pointed out that CDI ligands can establish several interactions accountable for the biorecognition of the biologically active pDNA isoform, namely hydrophobic interactions, van der Waals forces and hydrogen bonds, allowing the elimination of host impurities present in the lysate sample [52, 61]. In fact, it is important to refer that the chemical composition of chromatographic supports determines the interactions established with the target molecule, allowing its retention whereas undesirable molecules are eluted [50].

1.3.4.2 Histamine Monolith

Histamine monolithic support consists in a CDI monolith modified with the histamine ligand. The histamine ligand is derived from the decarboxylation of $_{L}$-histidine amino acid, containing an imidazole ring and a carbon spacer arm, as seen in Figure XII.

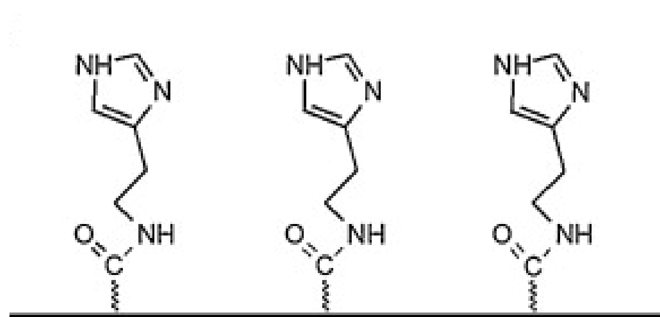

Figure XII - Schematic representation of immobilized histamine ligands (adapted from [56]).

This monolithic support has been successfully used also in the pDNA purification due to physicochemical properties and versatility of the ligand [55, 56, 59]. For instance, the Histamine monolith showed to be a multifaceted column to purify the sc pDNA from a lysate sample by different strategies (the simple purification strategy with ascending sodium chloride gradient and the combined purification strategy with ascending sodium chloride and then descending ammonium sulphate gradient) [56].

Although the histamine ligand has not yet been tested in the purification of proteins, the $_L$-histidine amino acid has been successfully used as a selective and efficient ligand for purification of various proteins and peptides [40]. Properties such as mild hydrophobicity, weak charge transfer, asymmetric carbon atom, and wide pK_a values are characteristics that make histidine a potential ligand for protein purification [40, 56].

1.3.4.3 Agmatine Monolith

Agmatine monolithic support was also prepared from a CDI monolith modified with the agmatine ligand. Agmatine ligand is derived from the decarboxylation of arginine amino acid, containing basic guanidinium group, as seen in Figure XIII [56, 58, 62].

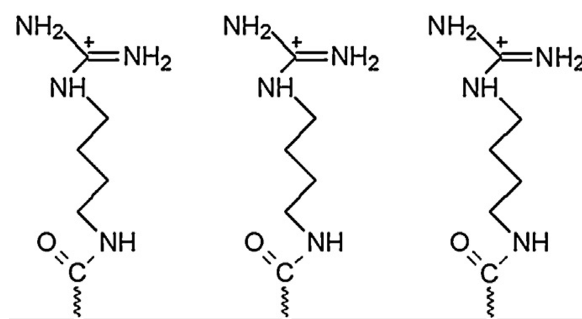

Figure XIII - Schematic representation of immobilized agmatine ligands (adapted from [56]).

This monolith has been used in microRNAs purification processes by exploiting the versatility of this ligand through three different binding and elution strategies based on increased NaCl and decreased $(NH_4)_2SO_4$ stepwise gradients, in order to obtain the final product with high purity degree [58]. It was also used in the pDNA purification, working under two elution strategies, by descending $(NH_4)_2SO_4$ gradient and by ascending NaCl gradient, combining ionic and hydrophobic interactions [41, 52, 55, 60].

Justification and Objectives

The main objective of this work is to explore the chromatographic conditions more appropriate for the recognition of SCOMT_6His protein by manipulating the elution buffer composition (type and salt concentration), using monolithic supports for the first time. To reach this propose, two purification strategies were developed.

Firstly, the comparison and assessment of the chromatographic behaviour of SCOMT_6His using Q-Sepharose as anion exchanger was performed in order to analyse the Q-Sepharose performance in terms of binding and elution conditions for SCOMT_6His recovery and pre-purification. Thereafter, the behavior of the pre-purified sample was analysed in three monolithic columns (CDI, Histamine and Agmatine) to explore different elution strategies by increasing and decreasing of sodium chloride and ammonium sulphate concentrations and increase the final purity degree of the SCOMT_6His protein.

After choosing the most promising monolith, the lysate sample was filtered and directly injected in the monolith, in order to obtain a greater recovery of the target protein with satisfactory purity degree in comparison to the other strategy.

Chapter II

Materials and Methods

2.1 Materials

Ultrapure reagent-grade water for ÄKTA™ avant was obtained with a Mili-Q system (Milipore/Waters). The easy select expression kit for expression of recombinant proteins using pPICZα vector in *P. pastoris* and zeocin (200 µL) were obtained from Invitrogen (Carlsbad, CA). Yeast extract, glucose, agar, tryptone, sorbitol, yeast nitrogen base (YNB), biotin, methanol, Cysteine (L-), bovine serum albumin (BSA), S-(5'-adenosyl)-$_L$-methionine chloride (SAM) and epinephrine (bitartrate salt) were obtained from Sigma Sigma-Aldrich (St. Louis, MO). Glycerol and sodium chloride (NaCl) was obtained from Himedia (Mumbai, India) and from Panreac (Barcelona, Spain), respectively. NZYcolour Protein Marker II used for estimation of subunit molecular weight was purchased in NZYTech (Lisboa, Portugal). Anti-rabbit IgG alkaline phosphatase secondary antibody was purchased on GE Healthcare Biosciences (Uppsalla, Sweden). Monoclonal rabbit anti-COMT antibody was produced in BIAL (S. Mamede do Coronado, Portugal). Acrylamide 30%/Bis solution was obtained from BioRad (Hercules, CA). Tris(hydroxymethyl)aminomethane (Tris) and CAPS were obtained from Fisher Scientific (Epson, United Kingdom). All other chemicals were of analytical grade and used without further purification. The agmatine monolith, histamine monolith and CDI monolith were kindly prepared and provided by BIA Separations (Ajdovščina, Slovenia).

2.2 Plasmids, bacterial strains and media

Briefly, the DNA fragment coding for SCOMT was obtained from the pET101/D-hSCOMT plasmid by polymerase chain reaction (PCR) using specific primers (forward primer, 5' AAC TCG AGA AAA GAA TGG GTG ACA CCA AGG AGC AG 3' and reverse primer, 5' AAC TCG AGT CAG TGA TGG TGA TGG TGA TGG GGC CCT GCT TCG CTG CCT G 3') for cloning in which the reverse primer was designed in order to introduce a hexahistidine tag in SCOMT carboxyl-terminal. The PCR conditions were as follows: denaturation at 95 ºC for 5 min, followed by 30 cycles at 95 ºC for 30 s, 60 ºC for 30 s and 72 ºC for 1 min, and a final elongation step at 72 ºC for 5 min. The amplified DNA was purified by low melting agarose gel electrophoresis, digested with Sac I and cloned into the vector pPICZα (previously digested with Sac I) by T4 DNA ligase. This construct was transformed into *E. coli* TOP10F cells, grown overnight at 37 ºC

in plates with Low-salt Luria-Bertani containing 25 µg/mL Zeocin and colonies were screened for the presence of the construct pICZαA-hSCOMT_His6. Therefore, some colonies were inoculated in 2.0 mL of Low-salt Luria-Bertani and grown at 37 ºC and 250 rpm overnight. From these cultures, highly purified plasmids were prepared using NzyMiniprep (Nzytech, Lisboa, Portugal) and were then subjected to DNA sequence analysis to confirm the identity of the amplicon, orientation and frame. Since the sequence was confirmed to correspond to human SCOMT gene with the six histidines addition in its carboxyl-terminal, the cloned plasmid was digested with Sac I and introduced into freshly made *P. pastoris* X-33 competent cells by electroporation at 2.5 Kv (2500 V), 25 µF and 1000 Ω [11, 33]. After confirming that the X-33 integrant presented a methanol utilization phenotype plus (Mut^+), the stable occurrence of the expression cassette was verified in the colonies genomic DNA by PCR. This process was carried out according to manufacturer’s instructions and as previously described [11, 33].

2.3 Recombinant SCOMT_6His production and recuperation

Recombinant SCOMT_6His production was performed using *P. pastoris* X-33 cells containing the expression construct pICZαA-hSCOMT_His6 according to the following protocol: cells containing the expression construct were grown for 72 hours at 30 ºC in YPD plates containing 200 µg/mL Zeocin. A single colony was inoculated in 100 mL of BMGH medium (100 mM potassium phosphate buffer, pH 6.0, 1.34 % YNB, 4 × 10^{-5} % biotin and 1 % glycerol) in 500 mL shake flasks and grown overnight at 30 ºC and 250 rpm to a cell density at 600 nm (OD_{600}) of 6. Posteriorly, since the inoculation volume was fixed to achieve an initial OD_{600} of 1, an aliquot of the fermentation in the BMHH medium (125 mL) (100 mM potassium phosphate buffer, pH 6.0, 1.34 % yeast nitrogen base (YNB), 4 × 10^{-5} % biotin and 0.5 % methanol) was collected and centrifuged (500 x g, 5 min at room temperature). After centrifuging the cells and to ensure that all glycerol was removed, the cells were ressuspended in the induction medium and added to 500 mL shake-flasks to a total volume of 125 mL. The fermentations were carried out during 24 hours at 30 ºC and 250 rpm and were supplemented with methanol 1%. Then, the cells were harvested by centrifugation (1500 x g, 10 min, 4 ºC) and stored at – 20 ºC until use. Thereafter, cells were lysed in equilibrium buffer (150 mM NaCl, 10 mM DTT, 50 mM Tris, 1 mM $MgCl_2$, pH 8.0) at a ratio of 1:2:2 (1 g cells, 2 mL lysis buffer and 2 g glass beads). Lysis was accomplished through the application of a sequential procedure with glass beads of 7 cycles of vortexing for 1 min with 1 min of interval on ice. Subsequently, the mixture was centrifuged (500 x g, 5 min, 4 ºC) and the pellet obtained was ressuspended in the chromatographic binding buffer (500 mM NaCl, 50 mM Tris and 1 mM $MgCl_2$ at pH 7.8) [33].

2.4 Pre-purification by Anion Exchange Chromatography

Chromatographic assays were performed at temperature 6 ºC in ÄKTA Avant system with UNICORN 6 Software (GE Healthcare, Uppsala, Sweden) equipped with a 2 mL injection loop. All buffers pumped into the system were prepared with Mili-Q system water, filtered through a 0.20 µm pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically.
Q-Sepharose (GE Healthcare Biosciences) was packed according to company guide-lines (20 mL of gel volume) into a C 16/20 [16 mm (diameter) x 200 mm (length)] glass column purchased from GE Healthcare Biosciences. Screening experiments were performed at different salt concentrations in order to assess the ideal sodium chloride concentration required for SCOMT_6His retention. The column was initially equilibrated with 10 mM Tris-HCl buffer pH 7.8. Aliquots of recombinant SCOMT_6His containing supernatant (3 mL) were injected into column at 1 mL/min with 10 mM Tris-HCl pH 7.8. The elution of unretained species occurred with an increasing sodium chloride gradient from 0 mM to 100 mM (3 CV). After elution, sodium chloride concentration was gradually increased from 100 mM to 310 mM (3.5 CV) to ensure that the protein was completely bound to the column. Subsequently, sodium chloride concentration in mobile phase was increased to 450 mM in a step mode (2.5 CV). Finally, a washing step was applied with 1 M of sodium chloride (2 CV) in a step mode.
In all chromatographic runs, conductivity was continuously monitored, as well as absorbance at 280 nm. Fraction volumes of 3 mL were collected and pooled according to the obtained chromatographic profile. Finally, samples were concentrated and desalted with 1 mL 10 mM Tris-HCl pH 7.8 with Vivaspin concentrators (10.000 MWCO) and conserved at 4 ºC until further analysis.

2.5 Purification by Monoliths

All chromatographic experiments were carried out in an ÄKTA Avant system accomplish with UNICORN 6 Software (GE Healthcare, Uppsala, Sweden). All buffers were filtered through a 0.20 µm pore size membrane (Schleicher Schuell, Dassel, Germany), and degassed ultrasonically. The system was run at a flow rate of 1 mL/min, and monitored at 280 nm.
Initially, the CDI monolith was equilibrated with 3M $(NH_4)_2SO_4$. The sample clarified by Q-Sepharose was applied onto the column using a 200 µL loop at a flow rate of 1 mL/min. After the elution of unbound species, the ionic strength of the buffer was decreased to 0 M of $(NH_4)_2SO_4$ in 10 mM Tris-HCl buffer (pH 7.8). After that, the assay under ionic condition was also performed. The CDI column was equilibrated with 10 mM Tris-HCl buffer (pH 7.8) and after the sample injection the ionic strength was increased to 1.5 M of NaCl in 10 mM Tris-HCl buffer (pH 7.8). These preliminary assays under hydrophobic and ionic elution conditions were

repeated for the Histamine monolith. The Agmatine monolith was used only in ionic elution conditions. The Agmatine column was equilibrated with 10 mM Tris-HCl buffer (pH 7.8) and after the sample injection the sodium chloride concentration in mobile phase was increased to 1.5 M in a step mode. Finally, a washing step was applied with 3 M of NaCl in 10 mM Tris-HCl buffer (pH 7.8) in a step mode.
Finally, samples were concentrated and desalted with 3 mL 10 mM Tris-HCl applying Vivaspin concentrators (10.000 MWCO) and conserved at 4 ºC until further analysis.

2.6 Total protein quantification

The protein content in lysate and purified samples was measured by the Pierce BCA Protein Assay Kit (Thermo Scientific, USA) on a 96 well plate, and a specific volume of working reagent (WR) was prepared from the kit according to the number of existing samples. It was used BSA as the standard and calibration control samples (5–2000 µg/mL).

2.7 SDS-PAGE and Western blot

Reducing Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot trials were performed, respectively according to the method of Laemmli [64] and the literature described above [42]. Samples (30 µL) to analyse in SDS-PAGE were treated by adding 10 µL of a reduction buffer. Samples were boiled in a loading buffer (500 mM Tris-HCl (pH 6.8), 10% (w/v) SDS, 0.02% (w/v) bromophenol blue, 0.2% (v/v) glycerol, and 0.02% (v/v) ß- mercaptoethanol) and were denatured at 100 ºC for 5 min. The run on 4% stacking and 12.5% resolving gels containing 0.1% SDS, with a running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS) at 120 V for 1h 40 min. After electrophoresis, one gel was stained by Comassie brilliant blue and the other gel was transferred to a polyvinylidene difluoride (PVDF) membrane, in order to perform the western blot experiments.
For Western blot, the proteins were transferred over a 30 min period at 750 mA at 4 ºC in a buffer containing 10 mM CAPS. After the blotting, the membranes were blocked with TBS-T (pH 7.4) containing 5% (w/v) milk for 60 min at room temperature, washed 3 times during 15 minutes and exposed overnight at 4 ºC to a rabbit anti-rat SCOMT polyclonal antibody, that cross reacts with the human protein, at 1:2000 dilution in TBS-T 1%. The membranes were washed three times (15 min each) with TBS-T and adherent antibody was detected by incubation for 1 h with an anti-rabbit IgG alkaline phosphatase secondary antibody at 1:40000 dilution in TBS-T 1%. The PVDF membranes were air dried, incubating with 1 mL of ECF for 5 min and enhanced by exposure to chemiluminescence's detection.

2.8 SCOMT_6His enzymatic assay

The methylation efficiency of SCOMT_6His was evaluated by measuring the amount of metanephrine formed from epinephrine. After processing the samples, the metanephrine levels in the different samples obtained in the Q-Sepharose assays were determined using a HPLC with coulometric detection [65].

Briefly, the chromatographic analysis was performed using a HPLC model Agilent 1260 system (Agilent, Santa Clara, USA) equipped with an auto sampler and quaternary pump coupled to an ESA Coulchem III detector (Milford, MA, USA). Chromatographic separation was achieved on an analytical column Zorbax 300SB C18 RP analytical column (250 × 4.6 mm with i.d. of 5 μm) (Agilent, Santa Clara, California, USA). The chromatographic method was developed using as mobile phase (0.1 M NaH_2PO_4, 0.024 M citric acid monohydrate, 0.5 mM sodium octyl sulpahte and 9% (v/v) acetonitrile) and the column effluent was monitored with an electrochemical detector in a coulometric mode, which was equipped with high sensitivity dual electrode analytical cell (electrodes I and II) using a procedure of oxidation/reduction (analytical cell #1: +410 mV; analytical cell #2: −350 mV). The method sensitivity was set at 1 μA, the flow-rate applied was 1 mL/min and the column temperature was optimized to 30 ºC. The chromatograms were obtained by monitoring the reduction signal of the working electrode where metanephrine retention time was around 8 min. Finally, the metanephrine content in samples was measured using metanephrine standards as a calibration control. The calibration curve was used y= 3521,2871x + 367,0215 [65].

Chapter III

Results and Discussion

In recent years, several attempts have been performed to obtain a large quantity of enzymatically active and pure SCOMT protein. Therefore, it was important to develop an appropriate strategy to reach this objective, ie, to develop purification strategies that allow to recover high levels of pure recombinant enzymatic product in an active form [5, 7]. According to the literature, the SCOMT protein has been subjected to numerous purification procedures. In fact, SCOMT purification by AEC combined with others chromatographic steps led to successful resolving of its atomic structure [11, 16, 28, 31].
Therefore, this work intends to establish two chromatographic strategies to purify the SCOMT_6His protein through the use of Q-Sepharose column in order to clarify the cell lysates of *P. pastoris* and subsequently evaluate the applicability of affinity chromatography by the use of three monolithic supports (CDI, Histamine and Agmatine).

3.1 Production of SCOMT_6His

SCOMT_6His biosynthesis from *P. pastoris* X-33 cells, containing the expression construct pICZα A-hSCOMT_His6, was previously optimized in order to enhance COMT activity recovery [33]. The SCOMT_6His production was monitored by measuring cell density at 600 nm (OD_{600}) over time. Its maximum output was reached after 24 hours as seen in Figure XIV.

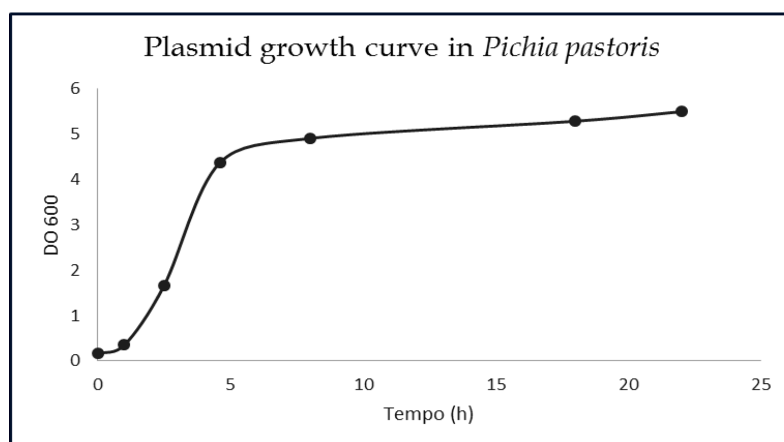

Figure **XIV-** A typical plasmid growth curve in *P. pastoris* from SCOMT_6His biosynthesis.

One of the major advantages in *P. pastoris* bioprocesses is that the recovery and purification steps are simplified due to the low levels of endogenous proteins that are secreted into the extracellular medium. In despite of this behavior, SCOMT_6His was accumulated in the intracellular space of *P. pastoris* [7]. The optimization of the production step was aimed to obtain larger amounts of protein so that purification was delicate as a straightforward method and so increase the chances of obtain significant quantities of protein in a highly pure, stable and conformational active state.

Afterward, a centrifugation and a suitable cell lysis stage employing glass beads were performed, in order to obtain a crude *P. pastoris* lysates. Subsequently, this lysate was injected into the Q-Sepharose column. Thus began the pre-purification processes, to clarify the sample.

In this work all the reviewed production conditions and lysis method were maintained in comparison with other works well established in the literature [6, 7, 11].

3.2 SCOMT_6His pre-purification assays on Q-Sepharose

The manipulation of soluble proteins is generally more easier than integral membrane proteins.

According to work done by our research group [11, 13] the SCOMT recovery strategy in Q-sepharose it is made at low ionic strength, specifically at 10 mM Tris-HCl at pH 7.8, followed by a step at 350 mM of NaCl and a final step at 1 M of NaCl. In this procedure, the major fraction of SCOMT is eluted at 350 mM NaCl. In relation to the main purification method apply for MBCOMT, the group proposed that it is necessary a linear adsorption gradient from 0 to 100 mM NaCl, followed by a step at 300 mM NaCl to remove some contaminants and a final salt increasing gradient from 350 mM to 1 M NaCl in 10 mM Tris-HCl at pH 7.8. In general, the recovery of COMT isoforms has performed by ionic strength manipulation, specifically in NaCl concentration onto mobile phase. A SCOMT complete retention was found at lower ionic strength, while MBCOMT required the application of a salt linear gradient for its adsorption. The elution of both isoforms was performed by an increase in ionic strength.

Equally, in this work, it was necessary to develop a new recovery strategy for SCOMT_6His by Q-sepharose. Thus, there was a need to adjust the chromatographic profile of the soluble isoform from the profile of MBCOMT, to clarify the SCOMT_6His lysate. The protein of interest differs from the enzyme previous work by having a six histidine tail and, not by the mutation of valine at position 108 that corresponds to the most studied genetic polymorphism. So, the main aim was to design a downstream process that reduce main protein interferences in SCOMT_6His active fractions recovered in primary isolation from recombinant host and analyse its bioactivity levels after an ionic chromatographic strategy.

AEC using Q-Sepharose as anion exchanger retains negatively charged molecules under low salt concentrations and NaCl is the typically used salt on elution buffers [11]. The presence of NaCl increases the protein solubility, which occurs due to a salting-in effect, i.e., a decrease in protein electrostatic interactions [66].
Several chromatographic experiments were conducted to establish mobile phase's conditions, improving the selectivity of the chromatographic process and analysing their effects on SCOMT_6His retention and elution behavior. Initially, it was tested the strategy previously described by our research group [11], as presented in Table II.

Table II - Adsorption and elution behaviour of SCOMT protein in the Q-sepharose column at NaCl concentrations described by our research group [11]. ↑: high; ↓: low.

[NaCl] (M)	0	0.35	1
Adsorption SCOMT	↑	↓	↓
Elution SCOMT	↓	↑	Moderate

The results published by our group (Table II) showed that the SCOMT recovery best strategy in Q-sepharose requires balancing column with 10 mM Tris-HCl at pH 7.8 at 1 mL/min and after the injection 2 mL of lysate SCOMT (1.5 g total protein), it was performed a step at 350 mM of NaCl followed a final step at 1 M of NaCl. According to the results of Table II the SCOMT is eluted at 350 mM NaCl. So, first we tested whether these same conditions would be reproducible for SCOMT_6His (Figure XV).

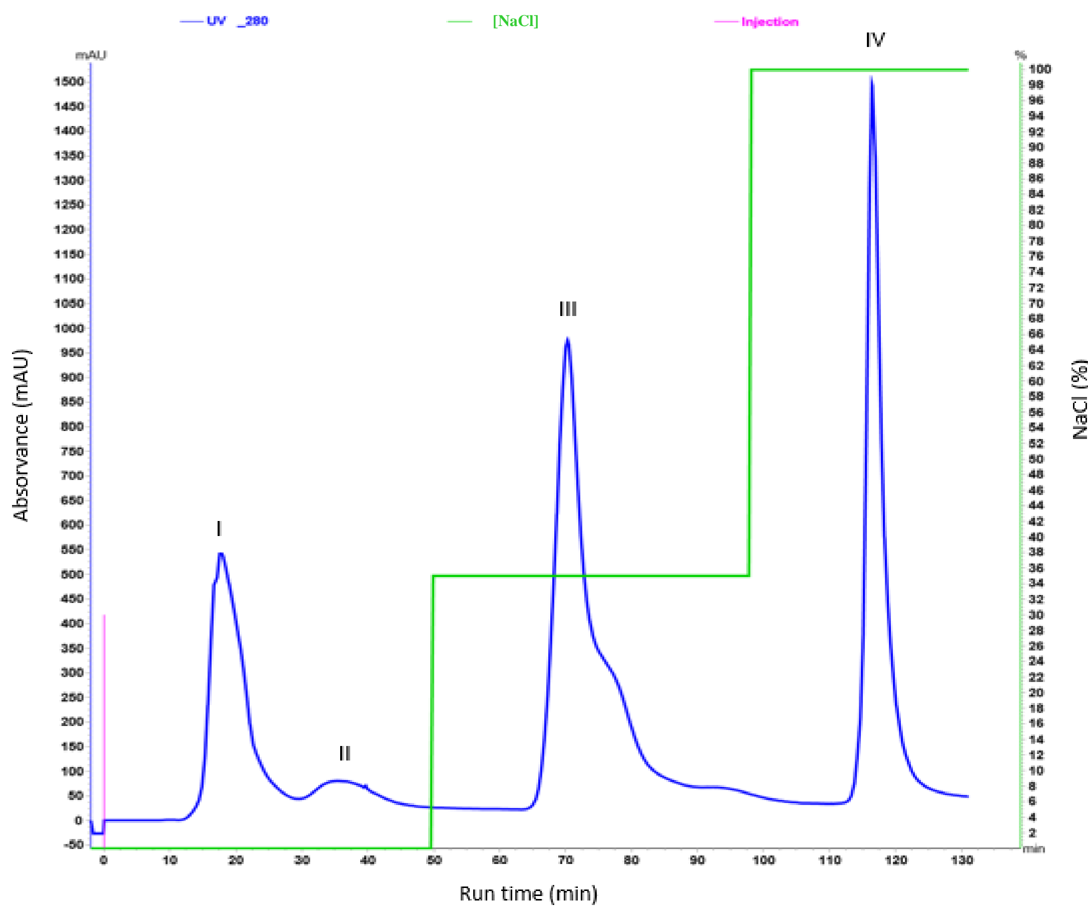

Figure XV - The SCOMT_6His chromatographic profile on Q-Sepharose. Adsorption was performed at 10 mM Tris-HCl buffer at pH 7.8, followed by an intermediate step at 350 mM NaCl and a final step at 1 M NaCl in 10 mM Tris-HCl at pH 7.8 buffer. Blue line represents absorbance at 280 nm and green line the NaCl concentration in the mobile phase.

By analysis of the recovered fractions from the chromatographic assay through SDS-PAGE gel and western blot (Figure XVI), it can be observed that SCOMT_6His was present in first, second and third peaks. The established elution gradient doesn't favor the selectivity the SCOMT_6His protein into Q-Sepharose column, a great percentage of the target protein was not retained on the column and another portion was eluted with 350 mM NaCl together with other lysate proteins (Figure XVI).

The explanation for this partial loss of protein during this initial trial might be due to the presence of a histidine tag which promotes some repulsion between the nitrogen of the imidazole ring of protein with nitrogen of column ligand.

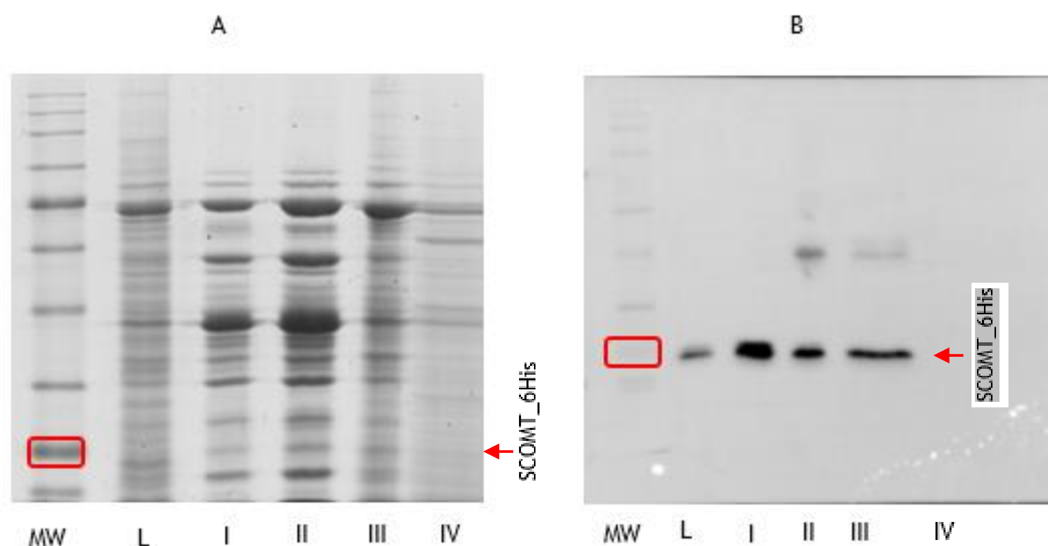

Figure XVI – SDS-PAGE analysis (A) and Western Bolt (B) of the recovered fractions from the SCOMT_6His chromatographic assay on Q-sepharose of figure XV. Lane MW – molecular weight standards; Lane L – Lysis supernatant injected on Q-sepharose; Fractions I and II - Peaks I and II, respectively obtained at 10 mM Tris-HCl buffer at pH 7.8; Fraction III - Peak III obtained at 10 mM Tris-HCl buffer at pH 7.8 at 350 mM NaCl; Fraction IV - Peak IV obtained at 1 M NaCl.

Comparing the obtained results with published results [11], the Q-Sepharose did not showed a good selectivity for SCOMT_6His, under these conditions. Thus, we have to tested and implemented a new chromatographic strategy for the recovery of SCOMT_6His from crude cell lysates.

So, it was required in Q-Sepharose to study the effect of salt on SCOMT_6His adsorption in Q-Sepharose. The adsorption strategy was studied by performing several increasing salt linear gradients, from 10 mM of Tris-HCl at pH 7.8 to a specific NaCl concentration. Thus, the elution strategy was optimized using, firstly a linear gradient of 0 - 100 mM NaCl during 3 CV (60 min) followed by a second linear gradient of 100 - 400 mM NaCl during 4 CV (80 min) and finally a linear gradient from 400 mM to 1 M NaCl during 2.5 CV (40 min) (Figure XVII). This strategy it was designed taking into account the results previously obtained by the research group [37].

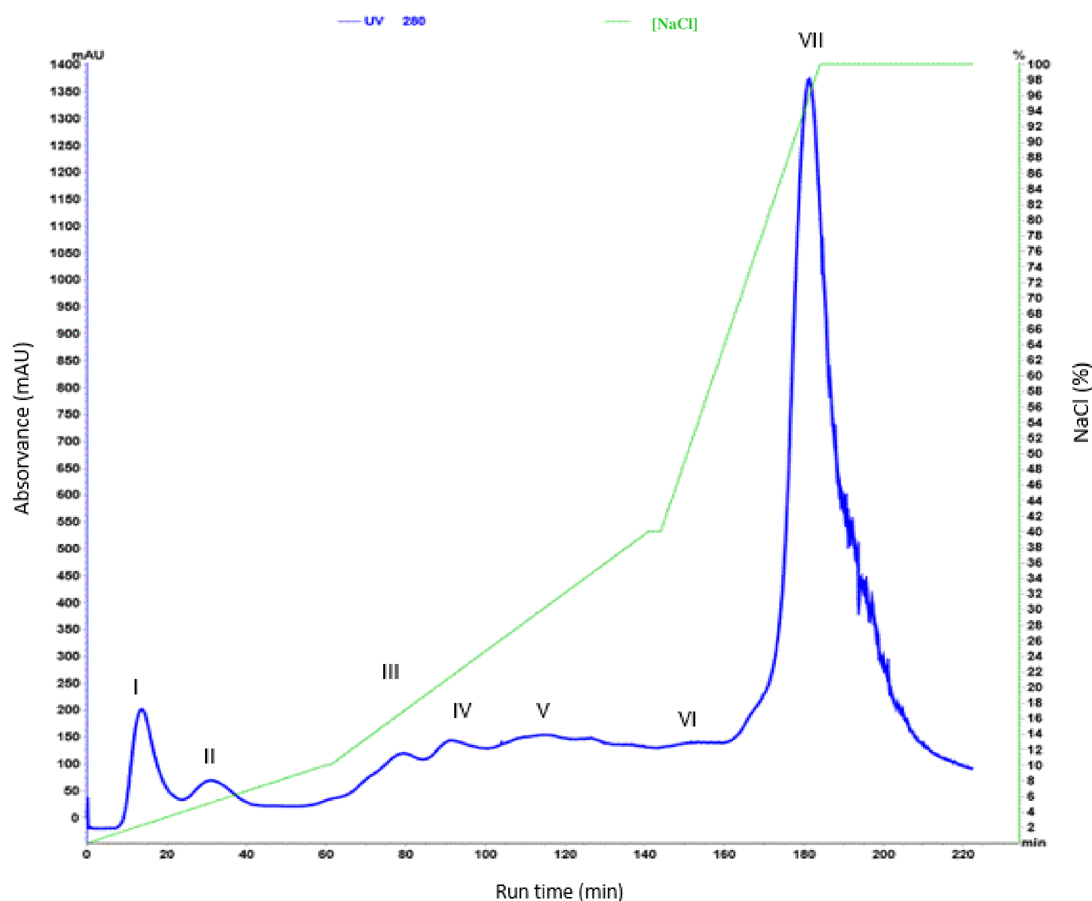

Figure XVII - The SCOMT_6His chromatographic profile on Q-Sepharose. Adsorption was performed at an increasing linear gradient from 0 to 100 mM of NaCl during 60 min, followed by increasing linear gradient from 100 mM to 400 mM NaCl during 80 min and finally a linear gradient from 400 mM to 1 M NaCl during 40 min in 10 mM Tris-HCl at pH 7.8 buffer. Blue line represents absorbance at 280 nm and green line the NaCl concentration in the mobile phase.

The results obtained by SDS-PAGE and western Blot (Figure XVIII) show that after the injection of 2 mL of lysate 1.5 g total protein of the SCOMT_6His was retained to Q-Sepharose column when a linear gradient from 0 to 100 mM NaCl was applied according to literature [67 - 69]. These results also indicate that specific percentage of SCOMT_6His protein was eluted during linear gradient from 100 to 400 mM NaCl, although the major content of the target protein was eluted in peak VI with the linear gradient from 400 to 1 M of NaCl, as it can be observed in Figure XVIII (B).

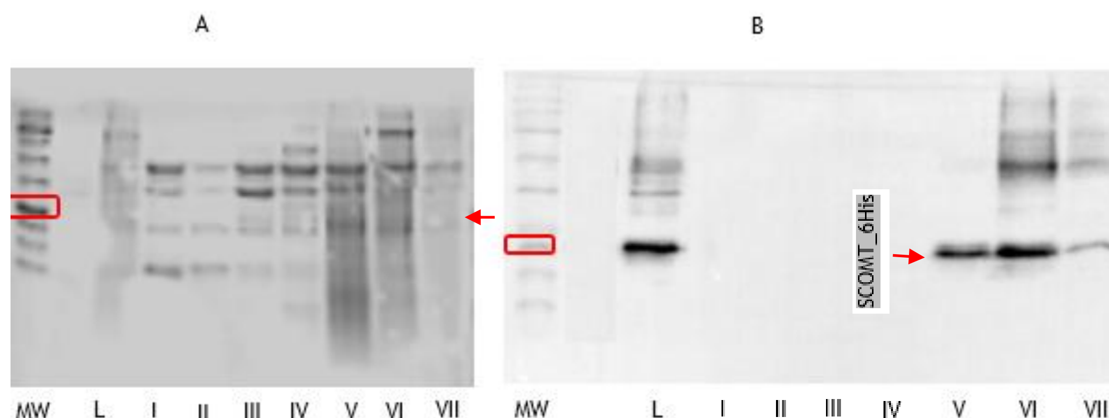

Figure XVIII - SDS-PAGE analysis (A) and Western Bolt (B) of the recovered fractions from the SCOMT_6His chromatographic assay on Q-sepharose of figure XVII. Lane MW – molecular weight standards; Lane L – Lysis supernatant injected on Q-sepharose; Fractions I and II - Peaks I and II, respectively obtained in the linear gradient from 0 to 100 mM NaCl in 10 mM Tris-HCl buffer at pH 7.8 from; Fraction III, IV and V - Peak III, IV and V, respectively obtained in the linear gradient from 100 to 400 mM NaCl; Fraction VI and VII - Peak VI and VII obtained with linear gradient from 400 mM to 1 M NaCl, respectively.

After the screening of an adequate SCOMT_6His adsorption strategy, it was tried to optimize a suitable chromatographic elution strategy for its isolation. Thus, in Figure XIX is presented the resultant chromatogram. Initially it was applied an increasing linear gradient from 0 to 100 mM NaCl during 3 CV (60 min) to promote the total SCOMT_6His adsorption, followed by an increasing linear gradient from 100 mM to 310 mM NaCl during 3.5 CV (70 min) with the aim of achieving greater retention of the SCOMT_6His subsequently was applied a step at 450 mM NaCl during 2.5 CV (50 min) in which SCOMT_6His should be eluted without significant levels of contaminants followed by a final washing step to 1M NaCl in 10 mM Tris-HCl buffer during 2 CV (40 min).

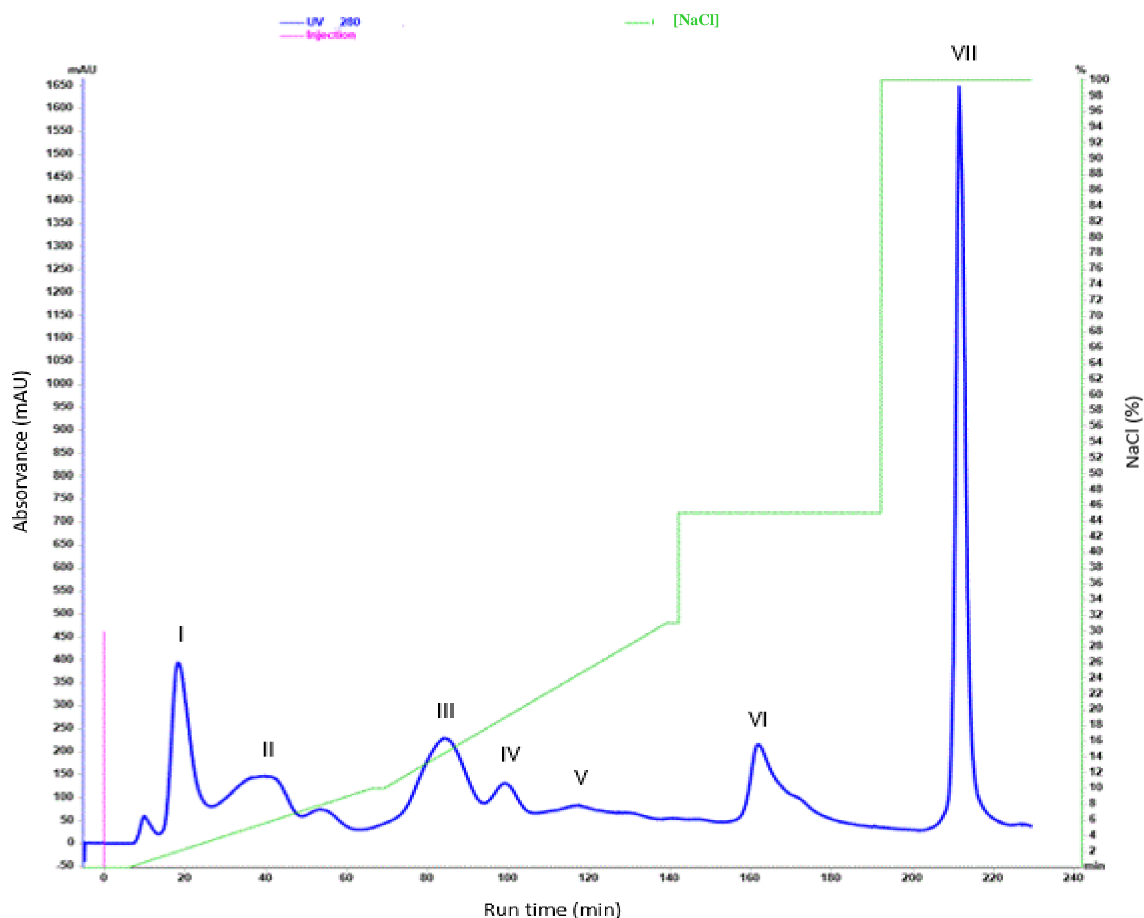

Figure XIX - The SCOMT_6His chromatographic profile on Q-Sepharose. Adsorption was performed at an increasing linear gradient from 0 to 100 mM of NaCl during 60 min, followed by increasing linear gradient from 100 mM to 310 mM NaCl during 70 min, followed by an intermediate step at 450 mM NaCl during 50 min and finally a final step at 1 M NaCl during 40 min. Blue line represents absorbance at 280 nm and green line the NaCl concentration in the mobile phase.

By analysis of recovered fractions in the SDS-PAGE gel and western blot (Figure XX), it can be observed that SCOMT_6His was eluted essentially in two different fractions at distinct NaCl concentrations. A basal fraction of SCOMT_6His was obtained on peak V at 310 mM of NaCl but the major fraction was eluted on peak VI at 450 mM NaCl as expected, resulting on an immunological active strong band of molecular weight 24.7 KDa.

Despite of most of impurities were immediately removed from column in first peaks (I, II and III), some contaminant proteins were retained with SCOMT_6His and eluted together in both peaks V and VI, as it is observed in SDS-PAGE result (Figure XX, Peak V and VI). Overall, the results showed that the elution strategy optimized on the Q-Sepharose column allowed the clarification of the SCOMT_6His protein mostly recovered in a single peak.

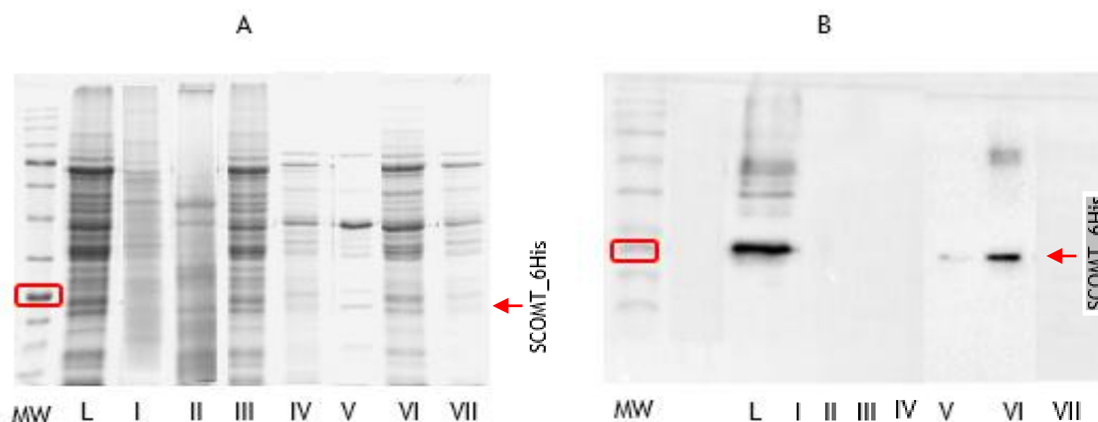

Figure XX - SDS-PAGE analysis (A) and Western Bolt (B) of the recovered fractions from the SCOMT_6His chromatographic assay on Q-sepharose of figure XIX. Lane MW – molecular weight standards; Lane L – Lysis supernatant injected on Q-sepharose; Fractions I and II - Peaks I and II, respectively obtained in the linear gradient from 0 to 100 mM NaCl in 10 mM Tris-HCl buffer at pH 7.8 from; Fraction III, IV and V - Peak III, IV and V, respectively obtained in the linear gradient from 100 to 310 mM NaCl; Fraction VI – Peak VI obtained with stepwise gradient at 450 mM NaCl; Fraction VII – Peak VII obtained with final washing step at 1 M NaCl.

In order to proceed the purification assays of the clarified sample with monolithic supports it is important first to evaluate the SCOMT_6His activity in the final of the Q-sepharose procedure.
According to several studies, the purified COMT is highly unstable during an isolation process and can lose up to 70% of its activity even at 4 °C [65]. However, the use of stabilizers such as $MgCl_2$, glycerol, cysteine allowed its stabilization and kinetic characterization [8, 13, 16, 29, 63]. So, the collected fractions from Q-Sepharose were stabilized with a suitable solution composed by 150 mM cysteine, 100 mM trialose and 10% of glycerol.
The SCOMT_6His activity was measured by quantification of metanephrine levels produced from epinephrine in COMT extracts, using a HPLC system coupled to a coulometric detector, according to the previously established method [65]. The amount of reaction product was quantified by converting peak area in metanephrine concentration using a calibration curve.
In Figure XXI, epinephrine and metanephrine are represented by peaks with a retention time of approximately 5.3 and 8.4 minutes, respectively. By HPLC chromatogram analysis it is noticeable that there is a marked decrease in amounts metanephrine resultant from purified extracts (peak VI) (Figure XXI, B) when compared to the values obtained for SCOMT_6His onto crude lysates (Figure XXI, A).

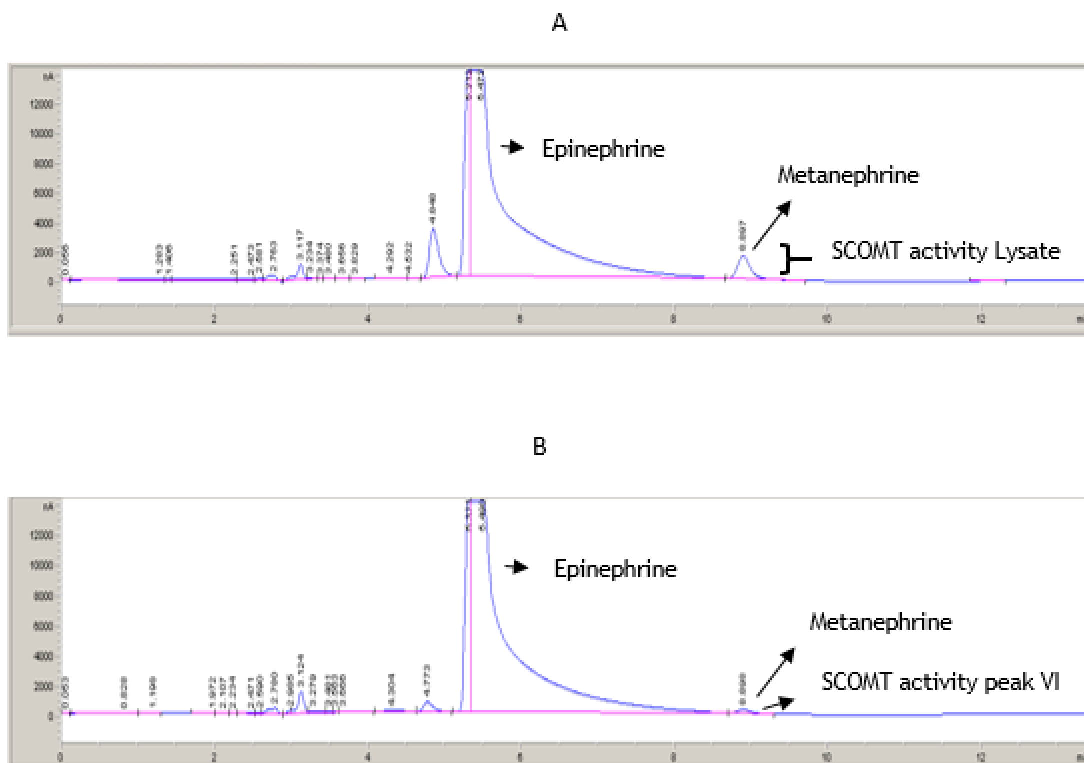

Figure XXI - Chromatographic profiles obtained by HPLC analysis corresponding to: SCOMT_6His lysate - Control (A) and peak VI of SCOMT_6His purified in Q-Sepharose (B). In each chromatogram is presented the peak of epinephrine (COMT substrate) and the peak of metanephrine (COMT product) where the last one allow the assess of SCOMT_6His activity.

In the table III we can observe the activity values for the sample of interest (peak VI) and the lysate.

Table III - Recombinant SCOMT_6His activity levels after recovery by AEC using Q-Sepharose as anion exchanger.

SCOMT activity assays							
Incubated samples	Average Area	Average Height	Metanephrine (nmol/mL)	Total Activity (nmol/h)	Real concentration (mg/mL)	Protein (mg)	Specific Activity (nmol/h/mg)
Lysate	17162.55	1440.90	4.77	14.28	24.30	6.08	2.35
Peak VI	1883.90	174.93	0.43	1.29	9.15	2.29	0.56

These results indicate that the SCOMT_6His protein recovered from the anion exchange process loses part of the specific bioactivity, being obtained around 25 % of the specific bioactivity comparing to the injected lysate [70]. These results suggest that the specific bioactivity lost can be related with the salt conditions used during the purification assay or with Q-sepharose column conditions, ie can be lost the binding ability over time, influencing consequently the SCOMT_6His retention behavior. Furthermore, the isolation procedure of SCOMT_6His is time-consuming and consequently there are quite significant protein activity losses.

3.3 SCOMT_6His purification assays on Monoliths

After the optimization of the elution gradient to recover most part of SCOMT_6His pre-purified in the Q-Sepharose column, further chromatographic assays were tested in different monoliths. Monolithic supports have improved the resolution and capacity of conventional matrices due to the high quantity of accessible binding sites [52] becoming attractive columns for purification studies. One of the advantages of the monolithic supports is the high functional recovery for labile biomolecules such as live virus vaccines, DNA plasmids, and large proteins [52, 53, 56, 58].
Given all the beneficial advantages of monoliths and considering that they never were tested in the SCOMT_6His purification, arise a great interest in the development of suitable purification strategy for this protein. Initially, some preliminary experiments were conducted in different monoliths (CDI, Histamine and Agmatine) in order to establish the suitable mobile phase's conditions for the SCOMT_6His retention and elution and to compare the performance of each monolith used. The tests carried out to study the suitable mobile phase's conditions to retain and elute the SCOMT_6His from all monoliths were conducted under ionic and hydrophobic conditions by increasing and decreasing of sodium chloride and ammonium sulphate concentrations, respectively.

3.3.1 CarbonylDiImidazole Monolith

Initially, it was tested the CDI monolith in the retention and elution study of the SCOMT_6His protein. According to the literature [52], this support favours hydrophobic interactions in the presence of high ammonium sulphate concentrations. Ammonium sulphate is a salt that promotes hydrophobic interactions due to its high 'salting-out' power [41, 42]. However, this effect, can contribute for a significant decrease in target protein yield and bioactivity [42, 43]. Thus, the aim of the following step was to develop a purification strategy that did not strongly affect the bioactivity of the SCOMT_6His protein.

Initially, the CDI monolith was equilibrated with 3 M $(NH_4)_2SO_4$ in 10 mM Tris-HCl buffer at pH 7.8 and after the injection of all sample recovered in the peak VI of the Q-Sepharose assay (200 µL), the unbound species were eluted in the flowthrough peak. After that, a final step at 10 mM Tris-HCl buffer at pH 7.8 was applied to elute the retained species, as depicted in Figure XXII.

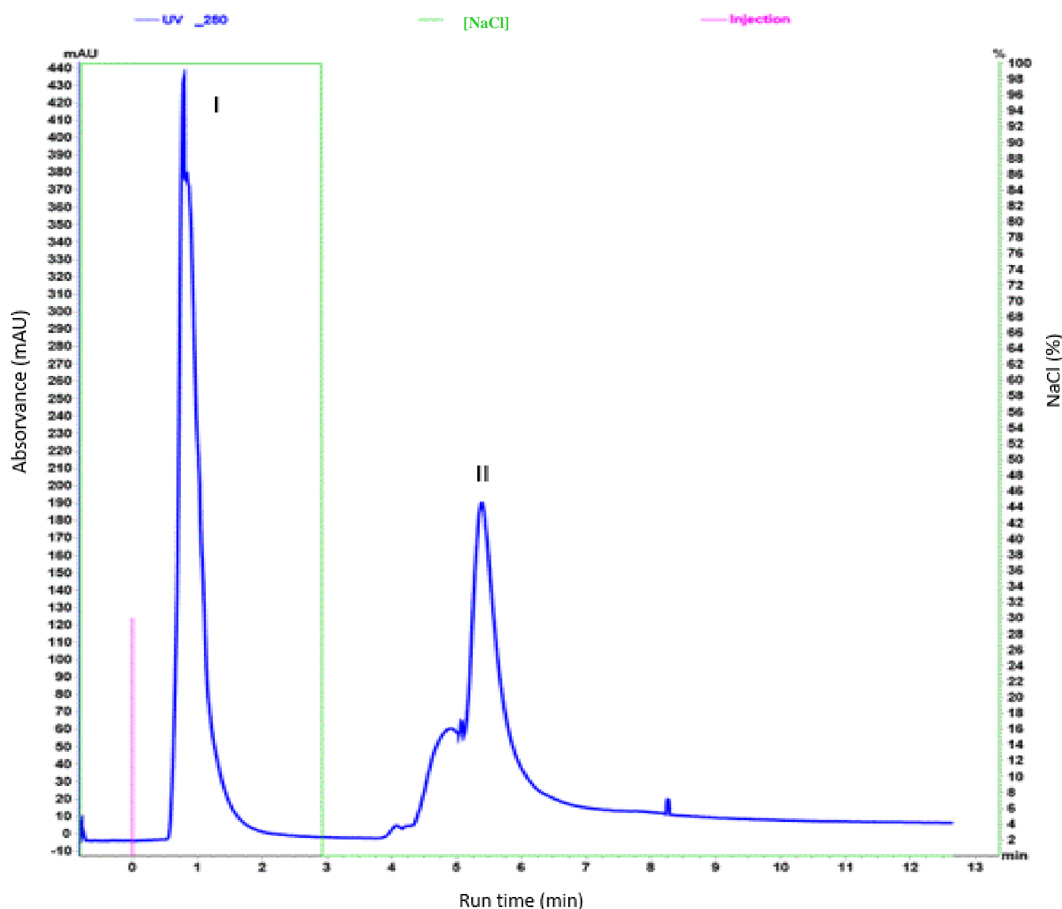

Figure XXII - The SCOMT_6His chromatographic profile on CDI monolith. This monolithic disk was equilibrated with 3 M $(NH_4)_2SO_4$ in 10 mM Tris-HCl buffer (pH 7.8) at 1 mL/min. Adsorption was performed at 3 M $(NH_4)_2SO_4$, followed by a final elution step at 10 mM Tris-HCl buffer at pH 7.8. Blue line represents absorbance at 280 nm and green line the $(NH_4)_2SO_4$ concentration in the mobile phase.

The result SDS-PAGE, shows that the SCOMT_6His wasn't retained to the CDI monolith in the presence of 3 M $(NH_4)_2SO_4$, since the protein band of interest (24.7 kDa) only it's present in the eluted fraction after sample injection together with other contaminating proteins, as shown in fraction I (peak I) of figure XXIII.

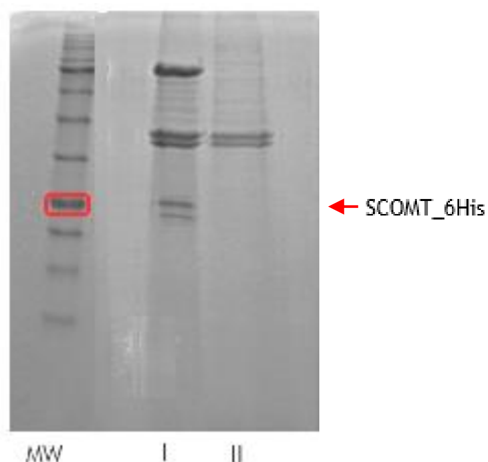

Figure XXIII – SDS-PAGE analysis of the recovered fractions from the SCOMT_6His chromatographic assay on CDI monolith of figure XXII. Lane MW – molecular weight standards; Fractions I and II - Peaks I and II, respectively obtained at 3 M and 0 M $(NH_4)_2SO_4$ in 10 mM Tris-HCl buffer at pH 7.8.

Posteriorly, the chromatographic behavior of the SCOMT_6His protein was analysed under ionic conditions, knowing that these conditions can be more suitable for the protein activity maintenance. Thus, the CDI monolith was equilibrated with 0 M NaCl in 10 mM Tris-HCl buffer at pH 7.8 and after the injection of all sample recovered in the peak VI of the Q-Sepharose assay (200 µL), the unbound species were eluted. Posteriorly, a final step at 1.5 M NaCl was applied with the objective to elute the retained species, as depicted in Figure XXIV.

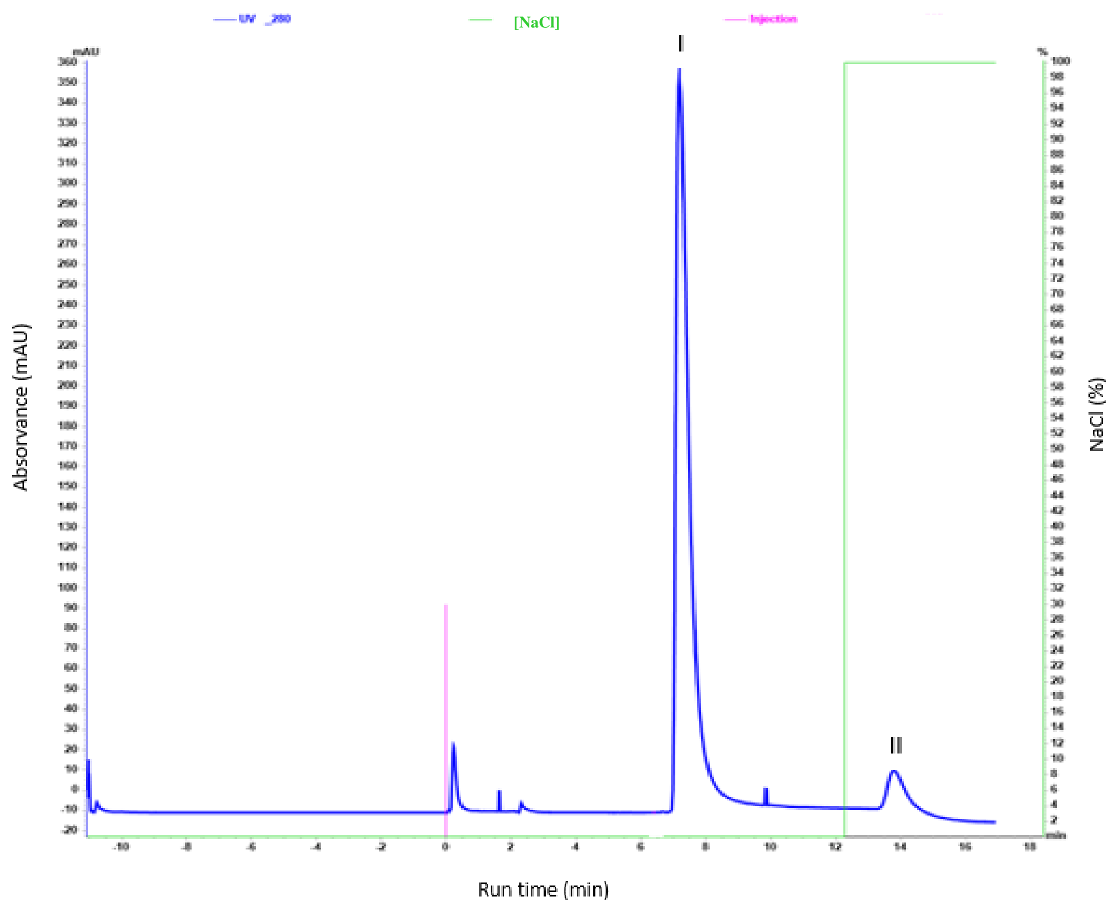

Figure XXIV - The SCOMT_6His chromatographic profile on CDI monolith. This monolithic disk was equilibrated with 10 mM Tris-HCl buffer (pH 7.8) at 1 mL/min. Adsorption was performed at 10 mM Tris-HCl buffer at pH 7.8, followed by a final elution step at 1.5 M NaCl in 10 mM Tris-HCl buffer at pH 7.8. Blue line represents absorbance at 280 nm and green line the NaCl concentration in the mobile phase.

However, the results of the SDS-PAGE electrophoresis are similar to the results previously described, over again the protein band of interest (24.7 kDa) only it's present in the eluted fraction after sample injection together with other contaminating proteins, as shown in fraction I (peak I) of figure XXV.

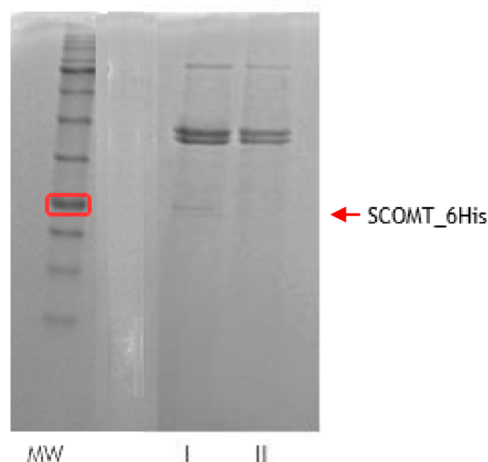

Figure XXV – SDS-PAGE analysis of the recovered fractions from the SCOMT_6His chromatographic assay on CDI monolith of figure XXIV. Lane MW – molecular weight standards; Fractions I and II - Peaks I and II, respectively obtained at 0 M and 1.5 M NaCl in 10 mM Tris-HCl buffer at pH 7.8.

According to the results obtained, it was found that the CDI monolith is not suitable to purify the protein SCOMT_6His under hydrophobic or ionic conditions.

3.3.2 Histamine Monolith

Histamine monolith is based on a CDI monolith modified with the histamine ligand (derived from the decarboxylation of $_{L}$-histidine amino acid). The interest in using this monolith is due to the multifaceted ability of this ligand in the purification of other biomolecules such as sc pDNA from a lysate sample by different strategies, under ionic and hydrophobic elution conditions [56].

As it was already performed with the CDI monolith, at this point were conducted studies to establish the retention and elution pattern of SCOMT_6His in the Histamine monolith by decreasing stepwise gradient of ammonium sulphate to promote mainly hydrophobic interactions and an increasing stepwise gradient of sodium chloride to favor mostly electrostatic interactions. Thus, the Histamine monolith was equilibrated with 3 M $(NH_4)_2SO_4$ in 10 mM Tris-HCl buffer at pH 7.8 and after the injection of all sample recovered in the peak VI of the Q-Sepharose assay (200 µL), the unbound species were eluted in the flowthrough. A final step at 10 mM Tris-HCl buffer at pH 7.8 was used to elute the retained species, as presented in Figure XXVI.

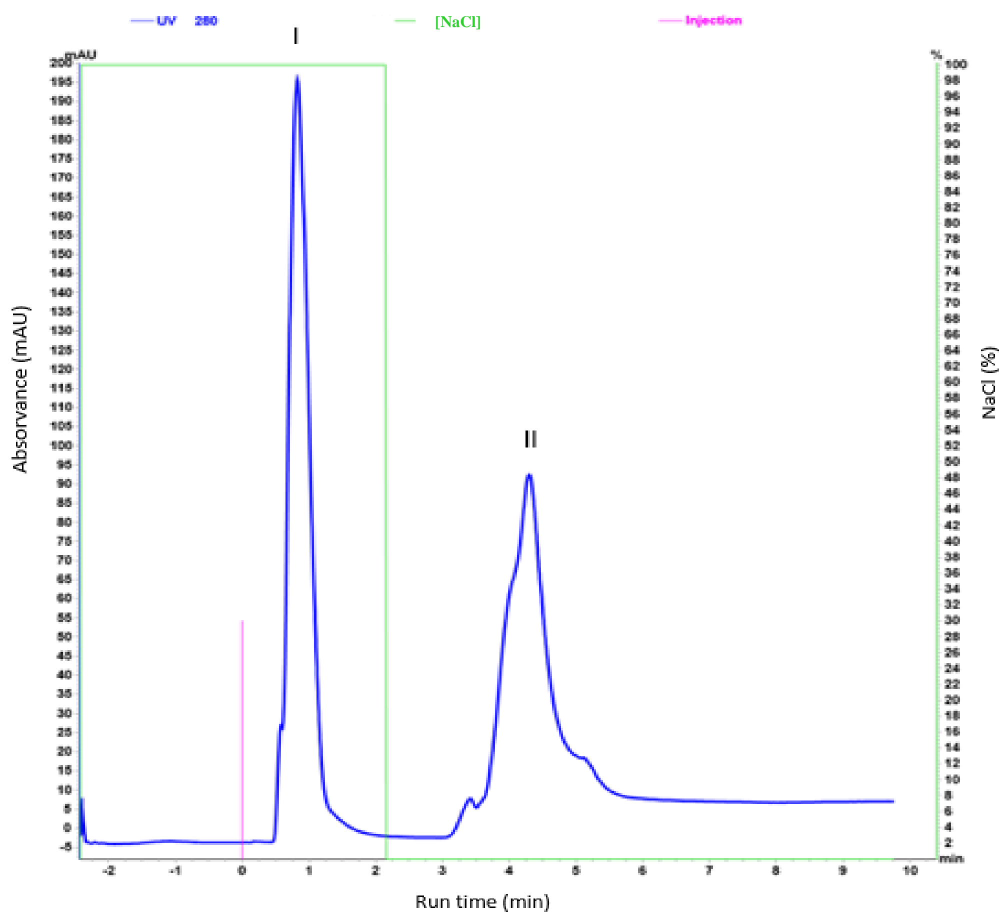

Figure XXVI - The SCOMT_6His chromatographic profile on Histamine monolith. This monolithic disk was equilibrated with 3 M $(NH_4)_2SO_4$ in 10 mM Tris-HCl buffer (pH 7.8) at 1 mL/min. Adsorption was performed at 3 M $(NH_4)_2SO_4$, followed by a final elution step at 10 mM Tris-HCl buffer at pH 7.8. Blue line represents absorbance at 280 nm and green line the $(NH_4)_2SO_4$ concentration in the mobile phase.

The recovered fractions were analysed by SDS-PAGE electrophoresis (Figure XXVII) revealing that there was no retention of the SCOMT_6His protein in the Histamine monolith under hydrophobic conditions, being eluted in the first peak together with other host proteins. On the other hand, these elution conditions can compromise the protein integrity and bioactivity.

Figure XXVII – SDS-PAGE analysis of the recovered fractions from the SCOMT_6His chromatographic assay on Histamine monolith of figure XXVI. Lane MW – molecular weight standards; Fractions I and II - Peaks I and II, respectively obtained at 3 M and 0 M $(NH_4)_2SO_4$ in 10 mM Tris-HCl buffer at pH 7.8.

In addition, ionic conditions were also tested for the SCOMT_6His adsorption to the Histamine monolith. The column was equilibrated with 0 M NaCl in 10 mM Tris-HCl buffer at pH 7.8 and after the injection of all sample recovered from the peak VI of the three Q-Sepharose assays (500 µL), the unbound species were eluted. Thereafter, a final step at 1.5 M NaCl in 10 mM Tris-HCl buffer at pH 7.8 was used to elute the retained species, as observed in Figure XXVIII.

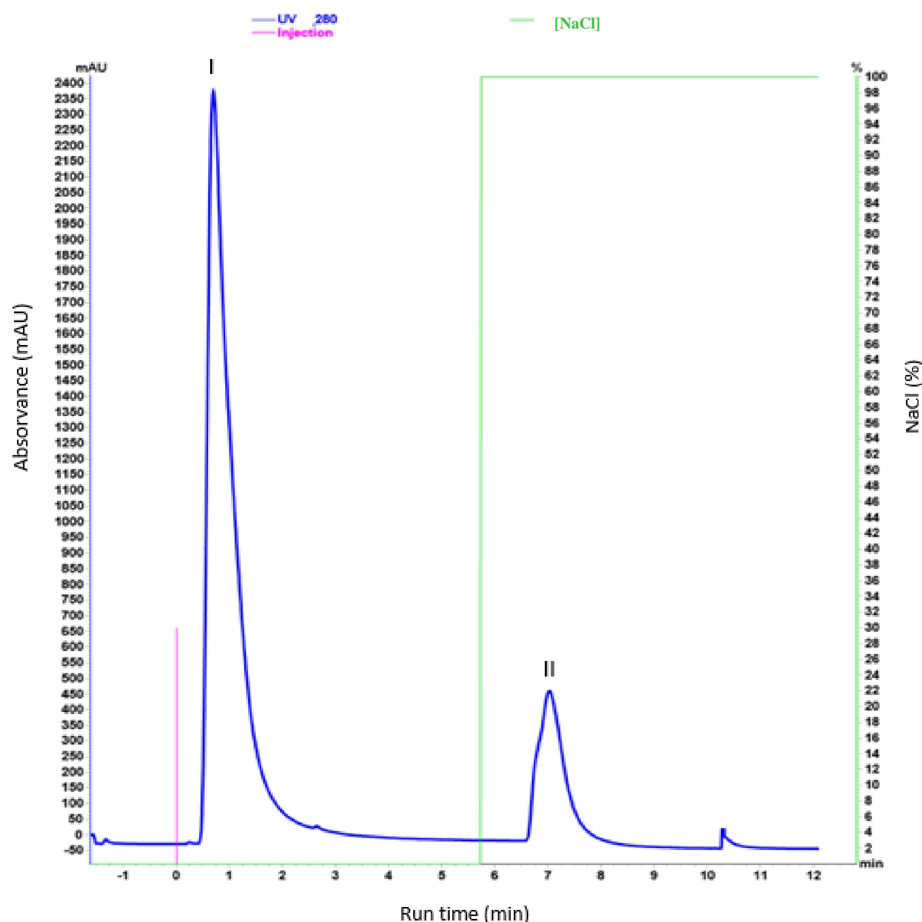

Figure XXVIII - The SCOMT_6His chromatographic profile on Histamine monolith. This monolithic disk was equilibrated with 10 mM Tris-HCl buffer (pH 7.8) at 1 mL/min. Adsorption was performed at 10 mM Tris-HCl buffer (pH 7.8) followed by a final elution step at 1.5 M NaCl in 10 mM Tris-HCl buffer at pH 7.8. Blue line represents absorbance at 280 nm and green line the NaCl concentration in the mobile phase.

By analysis of the SDS–PAGE electrophoresis, it seems that part of the SCOMT_6His protein was retained to the column, being eluted in the second peak with the increase of NaCl concentration (Figure XXIX, A). However, by analysis of western blot (Figure XXIX, B) it was concluded the target protein was eluted in the first peak together with unwanted proteins.

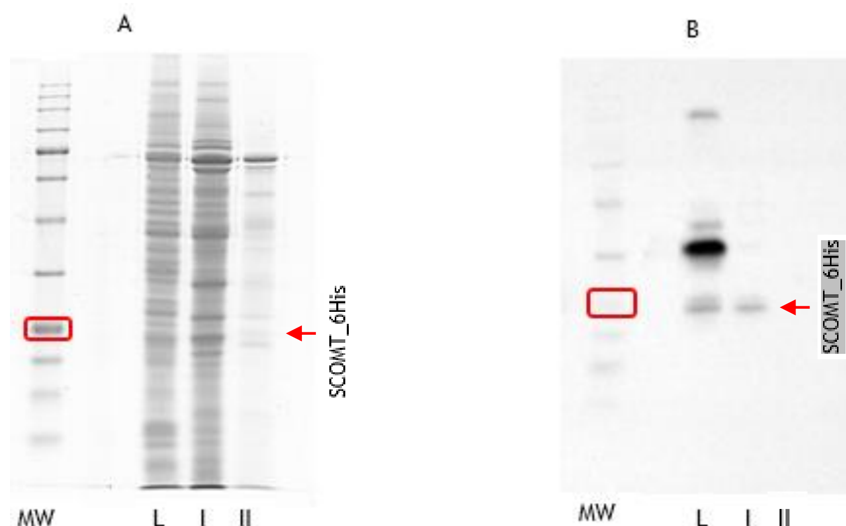

Figure XXIX – SDS-PAGE analysis of the recovered fractions from the SCOMT_6His chromatographic assay on Histamine monolith of figure XXVIII. Lane MW – molecular weight standards; Lane L- Lysis supernatant injected on Q-Sepharose; Fractions I and II - Peaks I and II, respectively obtained at 0 M and 1.5 M NaCl in 10 mM Tris-HCl buffer at pH 7.8.

A possible explanation can be the fact that SCOMT_6His protein has a six histidine tail, which can promote repulsion by the histamine ligands, derived from $_L$-histidine amino acid, under the explored chromatographic conditions.

3.3.3 Agmatine Monolith

Agmatine monolithic support was also prepared from a CDI monolith. Agmatine ligand is derived from the decarboxylation of arginine amino acid. Specifically, arginine-affinity chromatography has been successfully applied for the purification of plasmid DNA, RNA and proteins [62, 63]. Therefore, the affinity chromatography using the monolith agmatine was applied, for the first time, in the SCOMT_6His purification from *P. pastoris* lysates in the present work.

According to the literature, this monolith can retain the target biomolecule under hydrophobic or ionic conditions [56, 58]. Taking this into consideration and given that the hydrophobic conditions can affect drastically the bioactivity of the SCOMT_6His protein, the study of the agmatine monolith was started with ionic chromatographic conditions.

Therefore, SCOMT_6His pre-purified sample, obtained from the peak VI of the three Q-Sepharose assays (500 µL), was injected after the column be equilibrated with 10 mM Tris-HCl at pH 7.8. Then, the elution of bound species was achieved with the application of an increasing stepwise gradient of NaCl concentration to 1.5 M and 3 M NaCl in 10 mM Tris-HCl at pH 7.8, as depicted in Figure XXX. The washing step at 3 M NaCl was considered as a

"regeneration step" to remove all the bound species and assure that the monolith binding capacity is not compromised in subsequent assays.

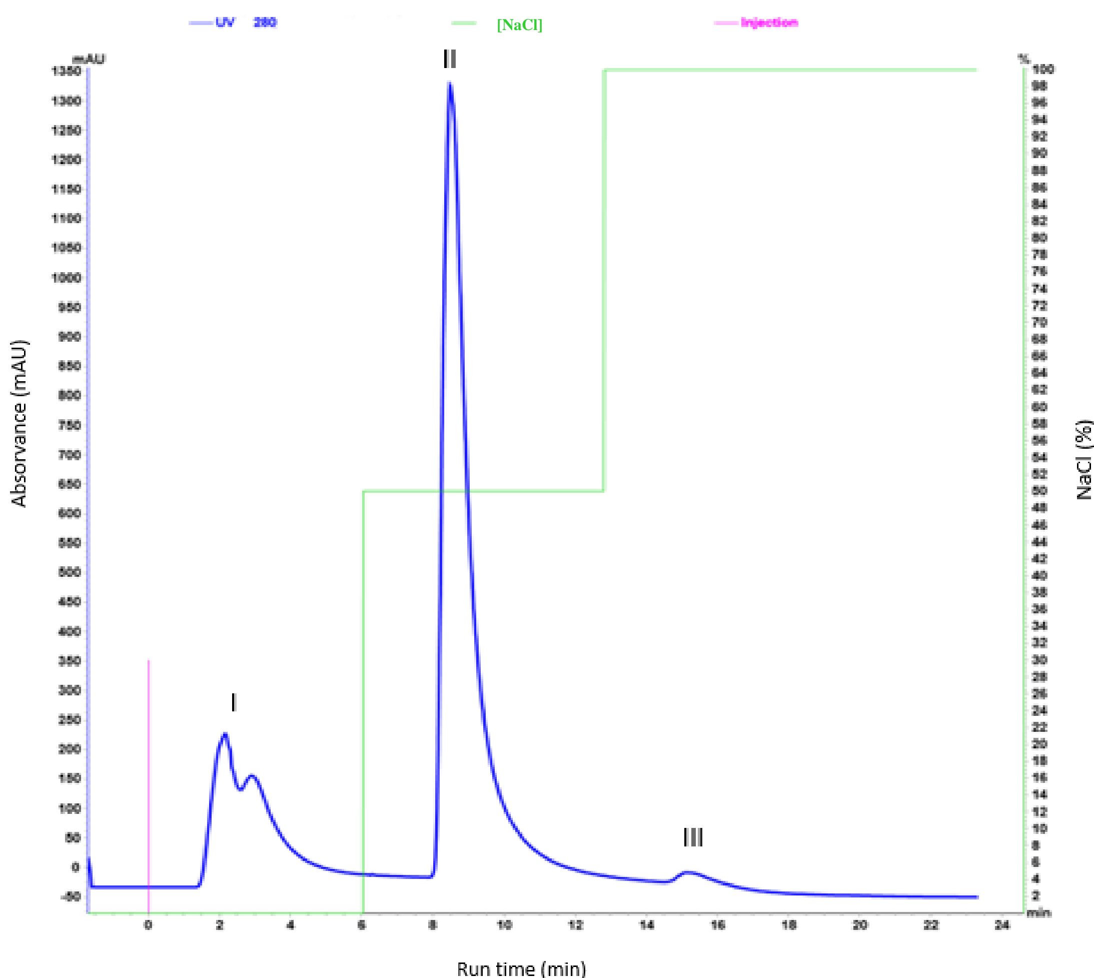

Figure XXX - The SCOMT_6His chromatographic profile on Agmatine monolith. This monolith was equilibrated with 10 mM Tris-HCl buffer (pH 7.8) at 1 mL/min. Adsorption was performed at 10 mM Tris-HCl buffer (pH 7.8), followed by stepwise salt gradient of 1.5 M and 3 M NaCl 10 mM Tris-HCl buffer (pH 7.8). Blue line represents absorbance at 280 nm and green line the NaCl concentration in the mobile phase.

The recovered fractions were analysed by SDS-PAGE electrophoresis revealing that most of the injected sample was retained in the Agmatine monolith, although some proteins have eluted directly in the flowthrough (Figure XXXI, A). It was also observed that the washing step at 3 M NaCl is needed since a great percentage of proteins were eluted.

By analysis of western blot it was confirmed the protein of interest was retained in the monolith, being eluted at 1.5 M NaCl together with a significant amount of contaminating proteins (Figure XXXI, B). These western blot results also show that the amount of protein recovered by Q-sepharose trials is low (as seen in fraction VI), however after injection in the

agmatine monolith appears to be fully recovered in peak II, suggesting that this monolith doesn't interfere with the recovery of the protein of interest.

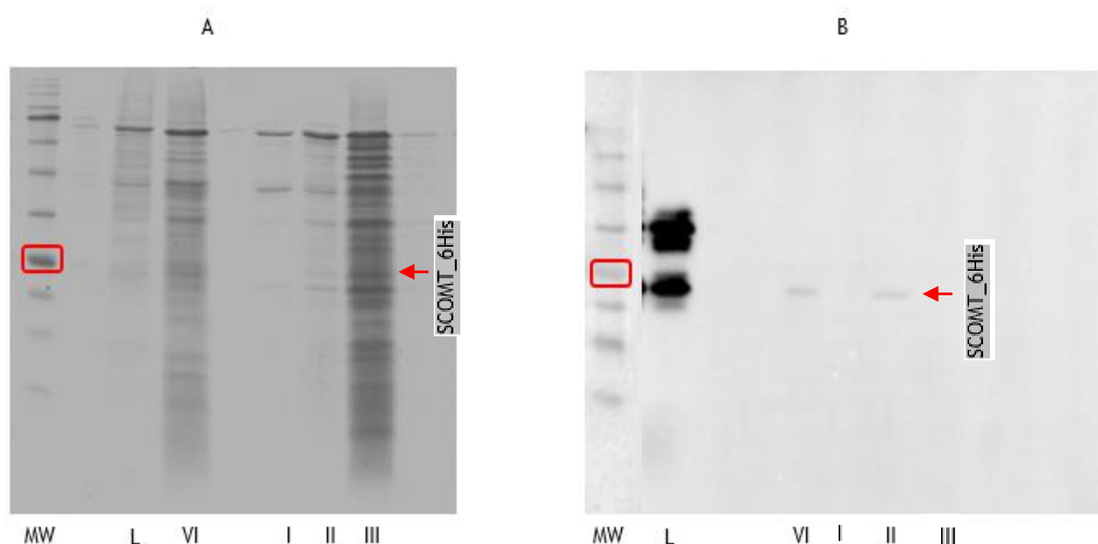

Figure XXXI - SDS-PAGE analysis (A) and Western Bolt (B) of the recovered fractions from the SCOMT_6His chromatographic assay on Agmatine monolith of figure XXX. Lane MW – molecular weight standards; (A) and (B) Lane L – Lysis supernatant injected on Q-sepharose; Fraction VI - Peak VI obtained by Q-Sepharose; Fraction I – Peak I obtained at 10 mM Tris-HCl buffer (pH 7.8); Fraction II – Peak II obtained by stepwise gradient at 1.5 M NaCl in 10 mM Tris-HCl buffer (pH 7.8); Fraction III – Peak III obtained by final step at 3 M NaCl in 10 mM Tris-HCl buffer (pH 7.8).

The protein of interest was retained in the Agmatine monolith under ionic conditions, thus various tests were performed to optimize the elution step in order to improve the purity of SCOMT_6His. However, to detect the target protein in each testing of the Agmatine monolith were required three Q-Sepharose assays due to the low recovery obtained on this column. Therefore, a new strategy was explored that consisted in the direct injection of *P. pastoris* lysate in the Agmatine monolith.

Given that the lysis of *P. Pastoris* cells with glass beads results in a complex lysate containing all the cell debris, a pre-treatment of the lysate sample is required before the direct injection in a monolithic column, otherwise the column will clog. Thus, the sample was subjected to different treatment processes including, filtration, centrifugation or combined both unitary operation.

By analysis of SDS-PAGE gel (Figure XXXII, A) and western blot (Figure XXXII, B) it is verified that the samples treated with centrifugation at 500 x g for 5 min at 4◦C loose the target protein in the pellet, together with cell debris. The lysate filtration through a 0.45 μm pore size membrane revealed the presence of SCOMT_6His in the filtered sample.

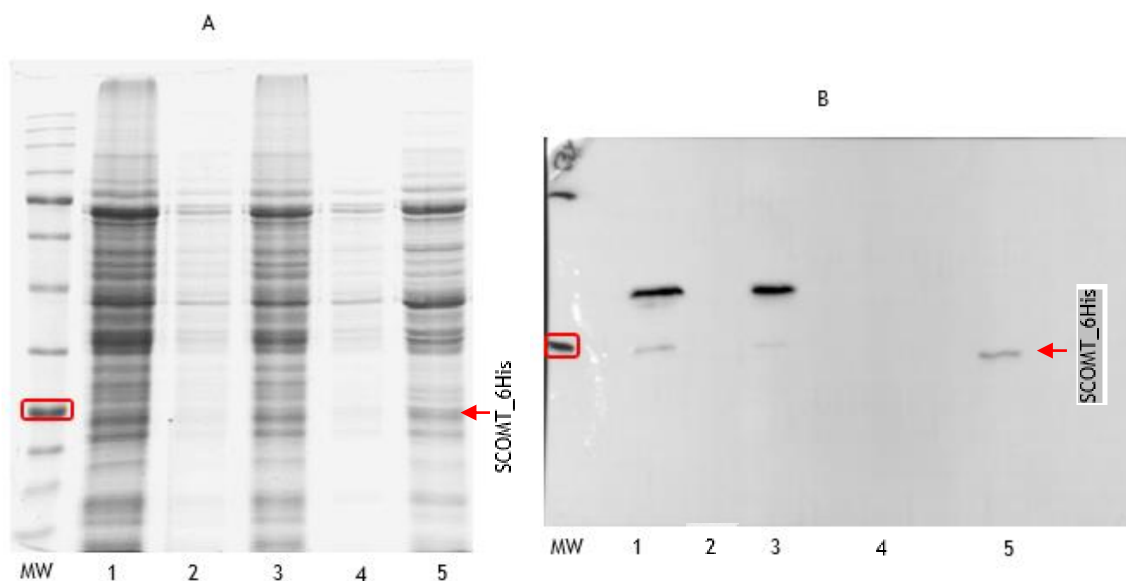

Figure XXXII - SDS-PAGE analysis (A) and Western Bolt (B) of the recovered fractions from the pre-treatment of *P. pastoris* lysate. (A) and (B) Lane MW – molecular weight standards; Lane 1 - Pellet resultant of centrifugation; Lane 2 - Supernatant resultant of centrifugation; Lane 3 - Pellet resultant of centrifugation with posterior filtration; Lane 4- Supernatant resultant centrifugation with posterior filtration; Lane 5 – Filtered lysate from *P. pastoris*.

After the filtration treatment, the same volume of lysate sample needed for the Q-Sepharose assay allowed to perform several assays in the monolith column. Therefore, the monolith was equilibrated with 10 mM of Tris-HCl at pH 7.8 and after the injection of filtered sample (500 µL), an increasing linear gradient from 0 M to 1.5 M NaCl 10 mM of Tris-HCl at pH 7.8 was conducted during 15 minutes (Figure XXXIII, A) and in the other assay, a stepwise gradient at 525 Mm and 1.5 M NaCl in 10 mM of Tris-HCl at pH 7.8 was performed (Figure XXXIII, B).

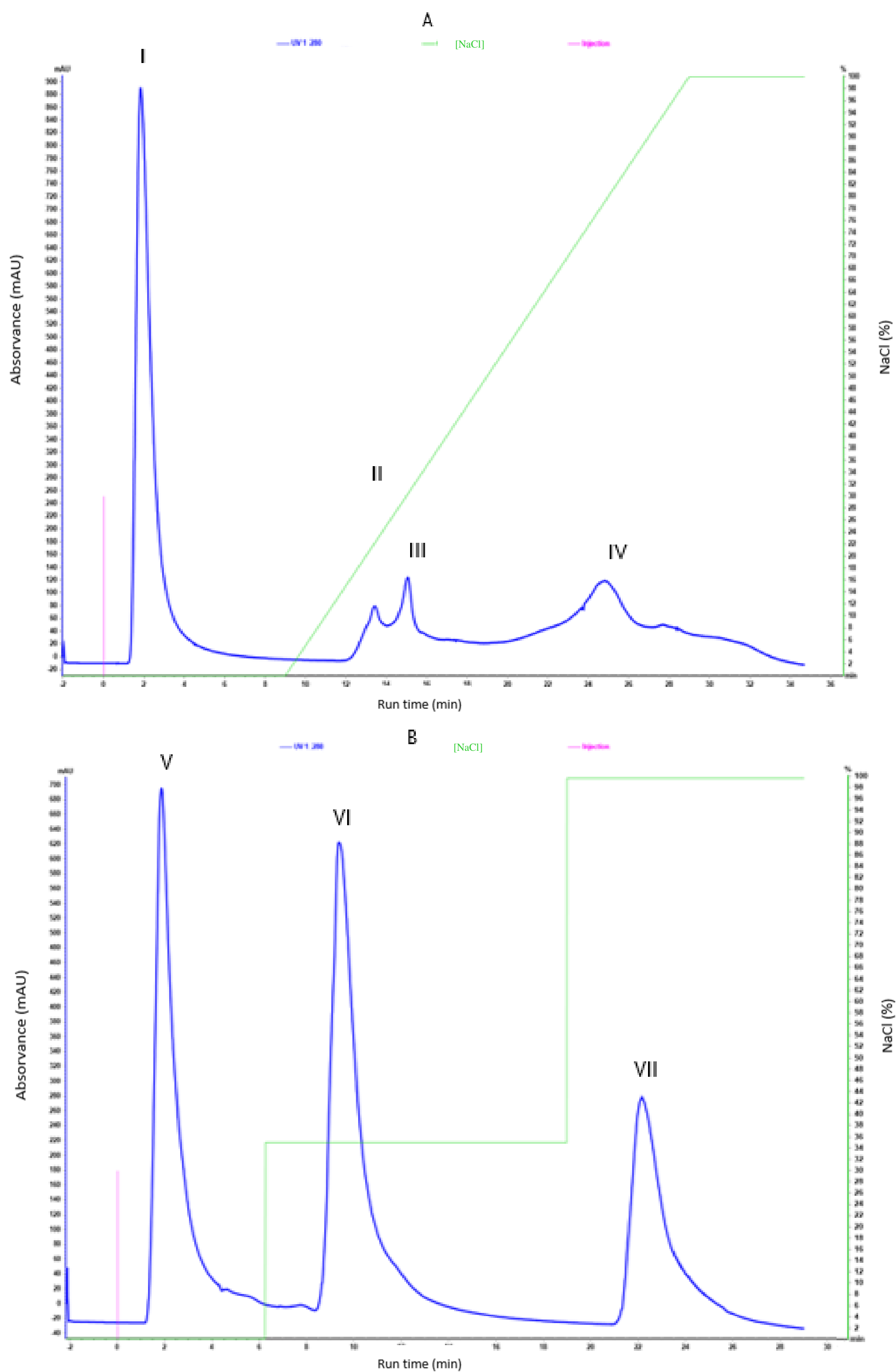

Figure XXXIII - The SCOMT_6His chromatographic profile on Agmatine monolith. This monolith was equilibrated with 10 mM Tris-HCl (pH 7.8) at 1 mL/min. (A) Adsorption was performed with 10 mM Tris-HCl, followed by a linear gradient at 1.5 M NaCl. (B) Adsorption was performed with 10 mM Tris-HCl buffer at pH 7.8 followed by two different steps at 525 mM NaCl and 1.5 M NaCl in 10 mM Tris-Cl buffer (pH 7.8). Blue line represents absorbance at 280 nm and green line the NaCl concentration in mobile phase.

By analysis of SDS-PAGE electrophoresis, it seems that part of the SCOMT_6His protein (molecular weight of 24.7 KDa) was not retained to the monolith and other part was eluted in peaks II and III by the linear gradient (Figure XXXIV, A), and in peak VI by the stepwise gradient the protein was eluted together with a considerable amount of contaminants (Figure XXXIV, B).

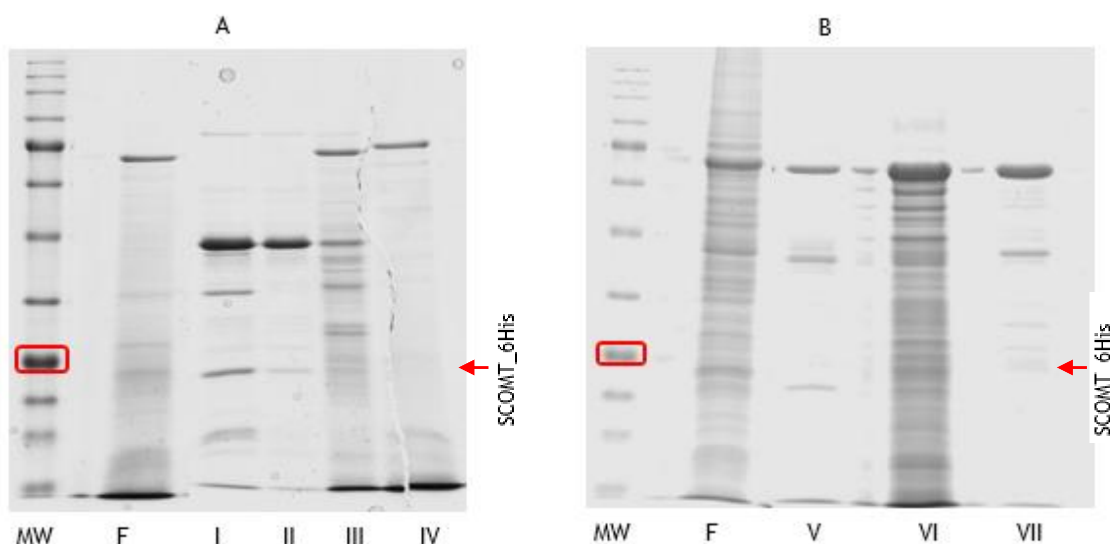

Figure XXXIV - SDS-PAGE analysis (A) and (B) from the SCOMT_6His chromatographic assay on Agmatine monolithic of figure XXXIII. Lane MW – molecular weight standards; Fraction F - Supernatant resultant of filtration; (A) Fraction I – Peak I obtained at 10 mM Tris-HCl (pH 7.8); Fraction II, III and IV – Peak I, III and IV respectively obtained by linear gradient at 1.5 M NaCl; (B) Fraction V – Peak V obtained at 10 mM Tris-HCl (pH 7.8); Fraction VI – Peak VI obtained by stepwise gradient at 525 mM NaCl; Fraction VII – Peak VII obtained by final step at 1.5 M NaCl in 10 mM Tris-HCl (pH 7.8).

According to previous results, a linear gradient will be improved after a washing step with 3 M NaCl (in order to avoid the loss of binding capacity due to bound proteins) and by increase of the injection volume for 2 mL of filtered lysate, which corresponds to lysate volume of one fermentation used in a Q-Sepharose assay. This adjustment in injection volume is needed because when the purity of the target protein is a critical issue, the initial sample concentration should be higher in order to increase the purification factor. After equilibration of the monolith with 10 mM of Tris-HCl at pH 7.8, a linear gradient from 0 to 1.5 M NaCl was performed during 20 minutes, followed by a final washing step at 3 M NaCl (Figure XXXV).

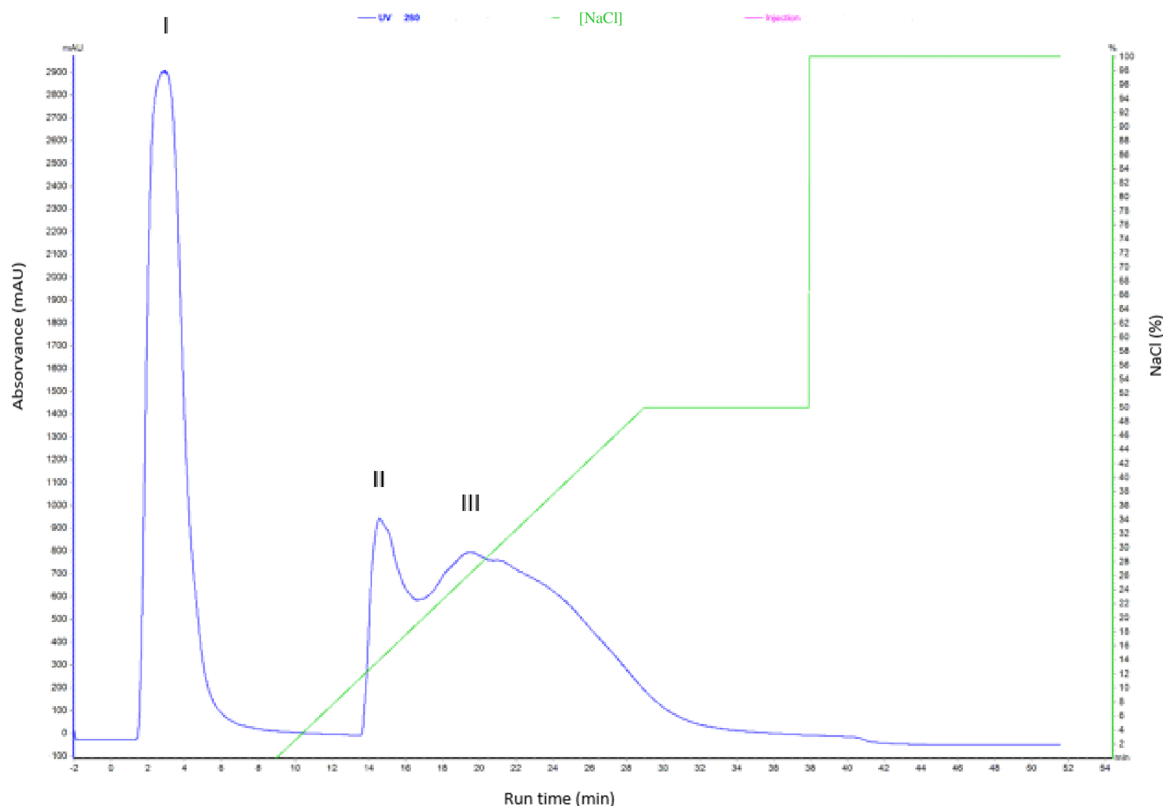

Figure XXXV - The SCOMT_6His chromatographic profile on Agmatine monolith. This monolith was equilibrated with 10 mM Tris-HCl (pH 7.8) at 1 mL/min. Adsorption was performed at 10 mM Tris-HCl buffer (pH 7.8), followed by increasing linear gradient from 0 M to 1.5 M NaCl during 20 minutes and a final washing step at 3 M NaCl in 10 mM Tris-HCl (pH 7.8). Blue line represents absorbance at 280 nm and green line the NaCl concentration in the mobile phase.

By analysis of recovered fractions in the SDS–PAGE gel electrophoresis (Figure XXXVI, A) and western blot (Figure XXXVI, B), it can be observed that SCOMT_6His was eluted essentially in peak III during the linear gradient from 0 to 1.5 M NaCl, resulting in an immunological active strong band of molecular weight 24.7 KDa (Figure XXXVI, B). Although some proteins interferents were eluted together with SCOMT_6His in the peak III, a huge amount of unwanted proteins were eliminated with this elution strategy, manly in the first peak and a part in the second peak (Figure XXXVI, A).

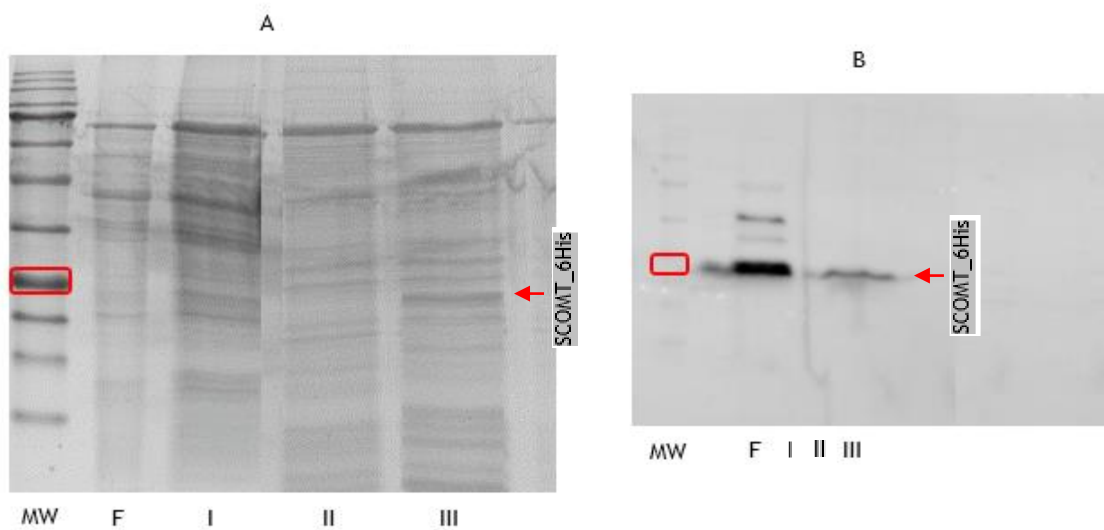

Figure XXXVI – SDS-PAGE analysis (A) and Western Bolt (B) from the SCOMT_6His chromatographic assay on Agmatine monolithic of figure XXXV. Lane MW – molecular weight standards; Fraction F - Filtered lysate; Fraction I – Peak I obtained at 10 mM Tris-HCl (pH 7.8); Fraction II and III – Peak II and III respectively obtained in the linear gradient at 1.5 M NaCl in 10 mM Tris-HCl (pH 7.8).

Chapter IV

Conclusions

Purification of recombinant proteins is still a challenge in biotechnology, which demand procedures to endow the purified protein with structural integrity and biological activity without contamination. The implementation of this study relies on the development of efficient chromatographic strategies to obtain an appropriate purification process. For the first time, two chromatographic strategies were proposed for the recovery of SCOMT_6His from *P. pastoris* lysate by using the Agmatine monolith after a pre-purification with the Q-Sepharose column as anion exchanger or directly after a filtration procedure.

In general, the pre-purification of SCOMT_6His in the Q-Sepharose column was performed after the combination and optimization of two chromatographic processes already described in the literature by ionic strength manipulation of NaCl onto mobile phase. Thus, the protein adsorption was obtained by a linear gradient from 0 to 100 mM NaCl revealing that a residual presence of NaCl was required to favour this mechanism. The SCOMT_6His elution was performed by a stepwise with high ionic strength (450 mM NaCl), being eliminated a great amount of contaminants in the other steps. In terms of recovery, the specific activity of the SCOMT_6His protein recovered in peak VI by Q-sepharose assay was 25% of the biospecific activity of the lysate sample injected probably because the Q-sepharose column over time can lost the binding ability and another factor that may be involved is long durability of the isolation procedure of SCOMT_6His. Overall, the Q-Sepharose has not shown a high selectivity for SCOMT_6His isolation, not being possible to maintain its biological activity with significant bioactivity high recovery rates after an anionic exchange chromatography strategy.

For improving the purity degree of the target protein obtained in the peak VI of the Q-Sepharose, several monolithic supports were tested. The elution strategies adopted for CDI and Histamine monoliths were based on the manipulation of hydrophobic and ionic conditions, however it was concluded that the SCOMT_6His was not retained to these monoliths, being eluted in the flowthrough together with other contaminant proteins. However, the retention of SCOMT_6His was obtained in the Agmatine monolith and its elution was possible by an increasing linear gradient of NaCl on the mobile phase. It is suggested that the affinity interactions promoted by the agmatine ligands involve multiple interactions, which can be greatly advantageous for the purification of SCOMT_6His.

The strategy of direct injection of filtered lysate in the Agmatine monolith showed to use less amount of sample and to be less time-consuming than the previous strategy. Moreover, also the SCOMT_6His recovery is higher than the strategy of the combination of Q-Sepharose and

Agmatine monolith, although the selectivity and purity degree obtained with the implemented elution strategy was not yet the required.

In conclusion, the comparison of these approaches demonstrated the complexity of SCOMT_6His purification processes. In spite of the requirement of structural studies, the monolithic supports clearly have advantages over the earlier methods published due of its simplicity and quickness in the SCOMT_6His purification assays. Therefore, the monolithic supports combined with affinity ligands can be a promising approach to obtain the SCOMT_6His protein with higher recovery and purity, in a biologically active form.

Chapter V

Future perspectives

The optimization of separation is a very complex process, however in order to improve protein stability and structure, it is necessary to develop a strategy that increases the level of SCOMT_6His purification in the monolith Agmatine. The flow-rate, pH and ionic strength are interesting variables/factors to be considered in a design of experiments to optimize the chromatographic procedure.

Thus, the elution strategy in the Agmatine monolith of both strategies (after a pre-purification with the Q-Sepharose or after a filtration process of the lysate sample) should be improved in order to verify the recovery, purity degree as well as the bio specific activity of SCOMT_6His obtained in each strategy. It is expecting that the shorter chromatographic process has the better performance, since the process combining two chromatographic procedures is quite time consuming and causes the protein lose bioactivity over time even with the addition of stabilizing solution.

In addition, an interesting future work can be the development of a membrane filtration technology as initial step in the lysate treatment before the purification step with the Agmatine monolith. This technique looks be an ideal candidate for large-scale purification and recovery of therapeutic proteins, because it removes biological macromolecules that can difficult hands on onto the purification processes.

Chapter VI

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Chapter VII

Appendices

Appendice I – High Range rainbow molecular weight marker from GE Healthcare.