UNIVERSIDADE DA BEIRA INTERIOR
Ciências

Purification of Recombinant Human Membrane COMT by Ionic Exchange Chromatography

Filipa Inês Feliciano Correia

Dissertação para obtenção do Grau de Mestre em
Biotecnologia
(2º Ciclo de estudos)

Orientador: Prof. Dr. Luís António Paulino Passarinha

Covilhã, Junho 2013

Para os Meus Queridos Pais

São a Razão da Minha Vida

Acknowledgments

A realização desta dissertação marca o fim de uma importante etapa da minha vida. Embora a tese seja, pela sua finalidade académica, um trabalho individual, há contributos de natureza diversa que não podem e nem devem deixar de ser realçados. Por essa razão, gostaria de agradecer a todos aqueles que contribuíram de alguma forma para a sua concretização.

Em primeiro lugar, agradeço ao professor Doutor Luís António Paulino Passarinha, meu orientador, pelo excelente acompanhamento, competência científica, disponibilidade e generosidade reveladas ao longo deste ano, assim como pelas críticas, correções e sugestões relevantes feitas durante a orientação. E ainda, agradecer pelo simples facto de nunca ter virado costas aos meus problemas no trabalho, mostrando eterna amizade e apoio para me ajudar a ultrapassá-los. Consegui em parte finalizar este meu projeto graças à motivação e confiança depositadas, que me deu força e vontade para conquistar todos os passos para estar aqui hoje a escrever palavra a palavra a minha dissertação.

Ao professor Doutor João Queiroz por todo o apoio científico e bibliográfico no decorrer no trabalho.

Agradecer do fundo do coração à minha família, por quem tenho o maior respeito e amor do mundo, o facto de terem estado presentes em toda a minha vida. Em especial, à minha mãe e ao meu pai que me deram ao longo da vida, com muito esforço, o possível e o impossível, para chegar onde cheguei e mostrar com o orgulho a pessoa que sou hoje. Não me cansarei de lhes agradecer porque aos meus pais devo tudo, são o meu pilar nesta vida. Um agradecimento também muito especial à Tia Gabriela, Tio Tó-Zé, Ana e Álvaro pelo apoio e ajuda prestado. Adoro-vos!

Ao João Farias, o meu mais que tudo, por todo o amor dado nestes últimos dois anos e por nunca ter desistido de me apoiar nos momentos mais difíceis. Se há coisa que vou guardar sempre é o teu apoio e confiança que não me permitiram em momento algum desistir dos meus sonhos. Realmente revelaste ter muita paciência, João. Obrigado meu amor por me permitires atingir o auge da Felicidade!

À Fátima Santos, minha “pseudo-co-orientadora”. Digo isto com toda a satisfação do mundo porque para além de me orientar no trabalho ao longo do ano, mostrou ser uma amiga incondicional que me ajudou em tudo o que precisei e sem hesitar partilhou os seus inestimáveis conhecimentos para que eu pudesse concretizar mais uma etapa da minha vida. O meu muito obrigado Fatucha!

A todos os meus colegas e amigos que me acompanharam e contribuíram para a minha chegada até aqui. Em especial, agradeço à Marta Esteves, Claúdia Mouro, Joana Vaz, Ana

Afonso e Filipa Pires pela amizade e paciência em aturar os meus devaneios, as minhas palermices e sobretudo pelo companheirismo e carinho prestado.

A todos os membros do CICS que graças a eles esteve sempre presente um bom ambiente de trabalho que proporcionou realizar as tarefas da melhor maneira possível. Em particular, ao Augusto, Margarida Grilo, Luís Miguel, Guilherme, Armanda, Luís Rocha e Sílvia Rocha, pelo apoio, companheirismo, espírito de equipa e amizade prestadas ao longo deste ano.

Resumo

A catecol-*O*-methyltransferase (COMT) é um alvo importante na engenharia das proteínas devido à sua função na actividade normal do cérebro e ao seu possível papel em desordens neurológicas humanas, como a doença de Parkinson’s, esquizofrenia, depressão e Alzheimer’s. Assim, o desenvolvimento de novos inibidores da COMT proporcionou uma melhoria no tratamento da doença de Parkinson’s. No entanto, apesar do facto da SCOMT humana ter sido extensivamente estudada, poucos estudos têm sido publicados relativamente à recuperação cromatográfica da MBCOMT e respectivos níveis de bioactividade. Portanto, neste trabalho foi comparado o perfil cromatográfico da hSCOMT e hMBCOMT usando a Q-Sepharose como ligando, de forma a avaliar os seus níveis de actividade e taxas de recuperação. Os resultados obtidos mostram que ambas as isoformas requerem diferentes condições de adsorção; A adsorção da isoforma solúvel é alcançada com a aplicação de baixa força iónica, enquanto que para a hMBCOMT torna-se necessário um aumento linear do gradiente de sal. Porém, a aplicação de 0,5% de Triton X-100 na fase móvel permitiu a adsorção da hMBCOMT a uma baixa força iónica. Aparentemente, o uso de detergentes aproxima os comportamentos das duas isoformas em termos de condições cromatográficas. Especificamente, as estratégias cromatográficas com e sem detergentes resultaram num índice de purificação respectivamente de 4,3 e 7, o que corresponde a valores de actividade específica de 331nmol/h/mg e 496noml/h/mg. Em relação ao valor de actividade específica da hSCOMT, este corresponde a 250nmol/h/mg e um grau de purificação de 3,6. Assim, apesar da instabilidade da COMT durante o seu manuseamento laboratorial, a aplicação da Q-Sepharose como permutador aniónico revelou ser eficiente na recuperação de ambas as isoformas, tornando-se um requisito transversal de aplicação cromatográfica para estudos cinéticos e farmacológicos.

Palavras-chave

Q-Sepharose; COMT solúvel; COMT membranar; Purificação; Cromatografia de Interacção Aniónica.

Abstract

Catechol-O-methyltransferase (COMT) is an important target in protein engineering due to their role in normal brain and their possible role in human neurological disorders such as Parkinson's, schizophrenia, depression and Alzheimer's disease. So, the development of new COMT inhibitors led to an improvement of Parkinson disease treatment. However, despite the fact that human SCOMT has been extensively studied, few studies have been published in relation MBCOMT chromatographic recovery and respective bioactivity levels. Therefore, in this work we compare the chromatographic profile of hSCOMT and hMBCOMT using Q-Sepharose as ligand, in order to evaluate activity levels and COMT recovery rates. Results show that both isoforms required different conditions for its adsorption; soluble isoform adsorption is perform at low ionic strength while for MBCOMT it becomes necessary an increasing linear salt gradient. However, the application of 0,5% Triton X-100 in mobile phase promotes hMBCOMT's adsorption at low ionic strength. Apparently, the use of detergents approaches both isoforms behavior in terms of chromatographic conditions. Specifically, the chromatographic strategies with and without detergents resulted in a 4,3 and 7-fold purification respectively, corresponding to specific activity values of 331 nmol/h/mg and 496 nmol/h/mg. Regarding to specific activity value of hSCOMT, this corresponding to 250 nmol/h/mg and a 3,6-fold purification. Thus, despite the instability of COMT during their handling laboratory, the application of Q-Sepharose as anion exchanger shown to be effective in recovery of both isoforms, it becomes a transversal requirement of chromatographic application for kinetic and pharmacological trials.

Keywords

Q-Sepharose; Soluble COMT; Membrane bound COMT; Purification; Anion Interaction Chromatography

Table of Contents

List of Figures

List of Tables

List of Acronyms

A	Adenine
AEC	Anion exchange chomatography
Ala	Alanine
AS	Ammonium sulphate
B. choshinensis	*Brevibacillus choshinensis*
BSA	Bovine serum albumin
C	Cytosine
CAPE	Caffeic acid phenethyl ester
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
Cl^-	Chloride ion
CMC	Critical micellar concentration
COMT	catechol-*O*-methyltransferase
CV	Column volume
DAGK	*E.coli* diacylglycerolkinase
DDAO	N-dodecyldimethyl amineoxide
DDM	N-dodecyl-β-D-maltoside
DEAE	Diethylaminoethyl
DFP	Diisopropylfluoro-phosphate
DHPC	L-a-1,2-dihexanoylphosphatidyl choline
DMPC	1,2-dimyristolysn-glycero-3-phosphocholine
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
DNC	Dinitrocatechol

DTT	Dithiothreitol
E.coli	*Escherichia coli*
ECG	(-)-epicatechin-3-gallate
EDTA	Ethylenediamine tetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
FPLC	Fast performance liquid chromatography
G	Guanine
HCl	Hydrogen chloride
HIC	Hydrophobic Interaction Chromatography
His	Histidine aminoacid
hMBCOMT	Human membrane bound catechol-*O*-methyltransferase
HPLC	High performance liquid chromatography
hSCOMT	Human soluble catechol-*O*-methyltransferase
IEC	Ion Exchange chromatography
IMAC	Immobilized metal-affinity chromatography
IPTG	Isopropylthiogalactosidase
Km	Michaelis- Menten constant
L-Dopa	Levodopa
Leu	Leucine
Lys	Lisine
MBCOMT	Membrane bound catechol-*O*-methyltransferase
Met	Methionine
Mg (II)/Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride

MP’s	Membrane proteins
nA	Nanoamperes
NaCl	Chloride sodium
NMR	Nuclear magnetic resonance
OD_{660}	Optical density at 660nm
OG	N-octyl-β-D-glucopyranoside
PD	Parkinson’s Disease
PI	Isoelectric point
PIS	Isoeletric points
Pro	Proline aminoacid
PVDF	Polyvinyl difluoride
SAH	S-adenosyll-homocysteine
SAM	S-adenosyll-L-methionine
SCOMT	Soluble catechol-*O*-methyltransferase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	Serine
SH-	Sulfhydryl group
T	Thymine
Tris	Tris(hydroxymethyl)aminomethane
Thr	Threonine
Trp	Tryptophan
UV	Ultra Violet
Val	Valine
Vmax	Maximum velocity

Chapter I

Introduction

1.1 Membrane proteins

The membrane system is one of the most important interfaces in biological systems and contain many types of receptor proteins, transport proteins and channel proteins that play critical roles in biological activity [1]. Membrane proteins (MPs) play a fundamental role in biological processes such as transport of molecules, cellular signaling, energy production and use and maintenance of cells and tissue structures [2, 3].

MPs represent 20-30% of all proteins encoded by the genomes of various organisms [2, 4] and can be classified as peripheral or integral [2, 5]. Peripheral MPs are poorly associated with the membrane and are usually water-soluble after being released from the lipid environment [2, 5]. On the other hand, the integral MPs are water insoluble and contain one or more transmembrane segments comprising polypeptide stretches that cross the membrane [2, 5]. The transmembrane fraction may be constituted by a single beam or multiple polypeptide stretches [2, 5].

Nowadays, the demand for therapeutic proteins has increased significantly due to scientific developments and continued growth of biotechnology and biopharmaceutical industries [3]. Specifically, the investigation of MPs has become a focus of interest for biochemical, biotechnological and biomedical researchers [6]. However most of experimental approaches require large quantities of proteins [6]. The main difficulty in handling MPs is to obtain sufficient quantities of the target protein since its level of expression is low in their native environment [4, 6, 7]. Thus, attempts are made to overexpress the protein of interest in order to achieve high cell concentrations of this protein [2, 4, 6, 7]. However, MPs aggregation has been reported in cytoplasm after its overexpression in heterologous expression systems such as *E. coli* [6–8]. As matter of fact, high yields of functional and stable proteins are rarely obtained [7]. MPs possess a great tendency to form aggregates even in the presence of detergents, which decreases its biological activity and reduces the efficiency of separation techniques [2, 6].

So, in order to improve MPs solublization several variables such as the type and concentration of detergents, ionic strength and pH should be considered. The accurate combination of this inputs contributes to achieve a homogeneously dissolved state of the protein, to increase its stability and to avoid the dissociation of labile protein complex structures [6]. Specifically, the use of detergents ensures the homogeneity and integrity of MPs interacting directly with its hydrophobic regions and allowing it solubilization by interacting with aqueous phase [6, 9]. The ionic strength should be adjusted to the minimum that allows a homogeneous solubilization and to a maximum which avoid the dissociation of labile protein structures [6]. Indeed, MPs solubilization can lead to a complex procedure which must be carefully optimized in order to avoid protein and activity losses [2].

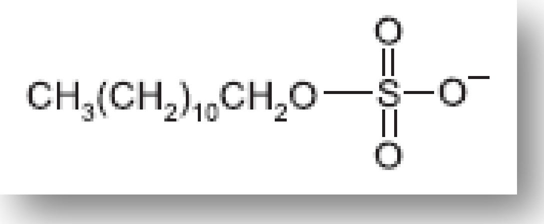
1.1.1 **Membrane proteins environment mimetization - Solubilization**

The *in vitro* studies such as crystallization are dependent of the successful solubilization or MPs reconstitution [7]. The use of detergent maintains the stability and function of the protein [4, 7]. A major difficulty in handling with MPs is that they are naturally incorporated into the lipid bilayer, which requires their removal [7]. This fact limits the application of conventional techniques used to determine and identify structure-function relationships such as NMR, X-ray crystallography and circular dichroism [6, 7]. Then, regarding the complexity of lipid bilayer is highly desirable to transfer proteins from the cellular membrane to an environment easier to handle, for further experimental studies [7]. So, the application of general methodologies are frequently restricted since they require the protein extraction from its native membrane and its subsequent solubilization with detergents or in a lipid native environment [7].

An effective solubilization with detergents or other hydrophobic compounds is necessary due to MPs requirement of be surrounded by a hydrophobic environment [7]. Indeed, solubilization is one of the most critical steps in the preparation of MPs and is effective only when occurs the dissociation of most lipid-protein and protein-protein interactions, separating the protein from its native environment [2]. In order to promotes this separation, the transference solution should include solubilizing components to satisfy the hydrophobic nature of transmembranar segments and to remove the MP of interest from their natural environment a aqueous medium [2, 6, 7].

Several approaches have been developed in order to fulfill these requirements [7]. The methods currently available to for solubilize and reconstitute MPs included typically the use of detergents. The detergents are amphipathic compounds that consist in a polar head group (hydrophilic) and a non-polar tail (hydrophobic). The polar part may present different ionic characters, providing specific classifications, namely, non-ionic, anionic, cationic or zwitterionic detergents [2, 7]. The hydrophobic surface areas of membrane proteins, and "tails" of the lipid are incorporated into the hydrophobic interior of the detergent micellar structures, while the hydrophilic parts of the protein are in contact with the aqueous medium [2, 7]. Thus, detergents act in order to disrupt the lipid bilayer, incorporating lipids and proteins into detergent micelles, mimicking its natural environment [2]. In general, the physical properties and behavior of detergents in solution can be described by several particular proprieties but the most relevant is the critical micellar concentration (CMC). The

CMC is defined as the concentration of detergent in aqueous medium in which detergent molecules are associated to form multimolecular complexes and micelles, with hydrophobic cores and hydrophilic surfaces [2, 7]. The CMC differs between detergents and also varies with temperature, pH and ionic strength [7]. So, the individual CMC values can be used to choose the optimum (minimum) concentration of a specific detergent [6]. In fact, all buffers used for solubilization, purification and storage of MPs should have a detergent concentration above the CMC [2]. Detergents can be divided in four major categories, the nonionic detergents, bile acid salts, ionic detergents and zwitterionic surfactants [2, 7, 10]. The ionic detergents contain a hydrophobic hydrocarbon chain and a polar head group, which has a net charge that can present a cationic or an anionic character [7]. Ionic detergents such as sodium dodecyl sulfate (SDS), represented in Figure 1, are highly effective in solubilization of membrane proteins but generally led to denaturation a great extension [6, 7, 11]. However, some proteins can be renatured after SDS step [7]. For example *E. coli* diacylglycerolkinase (DAGK) [11] was renatured by transfer the protein from SDS solution to a less denaturant buffer with others detergents or lipids [7]. However, occasionally the removal of SDS can often lead to an irreversible aggregation and precipitation of the protein under study [7].



$$CH_3(CH_2)_{10}CH_2O-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-O^-$$

Figure 1 – Structure of an ionic detergent type, Sodium Dodecyl Sulfate (SDS) (adapted from [7]).

The salts of bile acids, represented in Figure 2, are another ionic detergent but differ from SDS structure, once its backbone consists in rigid steroidal groups [7, 10]. As a result, bile acid salts form small kidney-shaped aggregates unlike the spherical micelles traditionally formed by detergents with ionic linear chains [7]. The bile acids are mild and, generally, non-deactivating detergents, offering a suitable alternative to ionic detergents with the same head group [7].

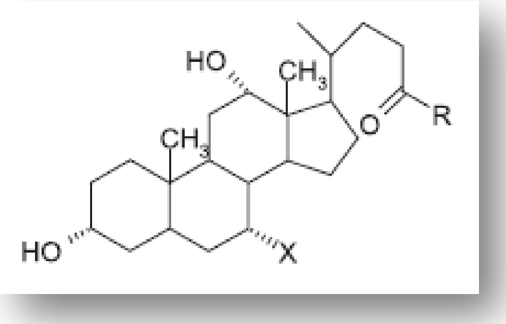


Figure 2 – Structure of bile acid salts (adapted from [7]).

The non-ionic detergents (Figure 3) contain an uncharged hydrophilic head group instead of a charged group [2, 7]. They are generally considered mild detergents and relatively non-denaturing because they break lipid–lipid and lipid–protein interactions rather than protein–protein interactions [7]. Thus, non-ionic detergents improve MPs solubilization without affecting its structural characteristics, allowing biomolecule isolation in its biologically active form [7, 10]. However, the nonionic detergents with short chains (C7-C10) such as n-octyl-ß-D-glucopyranoside (OG) can often lead to deactivation of MPs, unlike their intermediate (C12-C14), a chain derivative [7]. On the other hand, n-dodecyl-ß-D-maltoside (DDM) has become the non-ionic detergent more widely used for the solubilization of MPs, presenting good results in maintenance of MPs , namely, functional and structural properties [7].

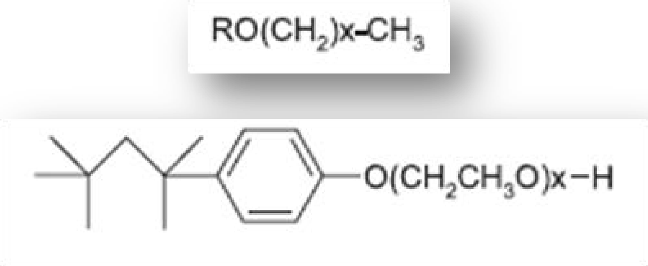


Figure 3 – Structure of non-ionic detergents (adapted from [7]).

The zwitterionic detergents (Figure 4) combine the properties of ionic and non-ionic detergents [6]. Despite zwitterionic detergents being more deactivating than non-ionic detergents, its application is common in MPs structural studies [6, 7]. Few examples include the use of N-dodecyldimethyl amineoxide (DDAO) in the crystallization of the reaction centre of *Rhodopseudomonas sphaeroides* [7].

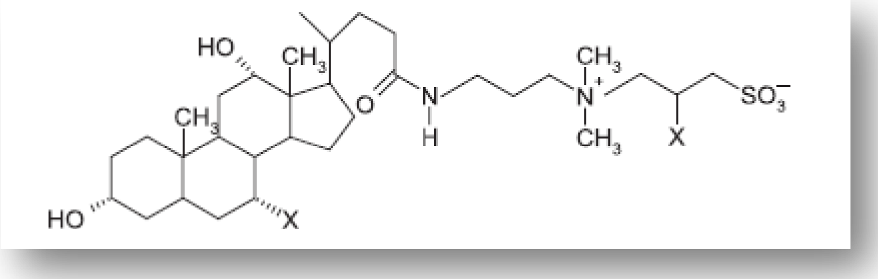


Figure 4 - Structure of zwitterionic detergents (adapted from [7]).

Instead of the application of traditional detergent in MPs solubilization, new solubilizing agents, with less destabilizing effects have been developed [7]. For this purpose, a new class of compounds such as tripod amphiphiles and amphipols or some mixed lipid–detergent systems have been tested for MPs solubilization. The tripod amphiphiles consist in a tetrasubstituted carbon atom carrying three hydrophobic tails and a polar head group [7, 12]. These compounds limit the length and flexibility of hydrophobic portions of MPs, which may be involved in its inactivation [7]. However, tripod amphiphiles has been successfully used in the solubilization of bacteriorhodopsin and bovine rhodops [12], maintaining the protein in its native monomer form for several weeks [7]. The amphipols, another class of new solubilizing agents, was designed to involve the hydrophobic portion of proteins and expose their hydrophilic backbone to the aqueous environment [7, 12, 13].

Also, MPs may be solubilized into detergent micelles/lipid mixtures [7, 14]. In these systems, hydrophobic regions of the protein are solvated by non-polar groups available in lipid dispersed solution [7,14]. When certain detergents, such as 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) or l-a-1,2-dihexanoylphosphatidylcholine (DHPC), are involved with short-chain lipids, such as 1,2-dimyristoylsn-glycero-3-phosphocholine (DMPC), with the right composition and temperature, bilayer structures known as bicelles can be formed [7, 15]. Bicelles formation required lower detergent concentrations than traditional mixed micelles and possess certain characteristics that makes it more advantageous for functional MPs solubilization [7]. However, this solubilizing agent can interfere in NMR, circular dichroism and crystallography studies [7, 15].

An ideal environment, for MPs *in vitro* studies should be as similar as possible to natural lipid bilayer that surrounds MPs *in vivo* [6, 7]. Despite this, due to the complexity of the bilayer, this environment becomes impossible to mimics [6, 7]. However, a suitable solubilization strategy should be designed to stabilize MPs, through the use of new solubilizing agents, lipids or appropriate detergents [7]. In fact, a combination of detergent and lipid can often prove to be useful in NMR and crystallization experiments [7].

1.2 Purification of membrane proteins

After a successful solubilization, it is recommended to proceed immediately to the purification step in order to minimize the decrease and loss of membrane protein activity due to aggregation, complete loss of terciary/quaternary structure or proteolytic degradation [2]. The high purity of a specific protein is crucial to resolve its molecular structure and assess its bioactivity [16]. Normally, the range of separation techniques available is limited and in the majority of experiments attempts for the full MPs isolation failed and only a partial purification can be achieved [6]. So, in order to design a successful chromatographic technique, the biochemical properties of target protein must be exploited [17]. The molecular adsorption in the surface of a solid is the basis of the adsorptive chromatography used for bioseparation [18].

Proteins adsorption/desorption is a complex process, generally constituted by five sequential steps: diffusion of the protein from the mobile phase into the ligands surface, protein adsorption on the surface transition, conformational changes of the adsorbed protein, protein desorption and diffusion of the protein back into mobile phase [18]. The orientation of the protein also has significant effects in its interaction with the surface, once protein surface is usually quite heterogeneous, with hydrophobic and hydrophilic regions [18, 19]. For example, when the hydrophobic region, located on protein surface, is close to the hydrophobic ligands, the protein is attracted to chromatographic support [18]. However, if the hydrophilic region of protein, is nears hydrophobic ligands the protein will be repelled [18].

1.2.1 Preparative Chromatography

The chromatography is a powerful separation method that was initially developed for extraction and purification of complex mixtures of vegetal origin [20]. Nowadays, many chromatographic techniques are available, such as Ion Exchange Chromatography (IEC), Hydrophobic Interaction Chromatography (HIC), Affinity Chromatography (AC), Reverse Chromatography and Gel Filtration [2, 20–23].

Currently, chromatographic processes can be differentiated in two types, the analytical and the preparative chromatography [1]. In analytical chromatography complex mixtures are separated in order to identify and quantify one or more specific components that after signals acquisition are discarded [20]. This purpose may involve the use of simple sensors (e.g. UV) or complex detectors (e.g. mass spectrometry) [20]. So, the principal aim of analytical chromatography is the rapid detection of specific components through the direct signal acquisition in order to calculate its concentration by a calibration curve [20] . On the other hand, in preparative chromatography a specific component is isolated from a complex mixture [20]. The preparative separation requires the injection of large quantities of sample in the column in order to obtain the maximum performance [20].

So, independently of the type of chromatography used, components of a complex mixture are preferentially distributed in stationary phase or in mobile phase, according to their physic-chemical properties [2, 24]. Typically, an insoluble matrix is used as stationary phase is packed into a column and in mobile phase is pumped through the system [24]. Generally, the chromatographic step compromise five major stages: phase equilibrium, injection of sample, washing of non-retained species, elution of molecules adsorbed in matrix and regeneration (Figure 5) [2, 24]. The equilibrium of the column ensures that initially all the stationary phase remains in same optimal conditions for binding the target biomolecule [24]. The sample injection consists in insertion of a certain quantity of a complex mixture, which contains the target protein, into a stationary phase [24]. After injection, sample crosses the column by the continuous addition of mobile phase and, according their affinity to each phase, the constituents may remain in mobile phase or be distributed in stationary phase [24]. In the washing stage, impurities that not interact within stationary phase are removed from the column using the same buffer of column equilibrium [24]. 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 [2, 24]. During this stage, the proteins strongly adsorbed move more slowly through the column than the weakly bound biomolecules, so their elution takes more time [24]. The regeneration step is a very important cleaning process that maintains the binding capacity, selectivity and lifetime of the chromatographic support [24]. After each chromatographic step, precipitated and denatured proteins and other biomolecules or salts may remain in matrix. So these molecules must be removed in order to maintain the native characteristics of the support [24]. The cleaning procedure depends on matrix type but in general are used highly acid or basic solutions, low or high-salt concentrated solutions, organic solvents or detergents [24].

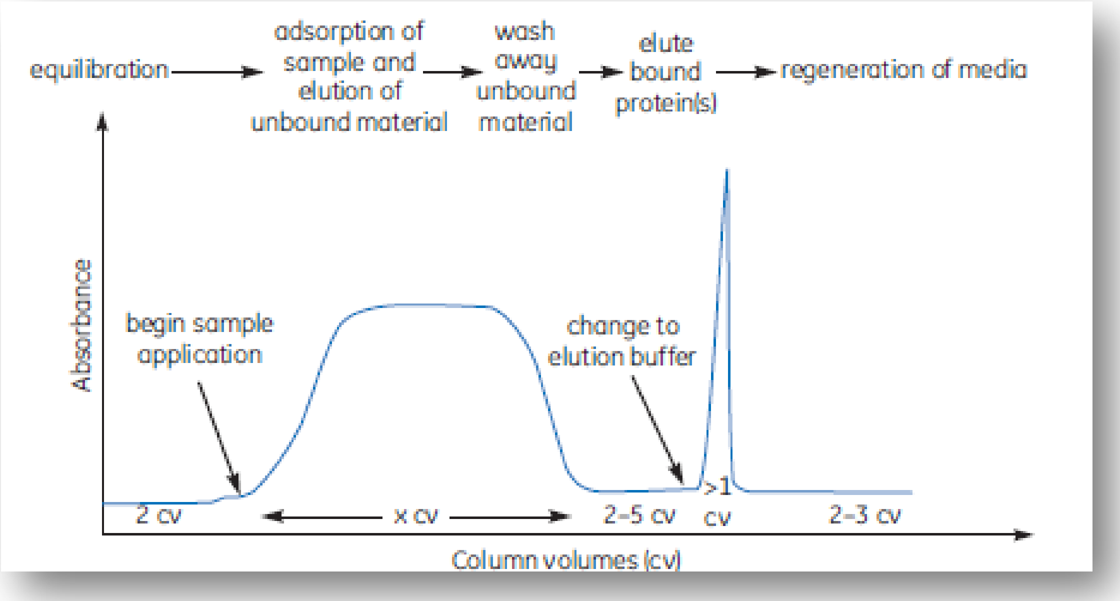


Figure 5 - Phases of a typical chromatographic procedure (adapted from [2]).

1.2.2 Chromatographic methods for MP's purification

The permanent requirement to understand the structure, function and properties of MPs encourages the design of new separation strategies [25]. The choice of a suitable purification technique depends on location, characteristics and desired purity of the target protein [26].

As is well known, each protein has unique physicochemical properties which make possible their isolation from complex mixtures if a suitable chromatographic purification process was designed [26]. However, the MPs purification is quite difficult, due to a great tendency to form aggregates even in the presence of detergents, reducing the efficacy of all separation techniques [6]. Moreover, remove MPs from their lipid environment can led to losses of catalytic activity during purification process [6]. Thus, it becomes important to maintain a minimum number of steps during a purification procedure to maximize the yields of the process, quality, purity and to avoid irreversible losses of activity [26].

Usually, various chromatographic procedures can be applied in MPs purification. The AC is a high-resolution technique commonly used in MPs isolation, separating proteins based in highly specific biological interactions between the protein and an affinity ligand, providing elevated selectivity [2, 6, 23]. These methods require often the addition of an affinity tag to protein during production step, which facilitates target protein binding to chromatographic matrix [26]. Consequently, affinity tag removal is necessary after the purification step, which usually implies a significant reduction in process yield and irreversible activity losses [26]. Nevertheless, there are characteristics that make AC an optimum bioseparation method for purifying MPs such as; allows use of a wide variety of buffers in order to obtain the stability of the protein, provides an optimal separation at moderate concentrations (NaCl), reducing the MPs aggregation MPs and permits the use of neutral detergent which does not interfere with the binding and elution of the protein [6].

The 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 [2, 27, 28]. This affinity results of reversible linkages formed between a metal ion chelated and certain groups in amino acids naturally presented or in residues of tags incorporated biotechnologically in target protein [27, 29].

Recently, a highly active membrane multiprotein complex was purified after use of Histidine-tagged [6]. Also, the purification of chloroplast ATP synthase (membrane protein complex) [6] and Mammalian Serine Palmitoyltransferase [8] was achieved through affinity techniques.

The HIC is a widely applied technique in the purification of biomolecules [26]. The HIC separates proteins with differences in hydrophobicity and is based on the reversible interaction between a protein and hydrophobic surface in a chromatographic medium [2, 16].

However, HIC applications to MPs isolation are uncommon, since detergents, binds very strongly to the matrix, preventing target protein binding [6]. However, some membrane proteins have been isolated by this technique, such as cytochrome F-450 [6] and hMBCOMT, evaluating the performance of different hydrophobic ligands [30].

In addition, size exclusion chromatography or gel filtration is also applied to MPs purification, in which fractionation is entirely based on molecular size [2, 16, 25]. The size exclusion chromatography is considered appropriate for a final purification step, such as polishing and final desalting in a downstream bioprocess [2, 6, 16].

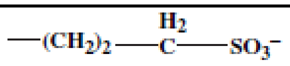
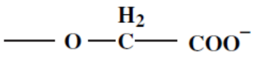
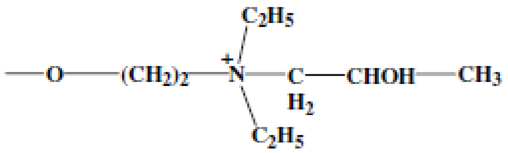
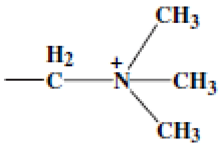
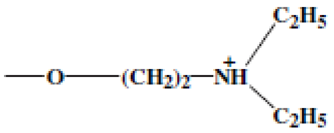
This technique was the main advantage that can be employed for the purification of any kind of protein and allowed that target protein retains its bioactivity since no molecular interactions are established [16]. But, the resolution is very low, and is able to distinguish proteins with small differences in their molecular weight [6, 16]. In literature, there are references to the application of size exclusion chromatography to cytochrome bc_1 complex as final polishing step [6].

1.2.3 Anionic Interaction Chromatography

The IEC is one of the most powerful and common techniques used in the purification of proteins, both for soluble proteins and MPs [1, 31]. The IEC allows the separation of biomolecules with high degree of resolution according to differences in their surface charge at specific pH value [2, 21, 31, 32]. This chromatographic procedure has revealed to be quite efficient, separating selectively proteins with minor differences in its charge, amino acids composition and with transactional modifications [16, 31]. Indeed, IEC is frequently a method of choice for isolating both soluble proteins and MPs for several reasons [6]. Firstly, a wide range of chromatography mediums, with different binding properties, are available at relatively low cost [6]. Additionally, high flow rates can often be achieved with mediums, allowing purifications in large scale [6]. Secondly, this chromatographic technique allows the use of any neutral detergents, which reduces the risk of MPs instability during the chromatographic step [6].

In general, one of the main advantages of this technique is the capacity to purify biomolecules with positive or negative charge [6, 21]. As most chromatographic techniques, IEC, requires a stationary phase, usually composed by hydrated insoluble polymers such as cellulose, dextran or Sephadex, to which is coupled an ion exchanger group, that can be cationic or anionic [21] . Some of ion exchanger groups commonly used IEC and respective chemical structures are shown in Table 1 [21].

Table 1 - Groups of ion exchangers used for the isolation of proteins (adapted from [21]).

Group	Chemical structure
Cation Exchangers	
S (Methyl Sulphonate)	 $-\overset{H_2}{C}-SO_3^-$
SP (Sulphopropyl)	 $-(CH_2)_2-\overset{H_2}{C}-SO_3^-$
CM (Carboxymethyl)	 $-O-\overset{H_2}{C}-COO^-$
Anion Exchangers	
QAE (Quartenary Aminoethyl)	
Q (Quarternary Ammonium)	
DEAE (diethylaminoethyl)	

Cation exchangers interact with positively charged molecules, due to the presence of anionic ligands in its surface. Anion exchangers are coupled ligands with positive charge capable to establish reversible interactions with negatively charged biomolecules and negatively charged counter ions (Figure 6) [6]. For example, counter ion Cl^- of the anion exchanger DEAE has a weak binding and may be substituted by anions with high affinity, such as negatively charged amino acids on protein surface (Figure 6) [6]. So, the basis of IEC is that charged ions can be freely exchanged by others ions with the same charge [21]. Other types of counter ions can be used depending of it level of affinity with anion or cation exchanger (Figure 7). The counter ions with high-affinity may be used for tightly binding proteins [6].

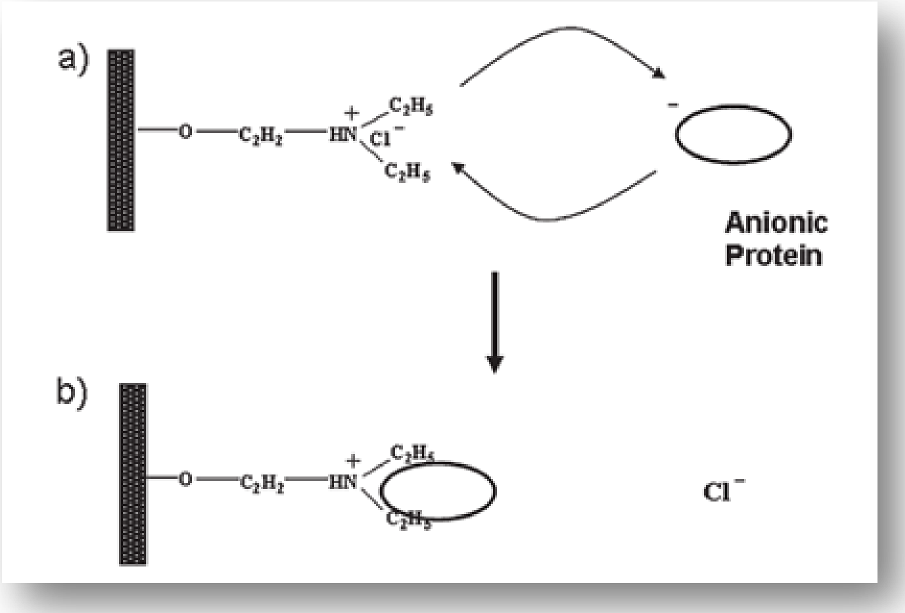


Figure 6 - Anionic Exchange Chromatography: a) Anionic proteins (negatively charged) state exchange with chloride ions; b) the protein establishes electrostatic bonds with groups DEAE - cellulose (adapted from [21]).

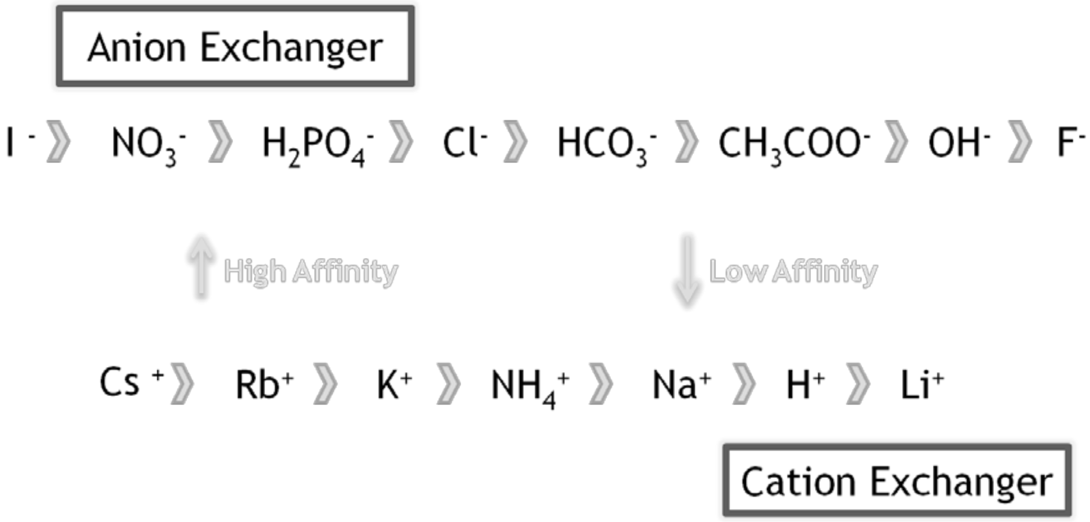


Figure 7 - Scale of counter ions with high affinity or low-affinity relatively to anion/cation exchanger applied (adapted from [6]).

The ion exchangers can be classified as strong and weak exchangers based on the difference between the functional groups [6]. 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 [6]. An example of a weak anion exchanger is usually diethylaminoethyl (DEAE), whereas a strong anion exchanger is, for example, the diethyl-2-hydroxypropylaminoethyl (QAE). Weak cation exchangers are constituted by carboxymethyl (CM) groups, while strong cation exchangers are characterized by sulfo, sulfomethyl, or sulfopropyl groups [6, 21].

Typically, DEAE has been used for purification of MPs such as the cytochrome bc1 complex using moderate salt concentrations [6]. Moreover, porins from *Mycobacterium smegmatis* [8], quinol: fumarate reductase [6], rat zinc transporter [8] and a rat MBCOMT (only partially) [8] were isolated using IEC.

Generally, in IEC the samples are loaded into column at low ionic strength and proteins charged interact with the oppositely charged ligands, while molecules with similar charge are eluted (Figure 8) [2]. Then, for elution of the adsorbed proteins, chemical conditions of mobile phase must be changed [2]. The elution is usually performed by increasing the salt concentration or suitable modification of pH [2], being salt application the most common [24]. This changes can be made gradually using a gradient as elution step, a stepwise strategy or combination of both [2].

An increased ionic strength reduces the degree of ionization of ion exchanger and protein, resulting in a lower effective binding. This effect is dependent of the counter ion applied [6]. For example, using NaCl, the counter ion Cl^- will compete with the protein for binding in the matrix and will progressively replace it, allowing protein elution [6]. In elution step, weakly bound proteins are remove firstly, followed by the strongly bound proteins, the proteins with greater net negative charge [21].

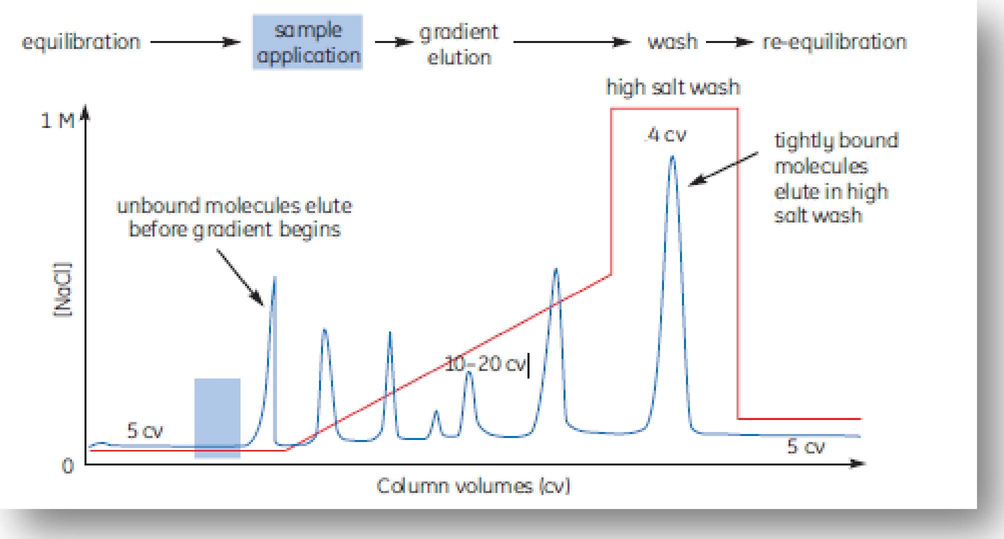


Figure 8 - Typical chromatographic profile obtained from anion exchange chromatography (adapted from [2]).

Typically in IEC the net surface charge of proteins vary with the operational pH [2]. When operating pH is above its isoelectric point (pI), the target protein will assume a negative net charge and will bind to an anion exchanger (figure 9) [2]. Consequently, the protein that has a more negatively net charge will be stay more time adsorbed on the column and will elute at last. On the other hand, if the pH is below of pI, the target protein will assume a positively net charge and will bind to a cation exchanger (Figure 9) [2]. Thus, the protein that has a more positively net charge in a protein mixture will be eluted at last, since that presents more affinity with the cation exchanger. So, different pH strategies can be applied to separate proteins with different pI values, as shown in Figure 9 [2].

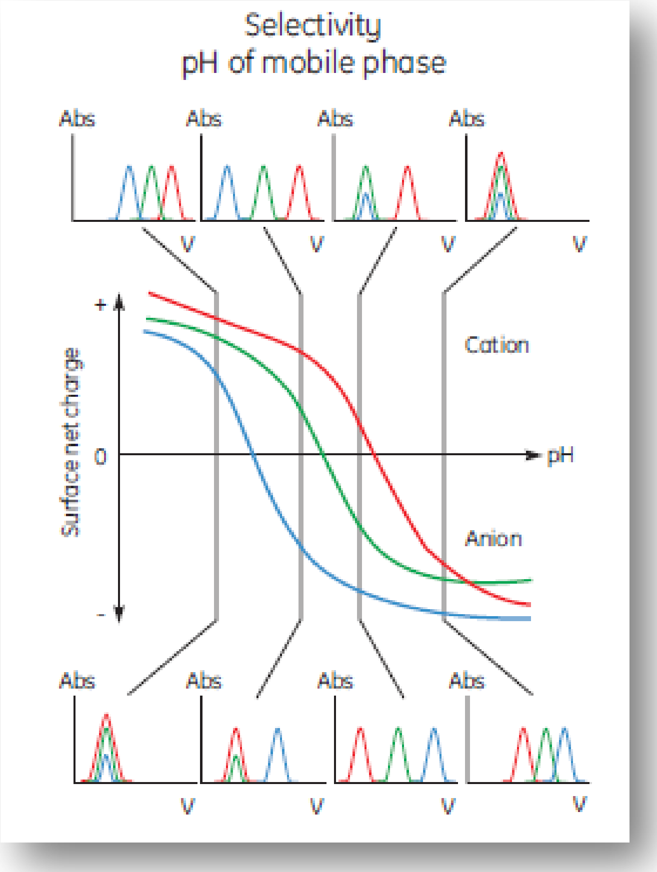


Figure 9 - Effect of pH on IEC elution strategies of various compounds (adapted from [2]).

1.3 The enzyme catechol-O-methyltransferase

The enzyme catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) was firstly discovered in rat liver extracts, and since then, it has been found in plants, fungi, invertebrates and vertebrates [8, 33, 34]. In mammals, the higher COMT activity levels have been found in liver, kidney and gut wall [33, 35].

COMT is a magnesium-dependent enzyme that catalyzes the transference of a methyl group from S-adenosyl-l-methionine (SAM) to a hydroxyl group on a catechol substrate, having as reaction products an *O*-methylated catechol and S-adenosyl-L- homocysteine (SAH) (Figure 10) [4, 8, 35–39].

According to Figure 10, in the first step of reaction SAM binds to COMT, forming a stable complex. In the second step, the divalent metal cation bind to the complex, specifically by coordination of various acid residues in COMT, no prevailing a direct interaction between the magnesium and cofactor SAM [36]. In the last step, catechol substrate coordinates with the metal and establishes a network of hydrogen bonds with the active site residues [36]. After the formation of this complex, the methyl group of the cofactor SAM is ready to be transferred towards the hydroxyl group of the substrate [36]. This reaction mechanism has been reinforced when the structure of soluble COMT (Figure 11) in rats and humans was crystallized in the presence of SAM, Mg (II) and the substrate analogue, 3,5-dinitrocatechol [34, 36].

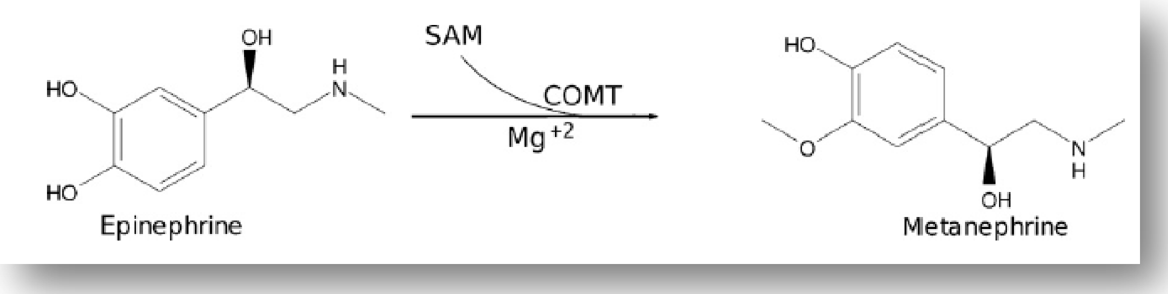


Figure 10 – A typical reaction catalyzed by catechol-O-methyltransferase (COMT). SAM - S-adenosyl-l-methionine; Mg^{2+} - Magnesium (adapted from [38]).

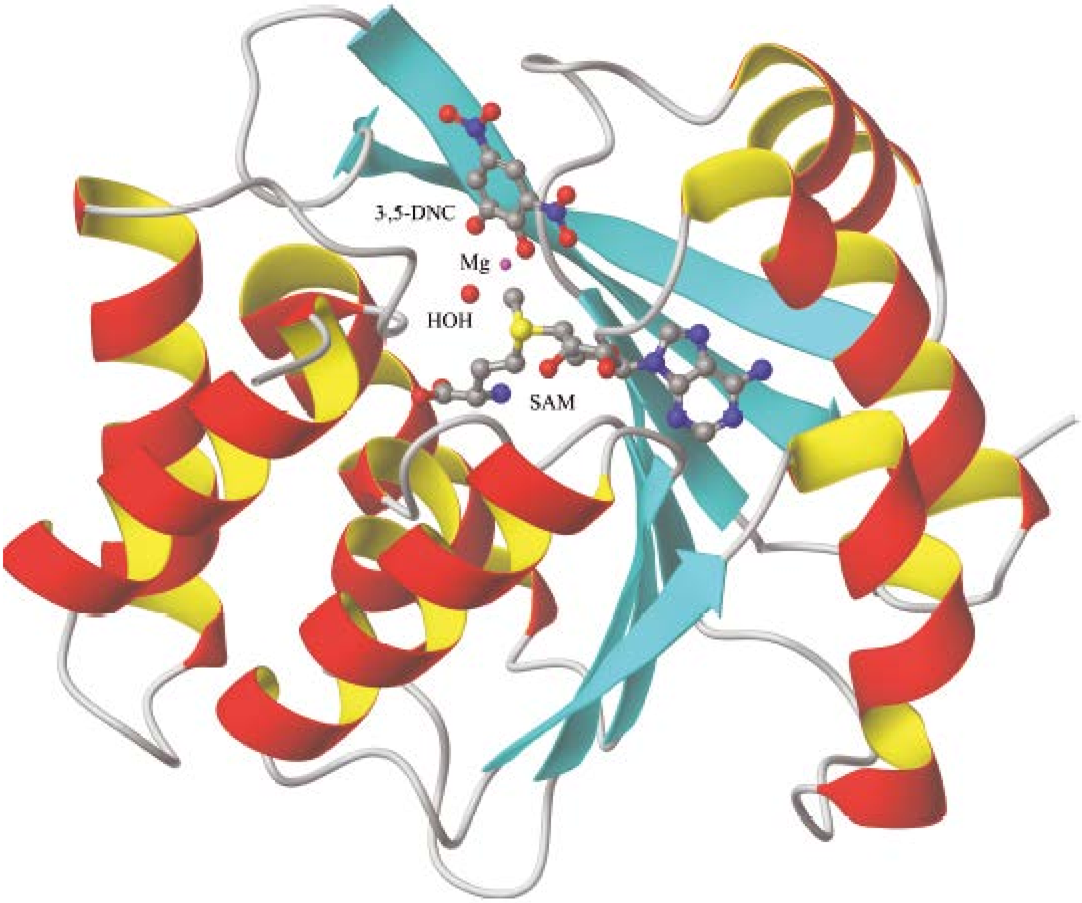


Figure 11 - Schematic representation of the three-dimensional structure of COMT. The S-adenosyl-L-methionine co-substrate (SAM), the 3,5-dinitrocatechol (3,5-DNC), the magnesium ion, and coordinated water molecules are depicted. SAM - S-adenosyl-L-methionine; Mg – magnesium; 3,5-DNC - 3,5-dinitrocatechol (adapted from [34]).

1.3.1 Physiological Functions

COMT is an important target for biotechnological engineering due to their role not only in normal brain function but also to their possible role in human neurological disorders such as Parkinson's disease (PD), schizophrenia, depression [26, 35, 38] and Alzheimer's disease [38].

In general, COMT physiological function is the inactivation of biologically active or toxic catechols [8]. Specifically, a major role of COMT includes its participation in the inactivation of the neurotransmitters in the central nervous system and regulation of both dopaminergic and noradrenergic systems. Additionally, COMT acts as enzymatic barrier, limiting the access of toxic catecholamines to certain biological compartments [33, 40]. The enzyme also acquires an important role in the metabolism of catecholestrogens and melanin and in the inactivation of toxic and carcinogenic compounds [8, 33]. The metabolic role of COMT is not restricted to the inactivation of neurotransmitters [18] but also includes the inactivation of neuroactive xenobiotics that have a catechol group, and regulation of its quantity in brain and other organs [36]. Indeed, catecholamines regulation is quite important to the survival and well-being of the body [36].

The malfunction of COMT and its polymorphisms has been associated to several medical conditions, such as Parkinson disease, anxiety, substance abuse and schizophrenia [26, 35, 36, 38]. Thus, COMT role in regulation of catecholamines levels of in tissues, becomes an important target for various pharmaceutical drugs [36].

1.3.2 Isoforms: SCOMT and MBCOMT

In nature, COMT can be found in two molecular forms: a soluble form (SCOMT) and a membrane-bound form (MBCOMT) [26, 35]. In humans, SCOMT, the predominant isoform, is a non-glycosylated protein that contains 221 amino acid residues and has a molecular weight of 24.7 kDa. The membrane isoform, associated to endoplasmic reticulum, has an additional peptide of 50 amino acids in its amino terminus besides of 221 residues of the soluble isoform, corresponding to a final molecular weight of 30 kDa [4, 35, 38, 41]. This additional peptide contains a stretch of 21 hydrophobic residues, which comprises a membrane anchor region of the enzyme [4, 35].

Despite of similar characteristics of both isoforms, MBCOMT has a higher affinity for catecholamines substrates and a lower reaction rate, when compared with soluble isoform [4]. Indeed, MBCOMT has a 100-fold higher affinity for catechol substrates than SCOMT [8]. Therefore, this suggests that MBCOMT plays an important role in metabolism of catecholamines at physiological concentrations *in vivo* [4]. However, for the catechol estrogens, both isoforms present similar km values [4].

Concerning its structure, COMT is composed by a β chain of seven sheets, trapped between two sets of helices α, as seen in Figure 11 [23, 34]. The enzyme active site consists in a S-adenosyl-l-methionine binding domain and some amino acids extremely relevant for the binding of substrate, water and Mg^{2+} [23, 34]. For example, amino acid residues such as Lys144 accept a proton of the hydroxyl group and residues such as Trp38, Trp143 and Pro174 form hydrophobic walls that define COMT selectivity for the substrate [23, 34].

1.3.3 Gene and localization of COMT isoforms

Both COMT isoforms are coded on a single gene but present different subcellular localizations [8, 33, 38]. Regarding its subcellular localization, SCOMT is present in cytoplasm while MBCOMT is associated to rough endoplasmic reticulum [8, 33, 38]. The MBCOMT, as an integral membrane protein, is associated to a membrane by its anchor region and with its catalytic portion oriented toward the cytoplasmic side of the membrane [8].

In humans and rats, a single gene with two distinct promoters encodes both COMT isoforms of COMT, and performs a translational regulation (Figure 12) [8, 33, 42]. The COMT gene is located on the long arm of chromosome 22 and is highly polymorphic [35, 38, 39, 42].

Through a translational regulation mechanism, COMT gene origins two primary transcripts using the two distinct promoters: an 1.5 kb transcript relative to MBCOMT and 1.3 kb transcript relative to SCOMT, from promoters P1 and P2, respectively.

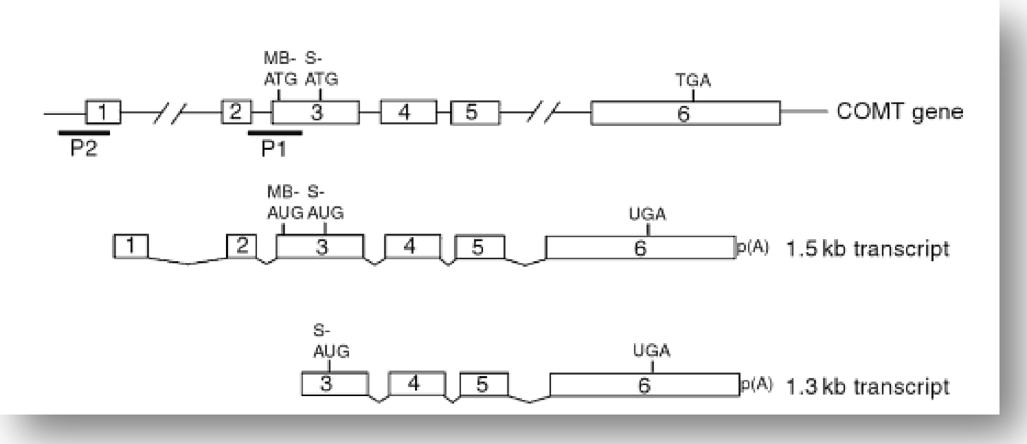


Figure 12 **-** The COMT enzyme gene and their transcripts (adapted from [42]).

1.3.4 Genetic polymorphisms

Genetic polymorphisms, both in upstream and downstream regions or in COMT gene itself, are been associated to a variety of neuropsychiatric phenotypes [38, 39]. The most studied and relevant is Val108Met polymorphism, associated to Parkinson's disease [41, 43], Alzheimer's disease [38] and others [35]. 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 [38].

Recent studies have reported the relation of the brain structure with COMT polymorphisms. It suggests that functional variants of COMT may affect the volume of the brain in patients with schizophrenia and even among healthy individuals [44].

Another COMT functional polymorphism is located at codon 158 causing a substitution of amino acid valine (Val) by methionine (Met) (Val158Met) [39, 40, 44–46]. Results in three genotypes (Val/Val, Val/Met and Met/Met) shows that polymorphism can affect differentially COMT stability and cognitive flexibility [40, 46]. The COMT-Met allele had an enzymatic activity 40% lower than the COMT-Val allele, which is associated to elevated dopamine levels [40]. Thus, the variant Val/Val developed higher enzymatic activity, increasing the efficiency of elimination of dopamine from synaptic cleft and, as result, decreasing it levels [40]. Therefore, COMT enzyme appears to play an important role in the metabolism of dopamine in prefrontal cortex and in other brain regions [39, 40].

1.3.5 Stability of COMT

The protein COMT is highly unstable and loses rapidly its activity during isolation and storage [22, 33, 35]. For example, the recombinant hSCOMT can lose 80% of its activity after 30 minutes at physiological temperature [47] and 50-70% when kept at 4ºC during 24 h [33]. Therefore, low COMT activities are associated with its thermal stability [48, 49]. In addition to the temperature, the pH is also essential to ensure maintenance of catalytic activity and specifically for COMT optimum pH is achieved between 7.5 and 8.0 [6]. Moreover, differences between both isoforms in terms of stability were been found in a variety of tissues [47].

The enzyme contains few cysteine residues in its primary structure and is suggested that in secondary structure hydrogen bonding with the serine hydroxyl group makes cysteine's particularly prone to oxidation [35]. Thus, 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 disulfide bridges [22, 35].

Some studies show that cofactor SAM and magnesium chloride ($MgCl_2$) reduce cysteine oxidation, preventing COMT inactivation, since these residues are essential to catalytic activity [35]. Additionally, experimental stability studies show that the combination of ethylenediaminetetraacetic acid (EDTA), ß-mercaptoethanol and $MgCl_2$ with a reducing agent such as dithiothreitol (DTT) have a stabilizing effect in all enzyme preparations [6, 22, 35]. Frequently, the protein stabilization is also achieved by the use of protease inhibitors such as EDTA and diisopropyl fluorophosphate (DFP), which are currently applied in a lab scale, since host proteases activity is a current problem during the purification of proteins [6]. Other types of stabilizers may have a stabilizing effect on proteins, such as sugars, divalent metals, glycerol and some amino acids (glycine and proline), protecting them against loss of activity and thermal denaturation [50, 51].

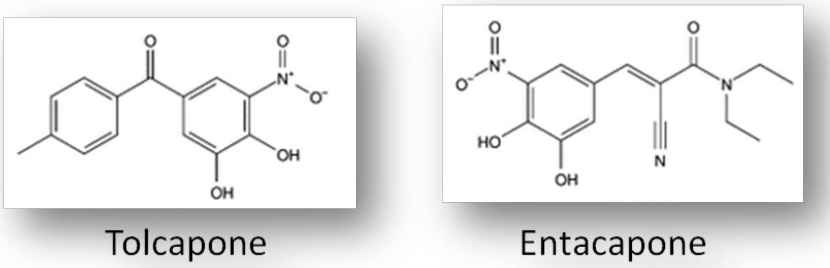
1.3.6 COMT inhibitors

The design of inhibitors has been investigated since COMT discovery in 1958 [52]. Nowadays, COMT inhibitors are used in Parkinson's disease therapy [26].

The first generation of COMT inhibitors such as tropolone, N-butylgallate and 2-hydroxylated estrogens presented a low efficacy and selectivity *in vivo*, little lasting effects and some toxicity [38]. The second-generation inhibitors include nitrocatechols as entacapone (OR-611), nitecapone (OR-462) and tolcapone (Ro 40-7592) [38]. The tolcapone and entacapone are currently used for the treatment of Parkinson's disease [26, 34]. These COMT inhibitors have beneficial effects in increasing the half-life of levodopa (L-Dopa), a drug used as substitute of dopamine [34, 38].

Since COMT discovery, many enzyme inhibitors have been developed and, nowadays, they are grouped as catechol-based COMT inhibitors (Type I), SAM-based COMT inhibitors (Type II), natural-products COMT inhibitors (Type III), bifunctional COMT inhibitors (Type IV), bisubstrate type of COMT inhibitors (Type V) and other types of COMT inhibitors (Type VI), according to similarities in its structure [38].

The type I class of inhibitors are based on catechol substructure and specific structural modifications resulted in a diverse range of compounds, including nitrocatechols, pyrogallol, salsolinol and polychlorinated biphenyl, among others [34, 38]. As mentioned above, tolcapone and entacapone (Figure 13) are clinically used for the treatment of Parkinson's disease [26, 53].



Tolcapone Entacapone

Figure 13 - Inhibitors of type I of class COMT inhibitors - Tolcapone and Entacapone, inhibitors currently used in the treatment of Parkinson's disease (adapted from [38]).

The inhibitors of type II are analogous to SAH, the demethylated product of SAM, which is a potent inhibitor of several methyltransferases (Figure 14) [38].

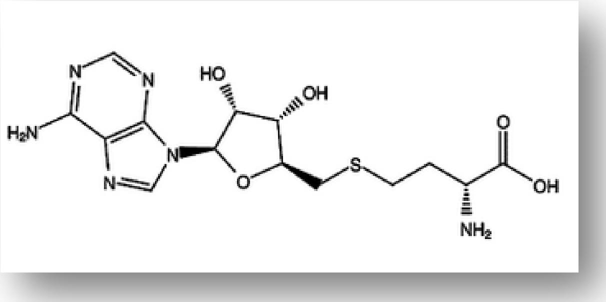


Figure 14 - Structure of S-adenosyl-L-homocysteine (SAH) (adapted from [38]).

The type III class of inhibitors (Figure 15) are natural product derivatives, including flavonoids that possesses great inhibitory action against COMT [38]. Also, four catechins found in green tea have showed inhibitory activity: epigallocatechin gallate (EGCG), (-)-epicatechin-3-gallate (ECG), epigallocatechin (EGC), (-)-epicatechin [38]. Also, coffee constituents such as chlorogenic acid, caffeic acid and caffeic acid phenethyl ester (CAPE) are effective inhibitors of COMT [38]. Additionally, natural products extracted from various plants, such as *Cistus parviflorus*, *Vitex agnus-cactus* and seeds of *Peganum harmala* have been reported due to their inhibition effects in COMT activity [54].

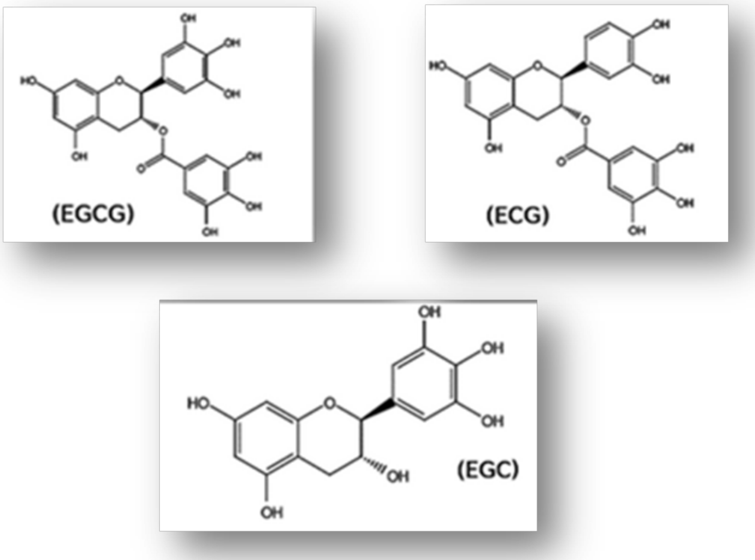


Figure 15 - Examples of Class III COMT inhibitors (adapted from [38]).

The type IV class of inhibitors (Figure 16) are duplicated substructures with capacity to interact with binding domains of the enzyme [38]. Some of this inhibitors are d-catechin, desmethylpapaverine and nordihydroxyguaiaretic acid [38]. Recently, bifunctional inhibitors were designed with dual substituted catechols and were mostly catechol derivatives such as 3,4- dihydroxybenzamide or 3,4,5-trihydroxybenzamide, linked by a spacer section consisting of various methylene units [34, 38].

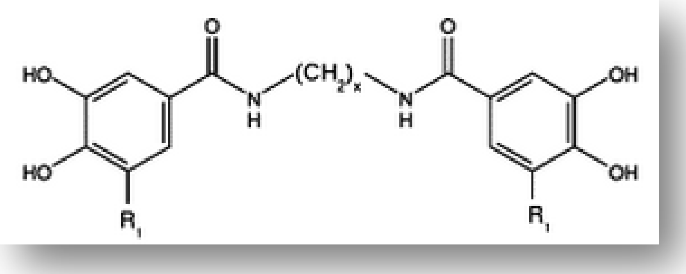


Figure 16 - Structure of two duplicates substructures - an example of Class IV inhibitors (adapted from [38]).

The type V class of inhibitors includes bisubstrate inhibitors, which are designed by replacing both the cofactor SAM and the catechol substrate (Figure 17) [34, 38].

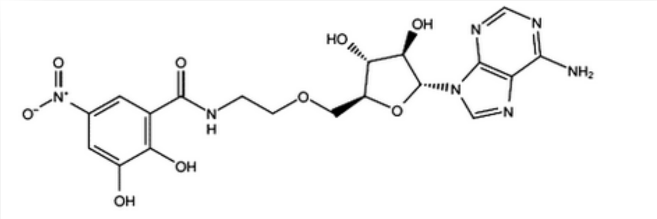


Figure 17 - Structure of the first substrate, inhibitor class of type V (adapted from [38]).

Finally, type VI of COMT inhibitors comprise all inhibitors that structurally are not related to any of the classes referred above [38]. These class includes tropolones (Figure 18), benzotropolones and many others inhibitors [38].

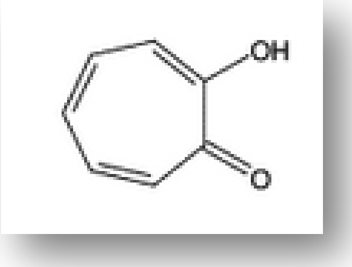


Figure 18 - Constitution of tropolone, an inhibitor of type VI (adapted from [38]).

1.4 Properties of COMT isoforms

The COMT has become an interesting biopharmaceutical target due to it association to some human disorders, particularly in Parkinson disease where the development of new COMT inhibitors lead to significant improvements in diseases prophylaxis [34, 53].

Initially, it was thought that MBCOMT was only an artifact, resulting from nonspecific binding of soluble form to cellular membrane [55]. However, MB-COMT represents a biochemically distinct molecular entity despite both isoforms share some similarities [55]. As referred above, MB-COMT has much lower capacity and Km value than the soluble form but a higher affinity to catecholamines [48, 55]. Thus, at low substrate concentrations, MB-COMT, a high affinity isoform, is probably the predominant isoform [55]. On the other hand, when substrate levels are raised above saturation, S-COMT activity increases and possibly prevails over MBCOMT activity [55].

Furthermore, there are other differences between the two isoforms such as pI values, which is 6.2 for MBCOMT and 5.2 for soluble form [55]. Although both COMT isoforms display considerable similarities specific differences in it kinetic and biochemical properties are evident [55].

1.4.1 Biosynthesis of hSCOMT and MBCOMT

The bacterial expression system is a challenging research area, although it has as advantages the relatively low cost and safety, when compared to eukaryotic systems such as mammalian cell cultures [26]. Indeed, expression systems such as *E. coli* have been extensively used as hosts for the expression of foreign proteins due to its rapid growth rate and capacity for continuous fermentation [26, 33].

As matter of fact, the recombinant hSCOMT was successfully produced in its active catalytically form in *E. coli* SG 13009 [33], DH-5 [33] and BL21-(DE3) Gold strains using

Isopropylthiogalactosidase (IPTG) as an inducible promoter [26, 56]. This recombinant system is an adequate host for expression of recombinant SCOMT since it is completely lacks endogenous COMT enzyme [33]. Indeed, with an optimized heterologous expression system in *E.coli* for recombinant hSCOMT production it was possible to produce and recover the active monomeric enzyme directly from cell crude culture using as lysis step freeze/thaw cycles or ultrasonication [26].

Furthermore, the expression of recombinant hSCOMT was also possible in insect cells using the *baculovirus* expression system [33].

Regarding hMBCOMT recombinant, several expression systems have been used to produce considerable amounts of the membrane enzyme for structural, function and pharmacological studies [4]. However, in some systems is impossible to express this recombinant protein, since its hydrophobic sequence can be toxic to bacteria [4]. Nevertheless, recombinant hMBCOMT was successfully produced in a catalytically active form and at relatively high levels in strains *E. coli* BL21 and SG 13009 [4].

Also other systems, have been described for recombinant expression of hMBCOMT, eukaryotic expression systems such as Sf9 insect cells [33], transfected human embryonic kidney fibroblast cell lines and hamster BHK-cells [4]. Despite of advantages associated, a drawback is the fact that hMBCOMT was expressed with low levels of biological activity [4].

The expression system based in *Brevibacillus choshinensis* cells is suitable to the production and secretion of heterologous proteins with high efficiency and presents a single membrane and low levels of protease activity [4]. *Brevibacillus choshinensis* is a gram-positive microorganism that shows an excellent capacity to produce several extracellular proteins [57]. In addition, this expression system allows the secretion of heterologous proteins with high efficiency and, as it produces a negligible amount of extracellular proteases, target bioproducts remain unscathed in the culture medium [57]. In sum, this system presents several advantages, becoming a system amenable to genetic engineering [57].

Nowadays, a rapid grow of knowledge in area of biogenesis of MPs has created new possibilities to design new strategies for improving yields in terms of host performance and protein production levels [4]. *Brevibacillus* recombinant expression system allows the production and recovery of human recombinant MBCOMT in a single procedure with a biological and immunological active form [4].

1.4.2 Purification of hSCOMT and MBCOMT

Over the past years, the human SCOMT has extensively studied and subjected to numerous purification procedures [22, 23, 26, 33, 47, 58, 59]. The SCOMT was subject to chromatographic processes such as hydrophobic interaction chromatography [22, 26, 47], bioaffinity chromatography [23, 33], size exclusion, anion and cation exchange chromatography [33, 58, 59].

Anionic exchange chromatography (AEC), a well-established technique widely used in isolation of soluble and membrane proteins (MPs) [6, 31], was one of the most usual chromatographic techniques applied to SCOMT isolation [33, 58, 59]. Although some of these purification procedures presented as drawback significant COMT activity and yield losses, some of their led to the successful resolving of SCOMT atomic structure [33]. Additionally, usual combination of AEC with others purification steps contributed to reinforce these issues [26, 33].

Regarding to hMBCOMT, some through kinetic and biochemical characterization has been carried out [34, 55], but few studies have been published concerning it isolation and so its structure remains unknown [4, 30, 34, 48, 60].

Recently, some work was developed on MBCOMT purification by AEC, where the authors describe MBCOMT recovery from microssomal fraction of rat liver homogenates [8, 60]. Specifically, hMBCOMT was partially isolated from the microsomal fraction of rat liver homogenates using a Resource Q column [8]. This strategy involved hMBCOMT solubilization with Triton X-100 and its purification was also performed using Triton X-100 and an increased sodium chloride concentration gradient for target protein elution [8].

Also, a new purification strategy were studied for recombinant human COMT (hMBCOMT) isolation from crude *Brevibacillus choshinensis* cell lysates, comparing different hydrophobic ligands such as octyl, butyl and epoxy [30]. In case of octyl and butyl ligands, the hMBCOMT's adsorption was performed at moderates salt concentrations, whereas on epoxy was used higher concentrations for their adsorption [30]. In addition, the hMBCOMT elution in octyl and epoxy was promoted using Triton X-100 and in butyl media besides the use of Triton X-100 was used a monosodium phosphate decrease [30].

In this work, chromatographic conditions required for each isoform isolation were compared and the results show that, using the same hydrophobic resins, lower ionic strength is required for MBCOMT's adsorption than for SCOMT [22, 30].

So, similarly to hMBCOMT's behavior on chromatographic hydrophobic adsorbents [30], it's likely that the additional amino acid residues in membrane anchor region domain, may contribute for the reinforcement of electrostatic interactions establish between MBCOMT and anion exchangers when compared with soluble isoform. Thus, it may be interesting to study the differences between both isoforms in terms of ionic interactions that can be established between each COMT isoform and an ionic exchanger, in a chromatographic procedure.

In this work it is intended to study, compare and assess the chromatographic behavior of both COMT isoforms using quaternary ammonium (Q-sepharose) as anion exchanger, in order to analyze Q-Sepharose performance in terms of binding and elution conditions for hSCOMT and hMBCOMT recovery. Furthermore, is also intended to determine enzyme recovery, activity and procedure yield by HPLC analysis.

Chapter II

Materials and Methods

2.1 Materials

Ultrapure reagent-grade water for Fast Performance Liquid Chromatography system (FPLC) and ÄKTA™ avant was obtained with a Mili-Q system (Milipore/Waters). Neomycin (trisulphate salt hydrate), Carbenicillin disodium salt, isopropylthiogalactosidase (IPTG), tryptone, Bacto yeast extract, Glucose, Calcium chloride dehydrate, lysozyme, Deoxyribonuclease (DNase), dithiothreitol (DTT), Cysteine (L-), Sucrose, DL-Metanephrine Hydrochloride (MN), citric acid monohydrate, 1-Octanesulfonic acid and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium chloride and sodium acetate (anhydrous) were supplied by Fluka (Buchs, Switzerland). Bacto soytone and polypeptone were obtained from Becton Dickinson (NJ, USA). Tris(hydroxymethyl)aminomethane (Tris) and Acetonitrile (HPLC grade) was obtained from Fisher scientific (Epson, United Kingdom). Sodium dihydrogen phosphate (NaH2PO4) and Glycerol were obtained from Riedel-de-Haën (Hanover, Germany) and Himedia (Mumbai, India), respectively. Sodium chloride (NaCl) and perchloric acid was purchased from Panreac (Barcelona, Spain). NZYcolour Protein Marker II used for estimation of subunit molecular weight was purchased in NZYTech (Lisboa, Portugal). Anti-rabbit IgG alkaline phosphatase secondary antibody were purchased on GE Healthcare Biosciences (Uppsalla, Sweden). Monoclonal rabbit anti-COMT antibody was produced in BIAL (S. Mamede do Coronado, Portugal) using purified recombinant rat COMT. All chemicals used were of analytical grade commercially available and used without further purification.

2.2 Plasmids, bacterial strains and media

The Champion pET101 Directional TOPO expression kit (Invitrogen Corporation, Carlsbad, CA, U.S.A.) was used for the expression of hSCOMT on *E. coli* BL21 (DE3) strain according to the manufacturer's instructions and as previously described [26]. The SOB medium [20 g/L triptone, 5 g/L yeast extract, 0.5 g/L NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ with 50 µg/mL cabenicillin] was used for *E. coli* growth.

Plasmid pNCMO2-hMBCOMT was used for hMBCOMT expression on Brevibacillus expression system [Takara Bio Inc. (Otsu, Japan)] according to the manufacturer's instructions and as previously described [4]. Typically, *B. choshinensis* cells were grown in 2SYNM medium (20.0 g/L glucose, 40.0 g/L Bacto Soytone, 5.0 g/L Bacto Yeast Extract, 0.15 g/L $CaCl_2.2H_2O$ and 50.0 µg/mL Neomycin) and MTNm plates (10.0 g/L glucose, 10.0 g/L polypeptone, 5.0 g/L

Bacto Yeast extract, 10.0 mg/L $FeSO_4.7H_2O$, 10.0 mg/L $MnSO_4.H_2O$, 1.0 mg/L $ZnSO_4.7H_2O$, 4.1 g/L $MgCl_2$ and 50 µg/mL Neomycin for MTNm liquid medium; 3.75 g/L Agar and 10.0 µg/mL Neomycin).

2.3 Recombinant COMT isoforms biosynthesis and recuperation

The biosynthesis of recombinant hSCOMT was performed according to the following protocol. *E. coli* cells transformed with the expression construct were grown overnight at 37°C in LB medium plates containing 50 µg/mL cabenicillin. A single colony was precultivated in 62.5 mL of SOB medium in 250 mL shake flasks. Cells were grown at 37ºC and 250 rpm until the optical density at 600 nm (OD600) reached 2.6, corresponding to middle of log phase. This bacterial preculture was then diluted using fresh SOB medium (250 mL) in a 1 L erlenmeyer flask, in order to achieve an initial OD600 nm of 0.2–0.3 units. When the OD600 nm of culture reached 0.7, the production of recombinant hSCOMT was induced by the addition of IPTG in a final concentration of 1 mM. After a 4 h growth at 37ºC, cells were harvested by centrifugation (7000×*g*, 15 min, 4ºC) and stored frozen at−80ºC until use [26, 56].

The supernatant obtained from centrifugation were resuspended in 5 mL of lysis buffer (150 mM NaCl, 10 mM DTT, 50 mM Tris pH 8.0) with protease inhibitors (5 µg/mL leupeptin and 0.7 µg/mL pepstatin) and COMT stabilizers (75 mM DTT, 250 mM sucrose, 150 mM cysteine and 20% glycerol). In cellular lysis, lysozyme (10 mg/ml) was added to extract for 15 minutes at room temperature, followed by six freeze (-196 ºC in liquid nitrogen)/thaw (42ºC) cycles. Then, DNAse (1,0 mg/ml) was added to the lysate and the supernatant resulting from centrifugation (16000xg, 20 min, 4ºC) was stored until use [26, 56].

Unless otherwise stated, recombinant hMBCOMT was carried out according to the following protocol. The *B. choshinensis* cells transformed with the expression construct were grown overnight at 37ºC in MTNm plates. A single colony of transformed cells was precultivated in 62.5 mL of 2SYNM medium in 250 mL shake flasks. Cells were grown at 30ºC and 120 rpm until cell density at 660 nm (OD660) reached 2.6. After grown time, an aliquot of pre-inoculum was diluted using 2SYNm medium on 500 mL shake flasks, corresponding to an initial OD660 of 0.2 units in a final volume of 125 ml. After a 48h growth at 30ºC and 120 rpm, cells were harvested by centrifugation (5000xg, 25 min, 4ºC) and stored frozen at -20.0ºC until use [4].

The bacterial cell pellet obtained was incubated with 5 mL of lysis buffer (150 mM NaCl, 10 mM DTT, 50 mM Tris, 1 mM MgCl2, pH 8.0) complemented with protease inhibitors (5.0 µg/ml leupeptin and 0.7 µg/ml pepstatin) and COMT stabilizers (75 mM DTT, 250 mM sucrose, 150 mM cysteine and 20% glycerol), disrupted by lysozyme treatment (10 mg/ml) for 15 minutes at room temperature and followed by six freeze (-196 ºC in liquid nitrogen)/thaw (42ºC) cycles. Then, DNAse (1,0 mg/ml) was added to the lysate and the soluble material removed by centrifugation (16000xg, 20 min, 4ºC). Full solubilization was carried out by pellet incubation with lysis buffer at 4ºC, overnight.

2.4 Anionic Chromatography

The chromatographic assays were performed at room temperature (25°C) in an ÄKTA Avant system with UNICORN 6Software (GE Healthcare, Uppsala, Sweden) equipped with a 2 mL injection loop and in a FPLC system (GE Healthcare Biosciences, Uppsalla, Sweden) consisting of a double-piston pumps P500, an MV 7 injection valve equipped with a 2 mL injection loop, a fraction collector FRAC 100, a dual path monitor UV-1 and a data processor 112, controlled by a LCC 501 Plus controller. 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 sodium chloride concentration required for hSCOMT and hMBCOMT's retention. Unless otherwise stated, column was initially equilibrated with 10 mM Tris HCl.

Aliquots of recombinant hSCOMT containing supernatant (2 mL with a protein concentration 3,65mg/mL) were injected into column at 1 ml/min with 10 mM Tris-HCl. After elution of unretained species, the concentration of sodium chloride was increased to 350 mM in a step mode for 2.5 column volume (CV). Subsequently, sodium chloride concentration in mobile phase was increased to 1M (2.5 CV).

Solubilized pellet containing hMBCOMT (2 mL with a protein concentration 2.7mg/mL) was injected into the column at 1 ml/min with 10 mM Tris-HCl. The elution of unretained species occurred with an increasing sodium chloride gradient from 0 mM to 100 mM (2 CV). After elution of unretained species, sodium chloride concentration was increased to 300 mM in a step mode (2.5 CV). Subsequently sodium chloride concentration in mobile phase was gradually increased from 350 mM to 1M NaCl in 10 mM Tris buffer (2,5CV). Finally, a washing step was applied with 1M of sodium chloride in Tris buffer 10 mM (1CV).

Otherwise, in an alternative chromatographic procedure was used 0.5 % Triton X-100 in 10 mM Tris HCl in adsorption step, followed by a step at 300 mM NaCl in 0.5% of detergent. Thereafter, a final salt increasing gradient from 350 mM to 1M NaCl and 0.5% Triton X-100 in 10 mM Tris buffer was applied.

In all chromatographic runs, conductivity was continuously monitored, as well as absorbance at 280 nm. Fractions volumes of 3 mL were collected and pooled according to the chromatograms profile obtained and stabilized in a suitable solution composed by 75 mM DTT, 150 mM of cysteine, 250 mM sucrose and 20% of glycerol in an adequate buffer. Finally, samples were concentrated and desalted with Macrosep® Advance centrifugal devices with Omega™ membrane from VWR (Carnaxide, Portugal) and conserved at 4°C until further analysis.

2.5 Total protein quantification

Protein contents in samples were measured by the Pierce BCA Protein Assay Kit (Thermo Scientific, USA) using BSA as the standard and calibration control samples (5–2000 µg/mL).

2.6 COMT isoforms enzymatic assays

The hMBCOMT and hSCOMT activity levels were evaluated in terms of enzyme capacity to convert epinephrine into metanephrine (MN). COMT isforms lysates and purify extracts were incubated at 37ºC, using epinephrine as substrate, during 15 min and 10 min, respectively. MN contents in samples were measured by HPLC analysis using MN standards (1-15 nmol/mL) as calibration control. Chromatographic analysis was performed using a HPLC model Agilent 1260 system (Agilent, Santa Clara, CA, USA) equipped with an autosampler and quaternary pump coupled to an ESA Coulochem III detector (Milford, MA, USA). The chromatographic method was developed using as mobile phase (0.1M NaH_2PO_4, 0.024M citric acid monohydrate, 0.5 mM sodium octyl sulphate and 9% acetonitrile, v/v), pH 2.9 on Zorbax 300SB C18 reverse phase analytical column (250 x 4.6 mm i.d. 5 µm) (Agilent, Santa Clara, CA, USA) with a column temperature of 30ºC and flow rate of 1 mL/min. Column effluent was monitored with an electrochemical detector by a coulometric mode using a procedure of oxidation/reduction (analytical cell #1: +410 mV; analytical cell #2: -350 mV).

2.7 SDS-PAGE, Western and Dot Blotting

Reducing Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot trials were performed, respectively according to the method of Laemmli [61] and as previously described [22].

SDS-PAGE samples were prepared by adding 10 µL of a reduction buffer, 30 µL of each fraction samples and to 10 µL of the starting material diluted in 20 µL of lysis buffer. Samples were boiled at 100^{o}C in a loading buffer containing 500 mM Tris-HCl (pH 6.8), 10% (w/v) SDS, 0.02% bromophenol blue (w/v), 0.2% glycerol (v/v), 0.02% ß-mercaptoethanol (v/v) for 5 min. Then, samples were run on 4% stacking and 15% resolving gels containing 0,1% SDS, using a running buffer containing Tris (25 mM), glycine (192 mM) and SDS (0.1% w/v) at 150 V for 90 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 Western blots experiments. Proteins were transferred over a 60 min period at 750 mA at 4ºC in a buffer containing 10 mM CAPS and 10% (v/v) of methanol. After the blotting, membranes were blocked with TBS-T (pH 7.4) containing 5% (w/v) non-fat milk for 60 min at room temperature, washed 3 times during 15 minutes and exposed, overnight at 4ºC, to a rabbit anti-rat MB-COMT monoclonal antibody, that cross reacts with the human protein, at 1:2500 dilution in TBS-T 1%. 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 1:10000 dilution in TBS-T 1%. The PVDF membranes were incubated with 200 µl of ECF for 5 min and enhanced by exposure to chemiluminescence's detection.

In alternative, we used a dot blot protocol with application of 20 µL of samples into a PVDF membrane previously activated with pure methanol and equilibrated with Mili-Q water and TBS (20 mM Tris-HCL, 150 mM NaCl, pH 7.8). Then, we let the membrane dry before blocking non-specific sites by soaking in 5 % (w/v) non-fat milk in TBS-T during 60 min. After the blocking step, membranes were incubated with the same primary and secondary antibodies applied for Western blotting. Membrane's analysis follows the same final steps as stated above for Western Blotting.

Chapter III

Results and Discussion

Over the past years, SCOMT 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 [33, 47]. Unfortunately, protocols used for purification of MPs are more demanding than for soluble proteins and, as a result, few studies have been published concerning the MBCOMT protein [30, 60].

In this work we try to establish new chromatographic strategies in order to recover both COMT isoforms from crude cell lysates and compare their chromatographic profiles using Q-Sepharose as the main isolation support. For this purpose, COMT isoforms biosynthesis was performed on recombinant expression systems developed previously: hSCOMT on *E. coli* BL21 (DE3) strain [26, 56] and hMBCOMT on *Brevibacillus* expression system [4] and cell lysates containing each isoform were loaded into Q-Sepharose under specific chromatographic conditions.

3.1. hSCOMT recovery assays on Q-Sepharose

The recombinant hSCOMT production and extraction from *E. coli* lysates were previously optimized in order to enhance COMT activity recovery [22, 26]. In this work all the reviewed production conditions and lysis method were maintained in comparison other works in the literature [22, 26].

After hSCOMT extraction, an ammonium sulphate (AS) precipitation was included in optimized procedure [22, 26]. Ammonium sulphate is a salt that promotes hydrophobic interactions due to its high ‘salting- out’ power [22, 62]. This effect promotes protein aggregation and, as result, can lead to activity and structural losses. Indeed, previous data show that AS precipitation step may lead to aggregates formation, promoting a decrease on hSCOMT activity levels [22, 26]. Furthermore, species adsorption on Q-Sepharose is promoted when low salt concentrations are used [6]. So, application of AS precipitation step implies its subsequent removal before purification step, which may contribute for a significant decrease in target protein yields. Concerning this, the strategy included a direct injection of supernatant resultant from crude *E. coli* lysates on Q-sepharose, without the AS precipitation step.

Initially, several control experiments were conducted to establish mobile phase’s conditions, improving selectivity of the chromatographic process and analyze their effects on hSCOMT’s retention and elution behavior (table 2).

Table 2 - Summary of salt concentrations used in adsorption and elution SCOMT's.

	[NaCl] (mM)	SCOMT Adsorption	SCOMT Elution
Chromatographic Conditions	0	High	Low
	150	Moderate	Moderate
	250	Low	Moderate
	350	Low	High
	500	Low	High

After these results, was tested a strategy that consisted in a complete retention of the target protein at 10 mM Tris HCl at pH 7.8, and its elution was optimized using, firstly, a sodium chloride increasing gradient strategy and after that a stepwise operation mode. Overall optimized hSCOMT recovery strategy in Q-sepharose included loading of crude *E. coli* lysates at low ionic strength, specifically at 10 mM Tris HCl at pH 7.8, followed by a step at 350mM of NaCl and a final step at 1M of NaCl, as depicted in Figure 19.

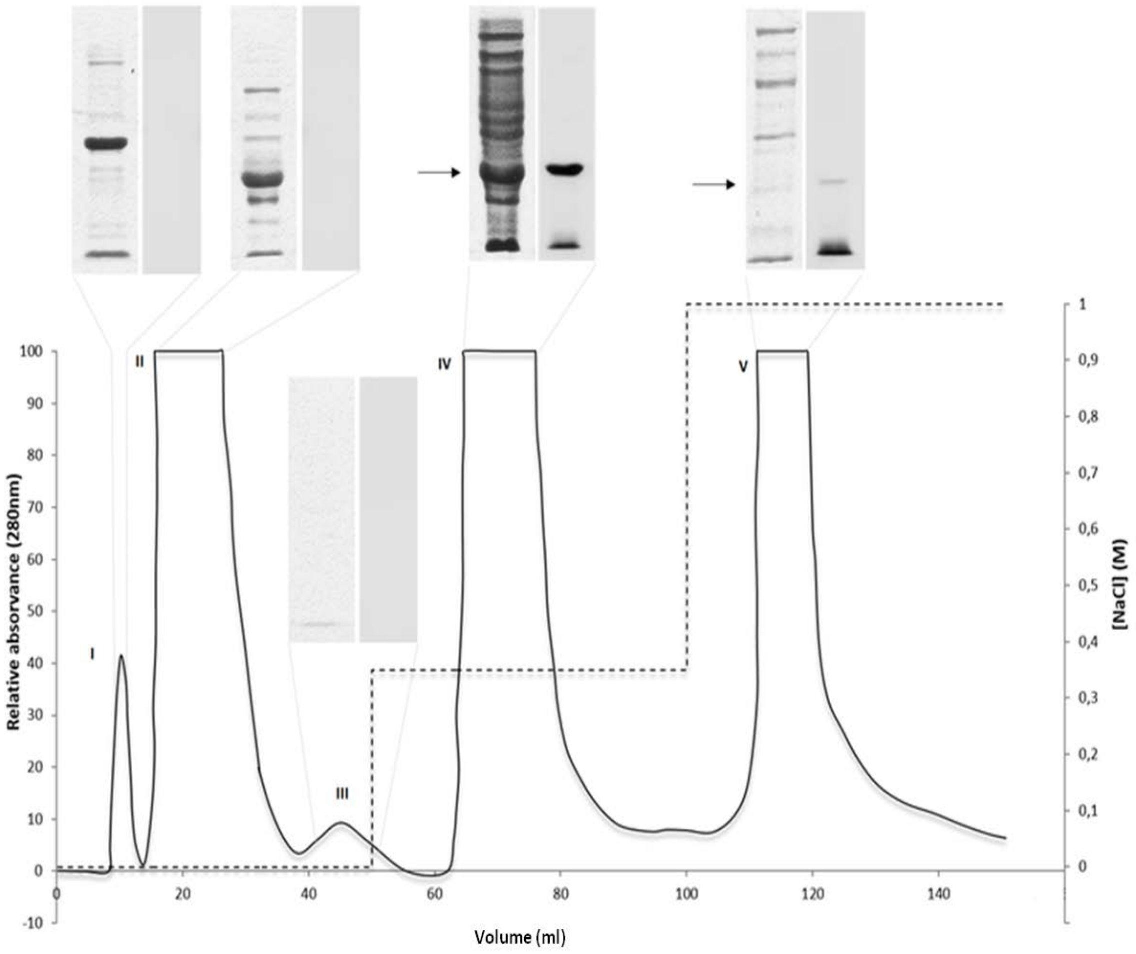


Figure 19 - The soluble COMT chromatographic profile on Q-Sepharose and respective SDS-PAGE/Western blot analysis of collected fractions. Adsorption was performed at 10 mM Tris HCl buffer at pH 7.8, followed by an intermediate step at 350 mM NaCl in 10 mM Tris HCl buffer and a final step at 1 M NaCl. Black line represents absorbance at 280 nm, black dashed line the NaCl concentrations in mobile phase. hSCOMT position in active fractions is represented by an arrow.

By analysis of elution profile with SDS–PAGE gel and western blot (Figure 19), it can be observed that hSCOMT was found to elute essentially at two different fractions at distinct NaCl concentrations. The major fraction is eluted on peak IV at 350 mM NaCl, resulting on an immunological active strong band and a basal fraction on peak V at 1M. Despite of most of impurities were immediately removed from column in first peaks (I and II), many protein contaminants were retained with hSCOMT and eluted together in both peaks as we can see in SDS-PAGE results (Figure 19, Peak IV and V). Thus, a considerable amount of contaminant proteins is still present in the enzyme recovery fractions. So, if highly pure COMT fraction is desired for further applications, a final polishment step is required after this strategy.

Another concern is the presence of nucleic acids contaminants in the target recovered fractions. Previous data showed that AS precipitation step allowed host cell impurities removal, including nucleic acids [22, 26]. However, since was removed AS precipitation from this strategy, nucleic acid impurities could be present in lysis supernatant injected on Q-Sepharose. Therefore, an agarose electrophoresis was performed to assess quality of recovered COMT fractions in terms of nucleic acid presence. The electrophoresis results demonstrated that RNA presence was only detected in peak V (Figure 20, lane V). Since RNA global charge is negative it's not surprising that strong interactions were established with positive ligands such as Q-Sepharose. On other hand, the absence of genomic DNA and plasmid DNA was due to Dnase enzymatic action during lysis procedure. So, even without the application of a AS precipitation step, it was possible to remove major nucleic acids, such as RNA, from the target protein fractions.

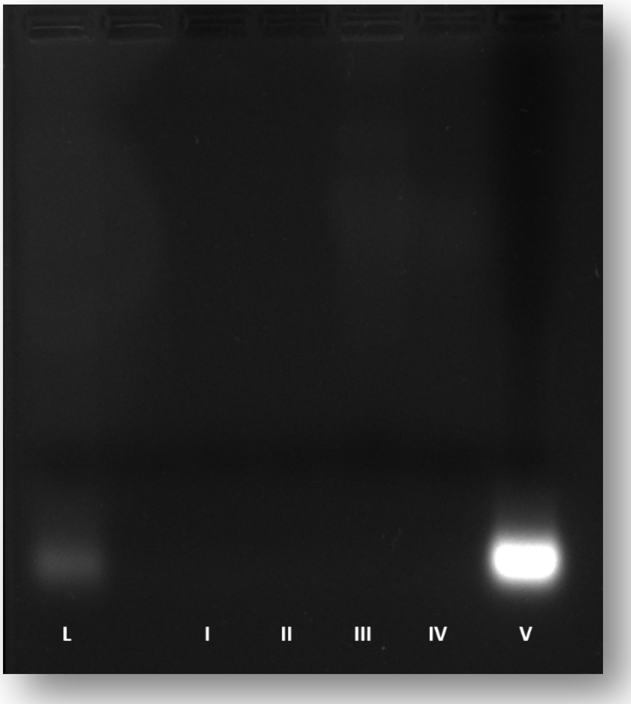
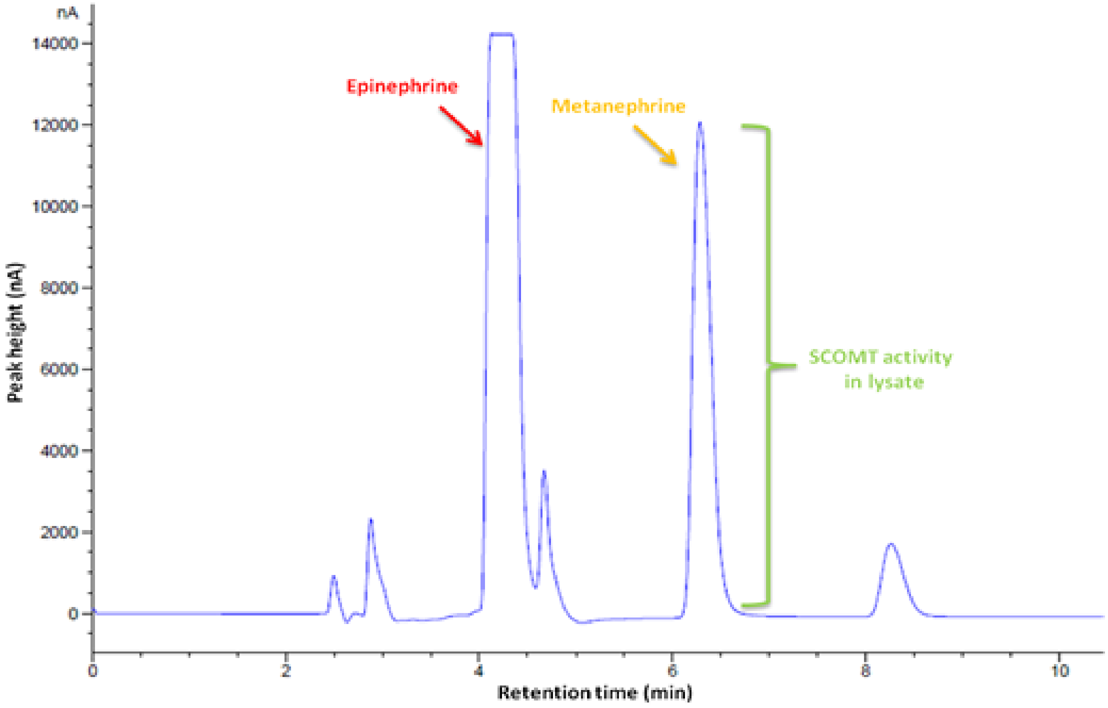


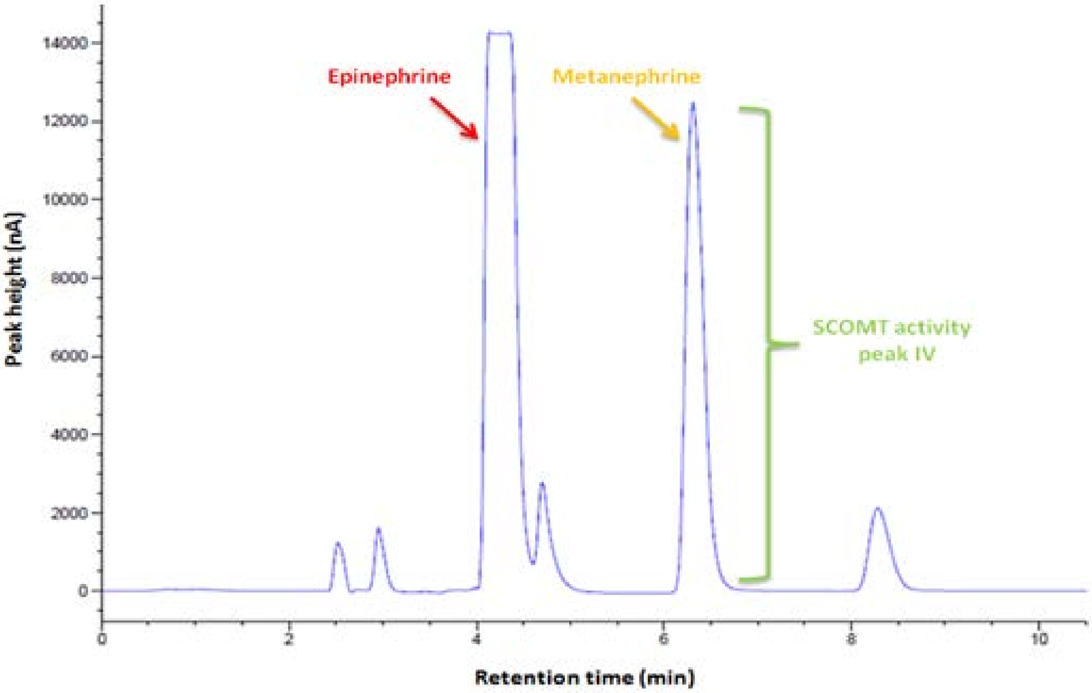
Figure 20 - Agarose electrophoresis analysis of recovered fractions from chromatographic assay on Q-sepharose for assessment of the quality of recovered fractions in terms of nucleic acids presence. Lane — lysis supernatant injected on Q-sepharose; Lanes I, II and III— Fractions I, II and II obtained at 10 mM Tris HCl buffer at pH 7.8 from peaks I, II and III, respectively; Lane IV— Fraction IV obtained at 350 mM NaCl from peak IV and Lane V — Fraction V obtained at 1M NaCl from peak V.

According to several studies, the purified COMT is highly unstable during an isolation process and may loses 50-70% of its activity even at 4°C [22, 33]. However, the use of stabilizers such as $MgCl_2$, glycerol, DTT, cysteine and other reducing agents stabilized the enzyme and allowed its purification and characterization [22, 26, 33, 35, 47, 63–66]. So, for activity assays the mobile phase’s buffers were supplemented with DTT (10 mM) and the collected fractions were stabilized with a suitable solution composed by 75 mM DTT, 150 mM cysteine, 250 mM sucrose and 20% of glycerol.

The measure of activity was performed by quantification of metanephrine levels produced from epinephrine in COMT extracts, using a HPLC system coupled to a coulometric detector. The amount of reaction product was quantified by converting peak area in metanephrine concentration using a calibration curve. The HPLC activity profiles from hSCOMT crude lysates and purified fractions collected from peak IV (Figure 19) are shown respectively in Figure 21-A and 21-B. In Figure 21, epinephrine and metanephrine are represented by peaks with a retention time of approximately 4.2 and 6.3 minutes. By HPLC chromatograms analysis is noticeable that both COMT extracts, the crude lysates (21–A) and the hSCOMT purified extract (21-B), produced similar levels of metanephrine, since they present identical peaks.



A)



B)

Figure 21 - Chromatographic profiles obtained by HPLC analysis corresponding to: SCOMT lysate - Control (A) and peak IV of SCOMT purified fraction (B). The chromatogram shows a peak of epinephrine (COMT substracte) and the peak of metanephrine (COMT product) where their area corresponds to SCOMT activity. Blue line represents the peak height at nA. Epinephrine position is indicated by the red arrow, metanephrine position is indicated by the orange arrow and SCOMT activity is represent by green keyway.

Activity assays results show that chromatographic procedure allows a bioactivity recovery of about 107%, whereas the total activity increase from 254 to 271 nmol/h, as seen in table 3.

Table 3 - Recombinant hSCOMT activity levels after recovery by AEC, using Q-Sepharose as anion exchanger.

SCOMT actiivty assays	**Incubated samples**	**Protein (mg)**	**Total Activity (nmol/h)**	**Specific Activity (nmol/h/mg)**	**Purification (fold)**	**Bioactivity Recovery (%)**
	Lysate	3.65	254	70	1	100
	Peak I	0.19	0	0	0	0
	Peak II	0.01	0	0	0	0
	Peak III	0.31	0	0	0	0
	Peak IV	**1.09**	**271**	**250**	**3.6**	**107**
	Peak V	0.42	17	41	0.6	7

Additionally and despite of the significant amount of contaminants present in COMT active fractions, it was verified a significant increase in purification levels (3.6-fold) and an increase in specific activity from 70 nmol/h/mg to 250 nmol/h/mg (Table 3).

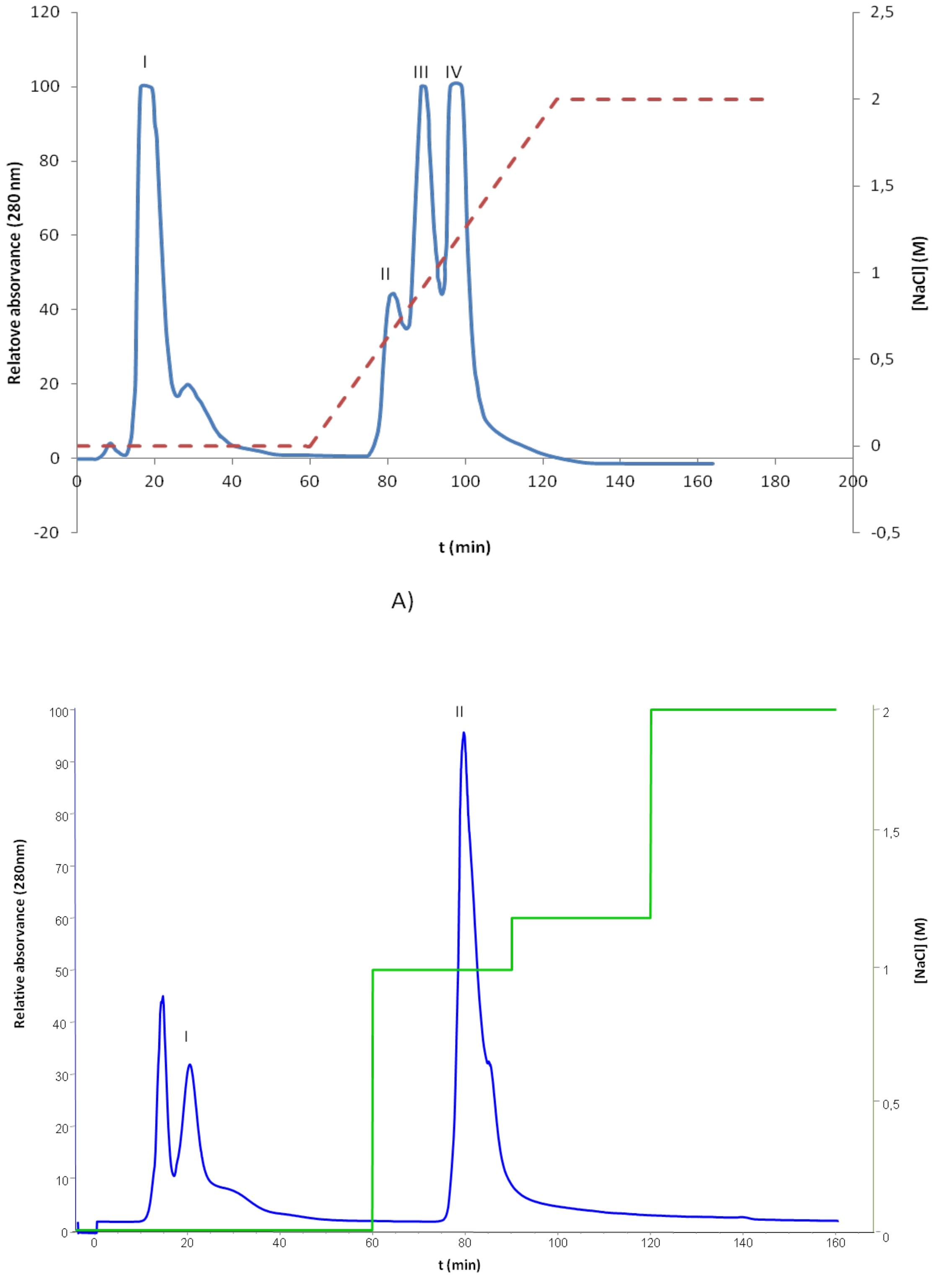
Previous activity results in hSCOMT partial purification by butyl-sepharose shows a purification degree of 3.9-fold [22], a value quite similar to the purification levels obtained after application of this strategy. Concerning bioactivity recovery, the applied strategy allow to recover 107% of total activity of injected lysates, while in hSCOMT purification on butyl there was total activity losses [22]. So, the activity results obtained suggest that the applied AIC strategy is suitable for recombinant hSCOMT recovery achieving satisfactory bioactivity recovery and purification folds.

3.2 hMBCOMT recovery assays on Q-Sepharose

The manipulation and isolation of integral membrane proteins (MPs) relatively to soluble proteins are generally more difficult [4]. Thus, it becomes essential to develop an adequate protocol for the hMBCOMT recovery from a complex mixture of proteins and other contaminants in order to avoid irreversible structural losses and to preserve its biological activity. Indeed to our best knowledge, there are no studies proving hMBCOMT catalytic activity recovery after a chromatographic procedure.

Recently, was developed a new expression system for recombinant hMBCOMT biosynthesis in its biological and immunological active form [4]. Therefore, in this work it is intended to design a suitable downstream process in order to reduce main protein interferences in hMBCOMT active fractions recovered in primary isolation from recombinant host and analyze its bioactivity levels after an ionic chromatographic strategy.

In anionic interaction chromatography, biomolecules are generally retained at low ionic strength and NaCl is the salt typically used on elution buffers [67, 68]. Thus, in an initial strategy was tested 10mM Tris-HCl buffer at pH 7.8 as adsorption buffer, followed by an increasing salt gradient from 0 to 2M NaCl and a final step at 2 M NaCl (Figure 22 - A). Moreover, a stepwise strategy was also exploited using the same adsorption strategy followed by three different steps at 1M, 1.2 M and 2M NaCl in 10mM Tris-HCl buffer at pH 7.8 (Figure 22 – B)). Indeed, high salt concentrations applied the as elution strategy allow us to find the lowest ionic strength that lead to removal of all the compounds retained on the column. Then, it was found that after a 1 M NaCl step no components were eluted from the column (Figure 22 – B).



A)

B)

Figure 22 - Initial purification trials on Q-Sepharose. Absorption was performed at 10mM Tris-HCl buffer at pH 7.8 followed by an increasing salt gradient from 0 to 2 M NaCl and a final step at 2 M NaCl (A) and adsorption with 10 mM Tris-HCl buffer at pH 7.8 followed by three different steps at 1 M, 1.2 M and 2 M NaCl (B) in 10 mM Tris-Cl buffer (pH 7.8). Blue line represents the absorbance at 280 nm and the dashed red (A) and the green lines (B) represent NaCl concentrations.

Concerning hMBCOMT recovery from Q-sepharose with a low levels of impurities, several trials were performed using a 10 mM Tris HCl at pH 7.8. Despite of recommend adsorption of biomolecules at low ionic strength, dot blot and SDS-PAGE analysis (figure 23) show that total hMBCOMT adsorption it it not observed and significant levels are immediately eluted (Figure 23 A) – Lane I). So, is suggesting that this effect can be promoted by a decrease in protein solubility or by aggregation. When removed from lipid bilayer, MPs can lose their structural integrity and become more flexible and unstable, which may contribute to the decrease of its solubility and lead to aggregation, reducing separation efficiency [6, 9, 30]. Indeed, previous results has demonstrated, by SDS-PAGE and Western analysis, that hMBCOMT has a tendency to form aggregates during purification [55].

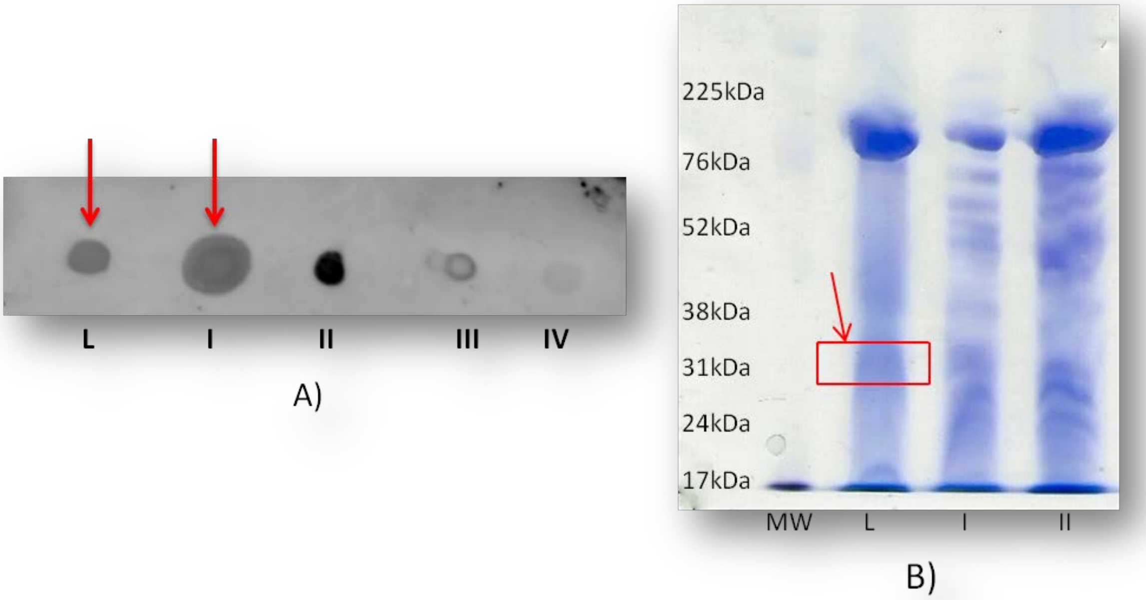


Figure 23 - Dot blotting (A) and SDS - PAGE analysis (B) of samples collected on chromatographic profiles of figure 22 (A) and (B), respectively. Lane L – solubilized lysis pellet injected on Q-Sepharose adsorbent; Lane I – Peaks I obtained at 10 mM Tris HCl; Lane II – Peak II obtained with < 1 M NaCl; Lane III – Peak III obtained with < 1,2 M NaCl concentrations and Lane IV – Peak IV obtained with < 2 M NaCl concentrations, in 10 mM Tris HCl at pH 7.8 (A). Lane MW – molecular weight standards; Lane L – solubilized lysis pellet injected on Q-Sepharose adsorbent; Lane I – Peaks I obtained at 10 mM Tris HCl and Lane II – Peak II obtained at 1 M NaCl in 10mM Tris HCl (B). Human MB-COMT position is indicated by the red arrow.

In sum, the initial control experiments are summarized in table 4, considering salt concentrations required for adsorption and elution of MBCOMT's.

Table 4 - Summary of salt concentrations used in adsorption and elution MBCOMT's

	[NaCl] (mM)	**MBCOMT Adsorption**	**MBCOMT Elution**
Chromatographic Conditions	0	Low	Moderate
	100	High	Low
	150	Low	Moderate
	300	Low	Moderate
	350	Low	High
	550	Low	High

In general, use of NaCl increases the solubility of proteins [69, 70] which occurs due to a salting-in effect, i.e., a decrease in protein electrostatic interactions [69]. In literature are also described the importance of ionic strength maintenance in MPs solubility [6]. It's common to include low or median NaCl concentrations to improve levels of total protein extraction and its solubility and to avoid MPs aggregation [17]. Considering that, was applied an initial NaCl gradient as adsorption strategy to increase MBCOMT solubility and thus to allow its adsorption on Q-sepharose [6].

In order to study the effect of salt on MBCOMT adsorption, trials were performed using several increasing salt linear gradients as adsorption strategy, from 10 mM of Tris HCl to a specific NaCl concentration. So, the used salt gradients were [0 – 50] mM NaCl, [0 – 74] mM NaCl, [0 – 80] mM NaCl, [0 – 100] mM NaCl and [0 – 150] mM NaCl. As a result of these strategies, was improved MBCOMT adsorption relatively to the application of 10 mM Tris HCl in the adsorption buffer (Figure 24 - I). Also, Dot blot analysis shows that it was obtained a greater retention when we applied a linear gradient from 0 to 100 mM NaCl (Figure 24 – V), while a lower retention was noted in the linear gradient from 0 to 50 mM of NaCl (Figure 24 – II). Then, as it is applied a higher salt concentration in linear gradient, most of the MBCOMT is retained, as we can observed in Figure 24. However, this effect only arises until the application of gradient from 0 to 100 mM of NaCl. Curiously, when was applied a linear gradient 0 to 150 mM as adsorption strategy, a reverse effect occurs, i.e., hMBCOMT adsorption decrease and its elution occurs in the first peak at significant levels (Figure 24 -

VI). Perhaps the salting-in effect, reinforced by NaCl addition, was beneficial for hMBCOMT solubility and its adsorption on Q-Sepharose until 0-100 mM NaCl gradient and thereafter it leads to a significant decrease in electrostatic protein-ligand interactions, leading to the enzyme elution.

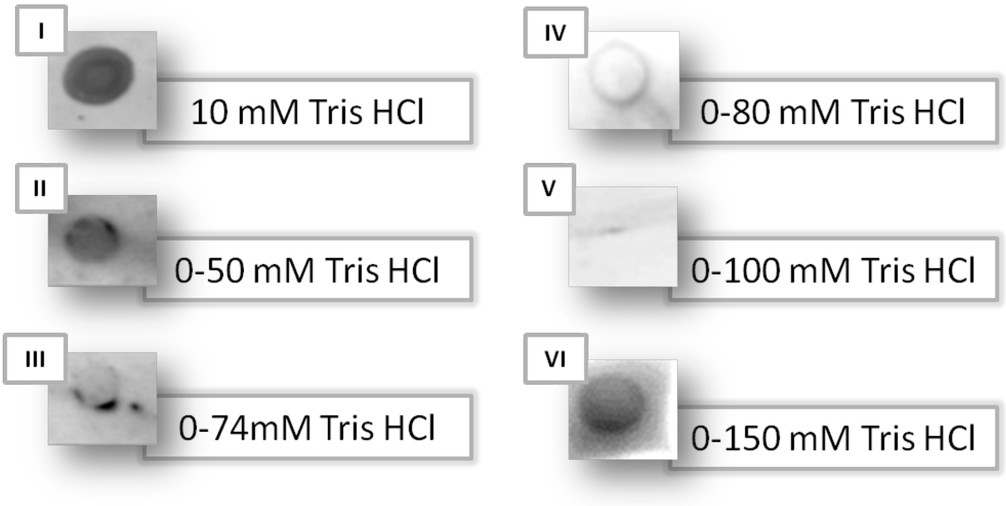


Figure 24 - Dot blot analysis of colleted fractions with no retained species on Q- Sepharose using several adsorption strategies. (I) Adsorption with 10 mMTris HCl, (II) Adsorption with linear gradient [0 – 50] mM NaCl, (III) Adsorption with linear gradient [0 – 74] mM NaCl, (IV) Adsorption with linear gradient [0 – 80] mM, (V) Adsorption with linear gradient [0 – 100] mM NaCl and (VI) adsorption with linear gradient [0 – 150] mM NaCl.

After the screening of an adequate hMBCOMT adsorption strategy, was tried to optimize a suitable chromatographic strategy for its isolation. So, in optimized hMBCOMT recovery assay was initially applied an increasing linear gradient from 0 to 100 mM NaCl, followed by an increasing NaCl gradient from 150 mM to 300 mM NaCl and a final step from 1 M NaCl in 10 mM Tris buffer (Figure 25 - A). The Dot blot analysis (Figure 25 - B) shows that hMBCOMT elution occurs, almost completely, in salt concentrations higher than 300 mM NaCl.

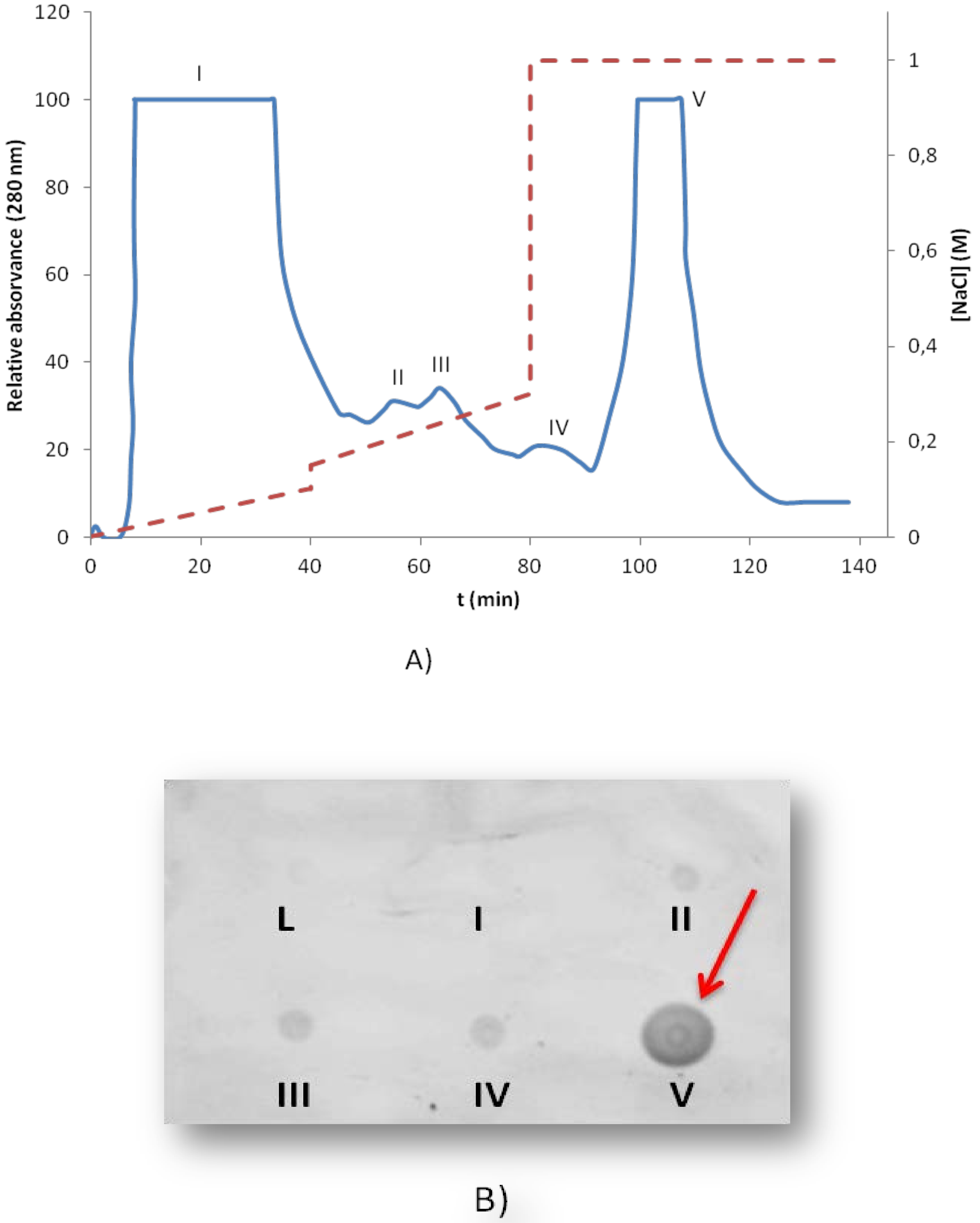


Figure 25 - Chromatographic profile for hMB-COMT isolation on Q-Sepharose (IEC) with respective dot blot analysis. Adsorption was performed at 0 to 100 mM NaCl. Desorption was performed at an increasing NaCl gradient from 150 mM to 300 mM NaCl and a final step from 1M NaCl in 10 mM Tris buffer (A). Lane L – solubilized lysis pellet injected on Q-Sepharose adsorbent; Lane I – Peaks I obtained at 0 - 100 mM NaCl in 10mM Tris HCl; Lane II, III and IV – Peak II, III and IV obtained at 150 – 300 mM NaCl and Lane V – Peak V obtained at 1M NaCl in 10mM Tris HCl at pH 7,8 (B).

After these results, several optimization trials, were designed. The new chromatographic strategy (Figure 26) was consisted in 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 1M NaCl in 10 mM Tris buffer, in which hMBCOMT should be eluted without significant levels of contaminants.

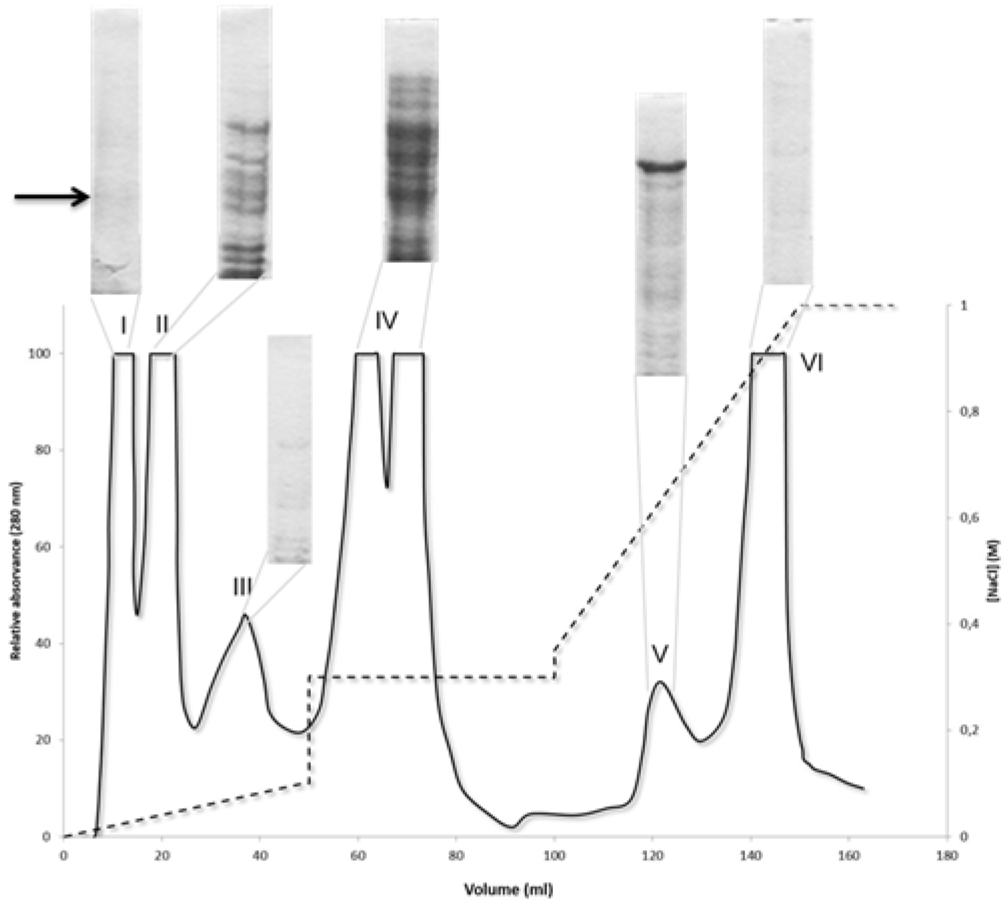


Figure 26 – The hMBCOMT chromatographic profile on Q-Sepharose by AEC and respective SDS-PAGE analysis. Adsorption was performed at an increasing linear gradient from 0 to 100 mM of NaCl. Desorption of retained species was performed at 300 mM NaCl and with an increasing linear gradient from 350 mM to 1 M NaCl. Black line represents absorbance at 280nm and black dashed line the NaCl concentrations in mobile phase. hMBCOMT position in major active fraction is represented by an arrow.

The presence of COMT in collected fractions was assessed by activity assays as described previously. In figure 27 - B is shown the HPLC profile corresponding to purified fraction collected from peak I (Figure 26), where MBCOMT is mostly eluted. HPLC chromatograms analysis evidence a decrease in metanephrine amounts produced from purified extracts (peak I) (27–B) when compared to the values obtained for hMBCOMT onto crude lysates (27-A).

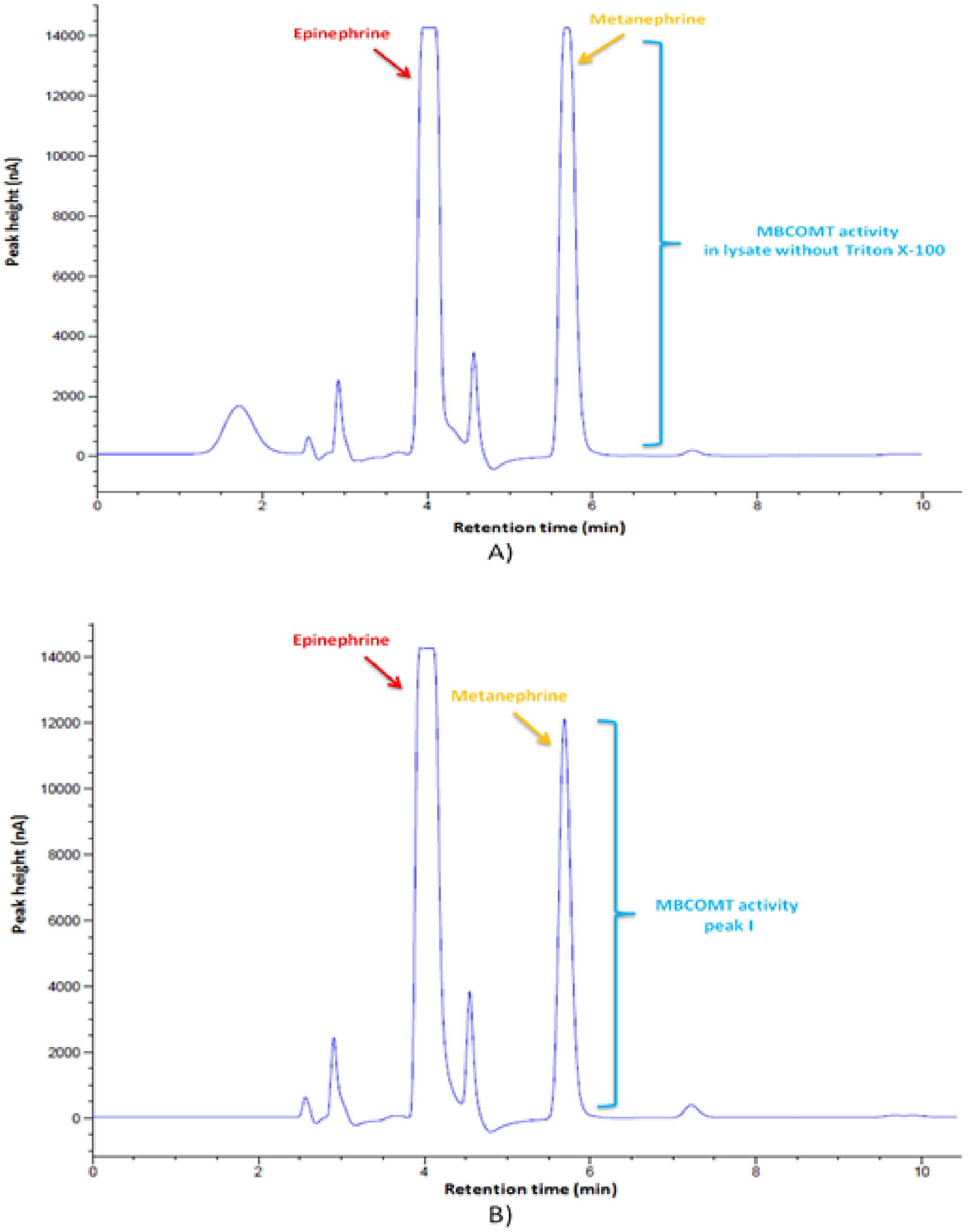


Figure 27 - Chromatographic profiles obtained by HPLC analysis corresponding to: MBCOMT lysate without Triton X-100 - Control (A) and peak I of MBCOMT purified fraction (B). The chromatogram shows a peak of epinephrine and the peak of metanephrine where their area corresponds to MBCOMT activity. Blue line represents the peak height at nA. Epinephrine position is indicated by the red arrow, metanephrine position is indicated by the orange arrow and MBCOMT activity is represented by the light blue keyway.

Suncitly, the fraction with the most significant levels of activity, as seen in Table 5, was collected in the first peak, with 133 nmol/h of total activity and a loss of 33% of bioactivity in relation to injected lysates with 201 nmol/h of total activity. Regarding the sum of total activity values of all collected fractions (187 nmol/h of total activity), there were no significant bioactivity losses, only 6% relatively to injected lysate.

Moreover, it was verified a great increase in specific activity from 73 nmol/h/mg to 496 nmol/h/mg, corresponding to a purification level of 7-fold (table 5). Residual activity levels were found in retained fractions. This indicates that enzyme was not significantly retained in the column with application of initial salt gradient from 0 to 100 mM and elute mostly in the first peak. Despite this, as can be seen in the results of SDS-PAGE analysis (Figure 26), the hMBCOMT's eluted in peak I without large quantities of contaminants.

Table 5 - Recombinant hMBCOMT activity levels after recovery by AEC using Q-Sepharose as anion exchanger (Strategies follow in Figure 26 and Figure 28).

	Triton X-100 (%)	Incubated samples	Protein (mg)	Total Activity (nmol/h)	Specific Activity (nmol/h/mg)	Purification (fold)	Bioactivity Recovery (%)
MBCOMT activity assays	0	Lysate	2.74	201	73	1	100
		Peak I	**0.27**	**133**	**496**	**7**	**67**
		Peak II	0.56	6	11	0	3
		Peak III	0.21	5	21	0.3	2
		Peak IV	0.21	17	83	1	9
		Peak V	0.33	18	54	0.7	9
		Peak VI	0.12	8	65	0.9	4
	0,5	Lysate	2.59	198	77	1	100
		Peak I	0.80	53	66	0.9	27
		Peak II	**0.55**	**181**	**331**	**4.3**	**91**
		Peak III	0.17	32	185	2.4	16
		Peak IV	0.08	6	83	1.1	3

Comparing with previously chromatographic approaches, in terms of adsorption strategy was applied 10 mM of DTT in the mobile phase and additional stabilizers were used during lysis process. Apparently, the application of DTT avoids the binding of hMBCOMT to the ligand. Interestingly, no similar cases were described in literature, but probably the use of DTT and other stabilizers can influence hSCOMT chromatographic profile by changing its conformation or even by direct competition with target protein in the binding process to Q-sepharose ligands.

Taking into account, probably the use of detergents during chromatographic process could changes hMBCOMT solubility and lead to an improvement in isolation yield, adsorption and bioactivity recovery. Since different MPs may have a distinct behavior in the presence of several detergents, it’s essential to identify the type of detergent and optimal concentration to be applied [4]. Thus, we selected Triton X-100 considering hMBCOMT previous studies focusing the influence of detergent in biological activity [4] and isolation by HIC [30]. Moreover, in the partial purification of rat MBCOMT from a microssomal fraction of rat liver homogenates by AEC, is referred the application of 0.5% in buffers [60].

In an attempt to improve the previously strategy, we performed cell lysis and hMBCOMT recovery with 0.5% Triton X-100 and mobile phase’s buffers were also supplemented with the same percentage of detergent and 10 mM of DTT. Concerning the chromatographic strategy was applied 0.5 % Triton X-100 in 10 mM Tris HCl as the main adsorption step, followed by a stage at 300 mM NaCl in 0.5% of detergent and a final salt increasing gradient from 350 mM to 1M NaCl and 0.5% Triton X-100 in 10 mM Tris buffer (Figure 28).

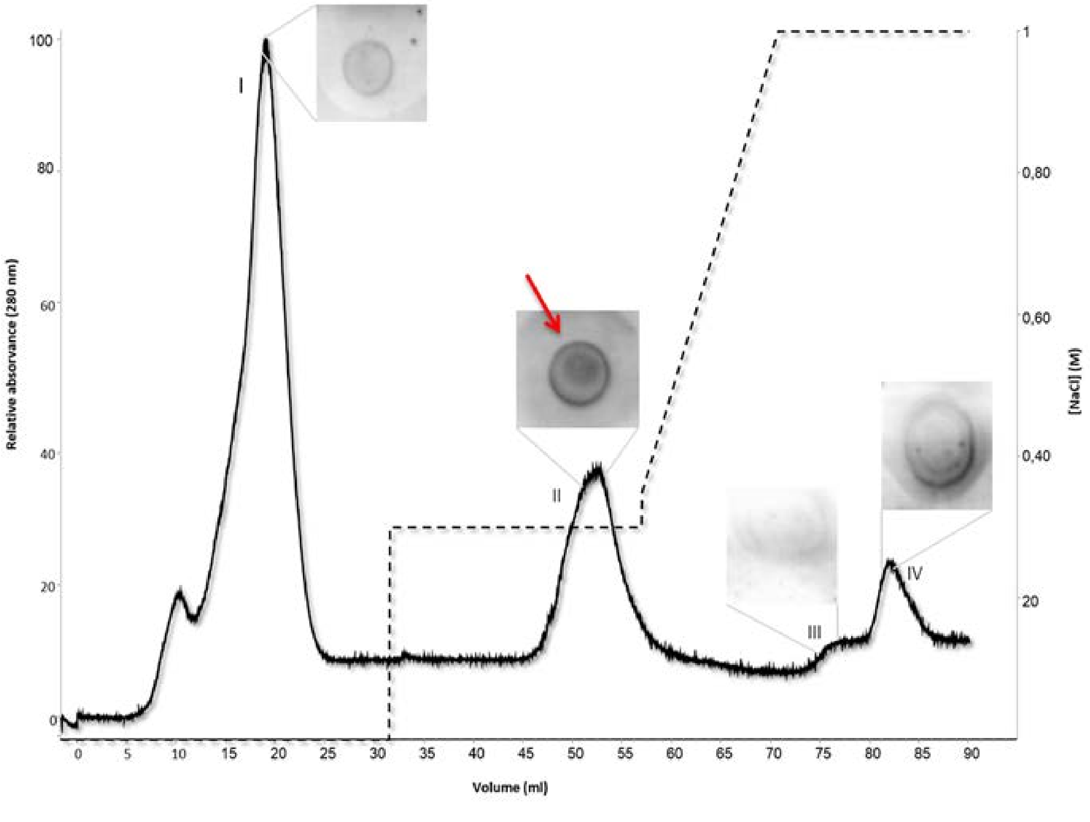


Figure 28 – The hMBCOMT chromatographic profile on Q-Sepharose by AEC and respective dot blot analysis. Adsorption was performed at 0,5% of Triton X-100 in 10mM Tris HCl at pH 7.8. MBCOMT desorption was performed in three different fractions; a major at 300 mM NaCl and a basal fractions at 10mM Tris HCl buffer and 1M NaCl respectively. Black line represents absorbance at 280nm and black dashed line the NaCl concentrations in mobile phase. hMBCOMT position in major active fraction is represented by a red arrow.

The analysis of elution profile by dot blot (Figure 28), it can be observed that hMBCOMT was found to elute essentially at three different fractions at distinct NaCl concentrations. The major fraction is eluted on peak II at 300 mM NaCl, resulting on an immunological active strong lane on dot blot. Also a basal fraction on peak I and IV was depicted at low strength ionic and 1M NaCl in 10 mM Tris HCl, respectively.

Additionally, Figure 29 shows the HPLC profiles of hMBCOMT crude lysates (A) and purified fraction collected from peak II (B) in chromatographic profile represented in Figure 28.

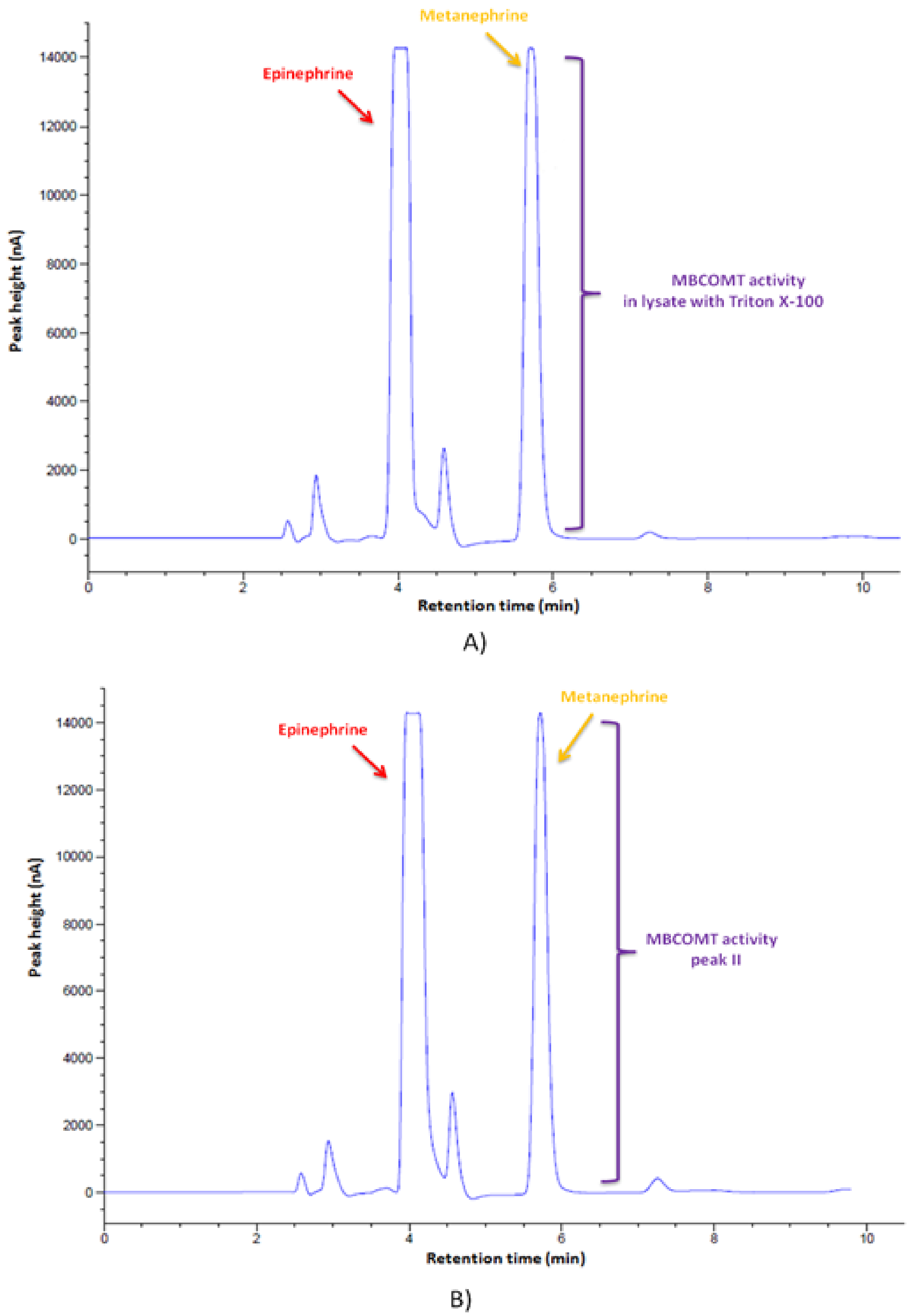


Figure 29 **-** Chromatographic profiles obtained by HPLC analysis corresponding to: MBCOMT lysate with Triton X-100 - Control (A) and peak II of MBCOMT purified fraction. The chromatogram shows a peak of epinephrine and the peak of metanephrine where their area corresponds to MBCOMT activity (B). Blue line represents the peak height at nA. Epinephrine position is indicated by the red arrow, metanephrine position is indicated by the orange arrow and MBCOMT activity is represented by the purple keyway.

Analysis of activity values from Table 5 show an increase in enzyme retention since there is a decreasing in total activity of non-retained species collected fractions. Moreover, MBCOMT's elution occurs mainly in second peak when the 300 mM NaCl step was applied and without significant losses of total activity relatively to injected lysate (from 198 nmol/h to 181 nmol/h). Regarding specific activity, there was a considerable increase in values from 77 nmol/h/mg to 331 nmol/h/mg, corresponding to purification levels of 4.3 fold. So, activity results are in agreement with Dot Blot results, demonstrating that enzyme is mainly eluted in peak II.

In particular, activity values of other collected fractions shows that COMT elute also in peak I by a ionic strength low step (total activity and specific activity, respectively, values of 53 nmol/h and 66 nmol/h/mg) and in salt increasing gradient from 350 mM to 1M NaCl but in minor amounts in peak III (total activity and specific activity values of 32 nmol/h and 185 nmol/h/mg) and in residual amounts in peak IV (total activity and specific activity values of 6 nmol/h and 83 nmol/h/mg). Indeed, the sum of total activity values of all collected fractions (272 nmol/h of total activity) shows there were significant bioactivity recovery, about 37% relatively to injected lysate. Therefore, these results suggest that membrane isoform maintains a multiple peak elution trends, but only elute in substantial levels in collected fractions at 300 mM NaCl, exhibiting biological activity in the final chromatographic step.

Comparing both hMBCOMT recovery strategies, with the use of detergent we are able to recover total activity from 133 nmol/h to 181 nmol/h. Despite this, losses in specific activity and purity levels from 496 nmol/h/mg to 331 nmol/h/mg and from 7 to 4.3 fold, respectively, were achieved. Thus, although the application of detergent in chromatographic step has contributed to a bioactivity recovery, there was a significant loss in purity levels.

3.3 Comparison of hMBCOMT and hSCOMT recovery

The strategies described allowed both isoforms to maintain its biological activity after an anionic exchange chromatography strategy, with considerable recovery rates. Nevertheless, these chromatographic strategies indicate that structural dissimilarities between COMT isoforms were reflected in chromatographic behavior of protein understudy.

Despite innumerous similarities between COMT isoforms, hSCOMT and hMBCOMT have some differences at the structural level, which are reflected in their kinetic behavior and their chromatographic profiles. In chromatographic techniques used in this study, the retention of each isoform was reached with different adsorption steps. In the case of soluble isoform, retention occurs at 10 mM Tris HCl pH 7.8 and elution of all the retained species onto the followed steps at 350 mM and 1 M of NaCl. Contrarily, membrane isoform retention needed a higher ionic strength in the adsorption stage.

These differences in chromatographic profiles are due to hMBCOMT tendency to aggregates formation, since it is more unstable outside of membrane environment. Moreover, structural differences of COMT isoforms are also reflected in their isoelectric point. In fact, membrane form exhibits an isoelectric point of 6.2, while SCOMT have an isoelectric point 5.2. So, at pH of chromatographic buffers applied in this work, SCOMT surface charge may be more positive than MBCOMT superficial charge. Then, as Q-sepharose is an anion exchanger, hSCOMT has more tendency to be absorbed than hMBCOMT.

Additionaly, hMBCOMT's adsorption at low ionic strength required the use of detergents. Therefore, it was possible to retain hMBCOMT in column at 10 mM Tris HCl accomplished by 0.5% Triton X-100 in adsorption buffer. Indeed, if we observed the hSCOMT chromatographic profile (Figure 19) and the hMBCOMT chromatographic profile with Triton X-100 (Figure 28), some similarities can be noted such as the NaCl concentration required for retained species elution. So apparently, use of detergents minimizes the experimental discrepancies between COMT isoforms chromatographic profiles.

Concerning the biological activity assays of the two isoforms, the developed chromatographic strategies allowed higher bioactivity recovery rates in case of soluble isoform. While in hSCOMT recovery assays the recovery rate was superior to 100%, in hMBCOMT isoforms case the recovery rate never reached 100%, even using detergents in mobile phase. Despite this, the use of 0,5% Triton X-100 allowed total activity recovery levels, in comparison to chromatographic strategy without detergents where bioactivity recovery near 67% was obtained.

In general, COMT presents a tendency to elute in a multiple peak pattern as previously described some research groups [22, 30]. For hSCOMT chromatographic strategy its elution occurs mainly in a single peak (peak IV) and only in residual levels in peak V (Table 2). However, in hMBCOMT chromatographic assays all recovered fractions, beyond the target fractions, present residual COMT activity levels as we can see in Table 5. Regarding this, apparently no significant bioactivity losses were observed in hMBCOMT recovery procedure despite of difficulties in manipulation and isolation of MPs relatively to soluble proteins. So, regarding the sum of total activity values of all collected fractions there were no significant bioactivity losses. The total activity of all hMBCOMT active collected fractions were 187 nmol/h relatively to strategy without detergents and 272 nmol/h in strategy with 0.5% Triton x-100, corresponding to a respective bioactivity recovery of 94% and 137% relatively to injected lysate (Table 5).

Concerning specific activity values, the developed chromatographic strategies were more effective for hMBCOMT than for SCOMT. While SCOMT presents a specific activity value of 250 nmol/h/mg (Table 2), MBCOMT has values of 331 and 496 nmol/h/mg (Table 5), relatively to assays with and without detergents, respectively. Moreover, purification levels are more significant in the case of hMBCOMT with values of 4.3 and 7 fold (Table 5) in contrast of 3.6 to SCOMT (Table 2).

The chromatographic behavior described for both isoforms is affected by its structural and biochemical characteristics and the use of detergents appears to minimize the experimental discrepancies between hSCOMT and hMBCOMT.

Chapter IV

Conclusions

The purification by AEC combined with others chromatographic steps that led to successful resolving of its molecular structure. Unfortunately, protocols used for purification of MPs are more demanding than for soluble proteins and, as a result, few studies have been published concerning hMBCOMT protein.

For the first time, our research group proposed and established a new chromatographic strategies for the recovery of both COMT isoforms from crude cell lysates in order to compare their chromatographic profiles using Q-sepharose as anion exchanger, assessing its bioactivity. In general, the recovery of COMT isoforms has performed by ionic strength manipulation, specifically in NaCl concentration onto mobile phase. An hSCOMT complete retention was found at lower ionic strength, while hMBCOMT required the application of a salt linear gradient for its adsorption. Also, hMBCOMT adsorption can be promoted with application of 0.5 % Triton X-100 in 10 mM Tris HCl. The elution of both isoforms elution was performed by an increase in ionic strength.

Also, all the chromatographic strategies described in this work doesn´t interfere with native isoforms atructure, since the biological activity was maintained after an anionic exchange chromatography strategy, with considerable recovery rates. For SCOMT, we are able to recover about 107% of bioactivity and to increase 3.6-fold the purification levels. Regarding hMBCOMT, bioactivity recovery rates were more significant with the application of detergents, 91% against 67% obtained in the strategy without detergents. Otherwise, the strategy without detergents allows high recoveries in specific activity and purity levels when compared with the strategy using Triton X-100. Specifically, the optimized procedures resulted in a 4.3 and 7-fold purification value respectively, in strategies with and without detergents, corresponding to a specific activity values of 331 nmol/h/mg and 496 nmol/h/mg. Comparing both isoforms studies, in terms of recovery, we conclude that different chromatographic conditions are required, but, some similarities can be detected. The hMBCOMT chromatographic profile obtained with Triton X-100 share some resemblances with hSCOMT chromatographic profile, suggesting that the use of detergents to minimize the effect of the structural and functional differences of both COMT isoforms in its recovery strategy. In conclusion, the comparative results shows that structural and functional variances between COMT isoforms can be reflected in the established ionic interactions and chromatographic conditions required for recovery of each isoform in an active form.

Also, although Q-Sepharose has not shown a high selectivity for hSCOMT and hMBCOMT isolation, it was possible to maintain its biological activity with significant bioactivity recovery rates after an anionic exchange chromatography strategy.

Chapter V

Future perspectives

Chromatography is a separation methodology widely used for the separation, identification, and determination of the chemical components in complex mixtures. However, optimization of separation is largely an empirical since it is impossible to predict the chromatographic behavior of target protein. Thus, an optimization of a chromatographic procedure for isolation of specific protein takes a long time and it's multifactorial. The temperature, pH, ionic strength and type of stabilizers are interesting variables to be considered in a factorial design applied to chromatographic procedure, in order to improve protein stability and structure. Factorial design is an important appliance to determine the effects of independent variables upon a single dependent variable. The factorial design studies may be applied in order to keep the physiological integrity of hMBCOMT and hSCOMT through the optimization of various conditions.

Regarding to protein purification, the membrane chromatography appears to be an ideal candidate for large-scale purification and recovery of therapeutic proteins and enzymes. This technique was designed to avoid the fundamental limitations of columns packed with beads, containing functional ligands attached in the pores as adsorbent sites. Until now, several proteins purification procedures apply membrane chromatography, as the capture step. Moreover, this technique can also be used as polishing step in bioprocess separation to remove biological macromolecules. Clearly, the binding capacity, pore size distribution, selectivity and flow distribution are improved if membrane chromatography meets the future separation needs of the biotechnology industry.

Chapter VI

References

[1] Y. Kashino, “Separation methods in the analysis of protein membrane complexes”, *Journal of Chromatography B*, vol. 797, pp. 191–216, 2003.

[2] G. E. Healthcare, *Purifying Challenging Proteins*. *General Electric Company*, 2007.

[3] B. V Bhut, K. A. Christensen, and S. M. Husson, “Membrane chromatography: protein purification from E. coli lysate using newly designed and commercial anion-exchange stationary phases”, *Journal of chromatography A*, vol. 1217, pp. 4946–57, 2010.

[4] A. Q. Pedro, M. J. Bonifácio, J. A. Queiroz, C. J. Maia, and L. A. Passarinha, “A novel prokaryotic expression system for biosynthesis of recombinant human membrane-bound catechol-O-methyltransferase”, *Journal of biotechnology*, vol. 156, pp. 141–6, 2011.

[5] T. Haltia and E. Freire, “Forces and factors that contribute to the structural stability of membrane proteins”, *Biochimica et Biophysica Acta*, vol. 1, pp. 295 – 322, 1995.

[6] C. Hunte, G. Von jagow, and H. Schagger, *Membrane Protein Purification and Crystallization*, 2nd Edition. 2003.

[7] A. M. Seddon, P. Curnow, and P. J. Booth, “Membrane proteins, lipids and detergents: not just a soap opera”, *Biochimica et biophysica acta*, vol. 1666, pp. 105–17, 2004.

[8] B. S. Selinsky, *Membrane Protein Protocols*, vol. 228. New Jersey:Human Press, 2003.

[9] E. P. Carpenter, K. Beis, A. D. Cameron, and S. Iwata, “Overcoming the challenges of membrane protein crystallography”, *Current opinion in structural biology*, vol. 18, pp. 581–6, 2008.

[10] S. Lund, S. Orlowski, B. De Foresta, P. Champeil, M. Le Maire, and J. V Møller, “Detergent structure and associated lipid as determinants in the stabilization of solubilized Ca2+-ATPase from sarcoplasmic reticulum”, *The Journal of Biological Chemistry*, vol. 264, pp. 4907–4915, 1989.

[11] F. W. Lau and J. U. Bowie, “A method for assessing the stability of a membrane protein”, *Biochemistry*, vol. 36, pp. 5884–5892, 1997.

[12] J. U. Bowie, “Stabilizing membrane proteins”, *Current Opinion in Structural Biology*, vol. 11, pp. 397–402, 2001.

[13] C. Tribet, R. Audebert, and J.-L. Popot, “Amphipols: polymers that keep membrane proteins soluble in aqueous solutions”, *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, pp. 15047–15050, 1996.

[14] P. J. Reeves, J. Hwa, and H. G. Khorana, “Structure and function in rhodopsin: Kinetic studies of retinal binding to purified opsin mutants in defined phospholipid ”, *Proceedings of the National Academy of Sciences USA*, vol. 96, pp. 1927–1931, 1999.

[15] J. A. Whiles, R. Deems, R. R. Vold, and E. A. Dennis, “Bicelles in structure–function studies of membrane-associated proteins”, *Bioorganic Chemistry*, vol. 30, pp. 431–442, 2002.

[16] G. Xindu and W. Lili, “Liquid chromatography of recombinant proteins and protein drugs”, *Journal of chromatography B*, vol. 866, pp. 133–53, 2008.

[17] R. Prasad, “Purification, reconstitution, and functional characterization of zinc transporter from rat renal brush border membranes”, *Methods in molecular biology*, vol. 228, pp. 195–221, 2003.

[18] L. Zhang and Y. Sun, “Molecular simulation of adsorption and its implications to protein chromatography: A review”, *Biochemical Engineering Journal*, vol. 48, pp. 408–415, 2010.

[19] R. A. Latour and L. L. Hench, “A theoretical analysis of the thermodynamic contributions for the adsorption of individual protein residues on functionalized surfaces”, *Biomaterials*, vol. 23, pp. 4633–48, 2002.

[20] G. Guiochon, “Preparative liquid chromatography”, *Journal of Chromatography A*, vol. 965, pp. 129–161, 2002.

[21] D. Sheehan, *Ion-Exchange Chromatography*. Molecular Biomethods Handbook, Totowa 2nd Edition, pp. 711–718, 2009.

[22] L. A. Passarinha, M. J. Bonifácio, and J. A. Queiroz, “Comparative study on the interaction of recombinant human soluble catechol-O-methyltransferase on some hydrophobic adsorbents”, *Biomedical chromatography*, vol. 21, pp. 430–438, 2007.

[23] S. R. Costa, M. J. Bonifácio, J. A. Queiroz, and L. A. Passarinha, “Analysis of hSCOMT adsorption in bioaffinity chromatography with immobilized amino acids: the influence of pH and ionic strength”, *Journal of chromatography B*, vol. 879, pp. 1704–6, 2011.

[24] R. Hatti-Kaul and B. Mattiasson, *Isolation and Purification of Proteins*. Marcel Dekker Incorporated, p. 676, 2003.

[25] M. Hutta, R. Góra, R. Halko, and M. Chalányová, “Some theoretical and practical aspects in the separation of humic substances by combined liquid chromatography methods”, *Journal of chromatography A*, vol. 1218, pp. 8946–57, 2011.

[26] L. A. Passarinha, M. J. Bonifácio, P. Soares-da-Silva, and J. A. Queiroz, “A new approach on the purification of recombinant human soluble catechol-O-methyltransferase from an Escherichia coli extract using hydrophobic interaction chromatography”, *Journal of chromatography. A*, vol. 1177, pp. 287–96, 2008.

[27] I. Tadeu, L. Bresolin, E. Alves, S. Maria, and A. Bueno, “Cromatografia de afinidade por ions metálicos imobilizados (IMAC) de biomoléculas: Aspectos fundamentais de aplicações tecnológicas”, *Quimica nova*, vol. 32, pp. 1288–1296, 2009.

[28] J. A. Bornhorst and J. J. Falke, “Purification of Proteins Using Polyhistidine Affinity Tags”, *Methods in enzymology*, vol. 326, pp. 245–254, 2000.

[29] G. S. Chaga, “Twenty-five years of immobilized metal ion affinity chromatography: past, present and future”, *Journal of biochemical and biophysical methods*, vol. 49, pp. 313–34, 2001.

[30] F. M. Santos, A. Q. Pedro, R. F. Soares, R. Martins, M. J. Bonifácio, J. A. Queiroz, and L. A. Passarinha, “Performance of hydrophobic interaction ligands for human membrane-bound catechol-O-methyltransferase purification”, *Journal of separation science*, vol. 36, pp. 1693–702, 2013.

[31] A. Porat, D. Winters, L. Cai, S. Smith, F. Abroson, L.-T. T. Tam, Z. Shen, and R. Hecht, “A novel anion-exchange resin suitable for both discovery research and clinical manufacturing purposes”, in *Preparative biochemistry & biotechnology*, vol. 42, pp. 304–21, 2012.

[32] J. C. Rea, G. T. Moreno, Y. Lou, and D. Farnan, “Validation of a pH gradient-based ion-exchange chromatography method for high-resolution monoclonal antibody charge variant separations”, *Journal of pharmaceutical and biomedical analysis*, vol. 54, pp. 317–23, 2011.

[33] C. Tilgmann and I. Ulmanen, “Purification methods of mammalian catechol-O-methyltransferases”, *Journal of Chromatography B*, vol. 684, pp. 147–161, 1996.

[34] M. J. Bonifácio, P. N. Palma, L. Almeida, and P. Soares-da-Silva, “Catechol-O-methyltransferase and its inhibitors in Parkinson’s disease”, *CNS Drug Reviews*, vol. 13, pp. 352–379, 2007.

[35] N. J. H. Cotton, B. Stoddard, and W. W. Parson, “Oxidative inhibition of human soluble catechol-O-methyltransferase”, *The Journal of biological chemistry*, vol. 279, pp. 23710–8, 2004.

[36] M. Sparta and A. N. Alexandrova, “How metal substitution affects the enzymatic activity of catechol-o-methyltransferase”, *PloS one*, vol. 7, 2012.

[37] P. Lautala, I. Ulmanen, and J. Taskinen, “Radiochemical high-performance liquid chromatographic assay for the determination of catechol O-methyltransferase activity towards various substrates”, *Journal of Chromatography A*, vol. 736, pp. 143–151, 1999.

[38] N. Jatana, A. Sharma, and N. Latha, “Pharmacophore modeling and virtual screening studies to design potential COMT inhibitors as new leads”, *Journal of molecular graphics & modelling*, vol. 39, pp. 145–64, 2013.

[39] A. V. Witte and A. Flöel, “Effects of COMT polymorphisms on brain function and behavior in health and disease”, *Brain research bulletin*, vol. 88, pp. 418–28, 2012.

[40] D. Pap, G. Juhasz, and G. Bagdy, “Association betweenthe COMT gene and rumination in a Hungarian sample”, *Neuropsychopharmacologia Hungarica*, vol. 14, pp. 285–292, 2012.

[41] K. Rutherford, I. Le Trong, R. E. Stenkamp, and W. W. Parson, “Crystal structures of human 108V and 108M catechol O-methyltransferase”, *Journal of molecular biology*, vol. 380, pp. 120–30, 2008.

[42] E. Nissinen, “Basic aspects of Catechol-O-methyltransferase and the clinical applications of its inhibitors”, in *Internacional Review of Neurobiology*, vol. 95I, pp. 1–336, 2010.

[43] H. Kunugi, S. Nanko, A. Ueki, E. Otsuka, M. Hattori, F. Hoda, H. P. Vallada, M. J. Arranz, and D. A. Collier, “High and low activity alleles of catechol-O-

methyltransferase gene: ethnic difference and possible association with Parkinson's disease", *Neuroscience Letters*, vol. 221, pp. 202–204, 1997.

[44] A. Batalla, C. Soriano-Mas, M. López-Solà, M. Torrens, J. A. Crippa, S. Bhattacharyya, L. Blanco-Hinojo, A. B. Fagundo, B. J. Harrison, S. Nogué, R. de la Torre, M. Farré, J. Pujol, and R. Martín-Santos, "Modulation of brain structure by catechol-O-methyltransferase Val(158) Met polymorphism in chronic cannabis users", *Addiction biology*, 2013.

[45] N. Landi, S. J. Frost, W. E. Mencl, J. L. Preston, L. K. Jacobsen, M. Lee, C. Yrigollen, K. R. Pugh, and E. L. Grigorenko, "The COMT Val/Met polymorphism is associated with reading-related skills and consistent patterns of functional neural activation", *Developmental science*, vol. 16, pp. 13–23, 2013.

[46] L. S. Colzato, F. Waszak, S. Nieuwenhuis, D. Posthuma, and B. Hommel, "The flexible mind is associated with the catechol-O-methyltransferase (COMT) Val158Met polymorphism: evidence for a role of dopamine in the control of task-switching", *Neuropsychologia*, vol. 48, pp. 2764–8, 2010.

[47] V. S. Nunes, M. J. Bonifácio, J. A. Queiroz, and L. A. Passarinha, "Assessment of COMT isolation by HIC using a dual salt system and low temperature", *Biomedical chromatography*, vol. 24, pp. 858–62, 2010.

[48] P. T. Männistö and S. Kaakkola, "Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors", *Pharmacological reviews*, vol. 51, pp. 593–628, 1999.

[49] K. Rutherford, E. Alphandéry, A. McMillan, V. Daggett, and W. W. Parson, "The V108M mutation decreases the structural stability of catechol O-methyltransferase", *Biochimica et biophysica acta*, vol. 1784, pp. 1098–105, 2008.

[50] A. Chefson, J. Zhao, and K. Auclair, "Sugar-mediated lyoprotection of purified human CYP3A4 and CYP2D6", *Journal of biotechnology*, vol. 130, pp. 436–40, 2007.

[51] E. K. Barbour, A. Abdelnour, F. Jirjis, O. Faroon, and M. T. Farran, "Evaluation of 12 stabilizers in a developed attenuated Salmonella Enteritidis vaccine", *Vaccine*, vol. 20, pp. 2249–53, 2002.

[52] J. Axelrod and R. Tomchick, "Enzymatic O-Methylation of Epinefrine and Other Catechols", *Journal of Biological Chemistry*, vol. 233, pp. 702–705, 1958.

[53] M. Vieira-Coelho and P. Soares-da-Silva, "Effects of tolcapone upon soluble and membrane-bound brain and liver catechol- O-methyltransferase", *Brain research*, vol. 821, pp. 69–78, 1999.

[54] D. Yalcin and O. Bayraktar, "Inhibition of catechol-O-methyltransferase (COMT) by some plant-derived alkaloids and phenolics", *Journal of Molecular Catalysis B: Enzymatic*, vol. 64, pp. 162–166, 2010.

[55] J. A. Roth, "Membrane-bound catechol-O-methyltransferase: a reevaluation of its role in the O-methylation of the catecholamine neurotransmitters", *Reviews of physiology, biochemistry and pharmacology*, vol. 120, pp. 1–29, 1992.

[56] R. Silva, S. Ferreira, M. J. Bonifácio, J. M. L. Dias, J. A. Queiroz, and L. A. Passarinha, "Optimization of fermentation conditions for the production of human soluble

catechol-O-methyltransferase by Escherichia coli using artificial neural network", *Journal of biotechnology*, vol. 160, pp. 161–8, 2012.

[57] TAKARA, *Brevibacillus expression System*. *TAKARA BIO INC*, pp. 1–19.

[58] K. Lundström, C. Tilgmann, J. Peränen, N. Kalkkinen, and I. Ulmanen, "Expression of enzymatically active rat liver and human placental catechol-O-methyltransferase in Escherichia coli; purification and partial characterization of the enzyme", *Biochimica et biophysica acta*, vol. 1129, pp. 149–54, 1992.

[59] C. Tilgmann and N. Kalkkinen, "Purification and partial sequence analysis of the soluble catechol-O-methyltransferase from human placenta: comparison to the rat liver enzyme", *Biochemical and biophysical research communications*, vol. 174, pp. 995–1002, 1991.

[60] M. J. Bonifácio and P. Soares-da-Silva, "Purification of Membrane – Bound Catechol – O – Methyltransferase", *Methods in molecular biology*, vol. 228, pp. 231–8, 2003.

[61] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4", *Nature*, vol. 227, pp. 680–5, 1970.

[62] J. A. Queiroz, C. T. Tomaz, and J. M. Cabral, "Hydrophobic interaction chromatography of proteins", *Journal of biotechnology*, vol. 87, pp. 143–59, 2001.

[63] J. D. Batchelor, A. Olteanu, A. Tripathy, and G. J. Pielak, "Impact of protein denaturants and stabilizers on water structure", *Journal of the American Chemical Society*, vol. 126, pp. 1958–61, 2004.

[64] R. L. Letsinger, R. Elghanian, G. Viswanadham, and C. A. Mirkin, "Use of a steroid cyclic disulfide anchor in constructing gold nanoparticle-oligonucleotide conjugates", *Bioconjugate chemistry*, vol. 11, pp. 289–91, 2000.

[65] A. K. Bera, A. Grinberg, and R. Bernhardt, "A step toward understanding the folding mechanism of bovine adrenodoxin", *Archives of biochemistry and biophysics*, vol. 361, pp. 315–22, 1999.

[66] M. Li, H. Zhou, H. Zhang, P. Sun, K. Yi, M. Wang, Z. Dong, and S. Xu, "Preparation and purification of l-cysteine capped CdTe quantum dots and its self-recovery of degenerate fluorescence", *Journal of Luminescence*, vol. 130, pp. 1935–1940, 2010.

[67] R. Monjezi, B. T. Tey, C. C. Sieo, and W. S. Tan, "Purification of bacteriophage M13 by anion exchange chromatography", *Journal of chromatography B*, vol. 878, pp. 1855–9, 2010.

[68] P. R. Levison, "Large-scale ion-exchange column chromatography of proteins - Comparison of different formats", *Journal of chromatography B*, vol. 790, pp. 17–33, 2003.

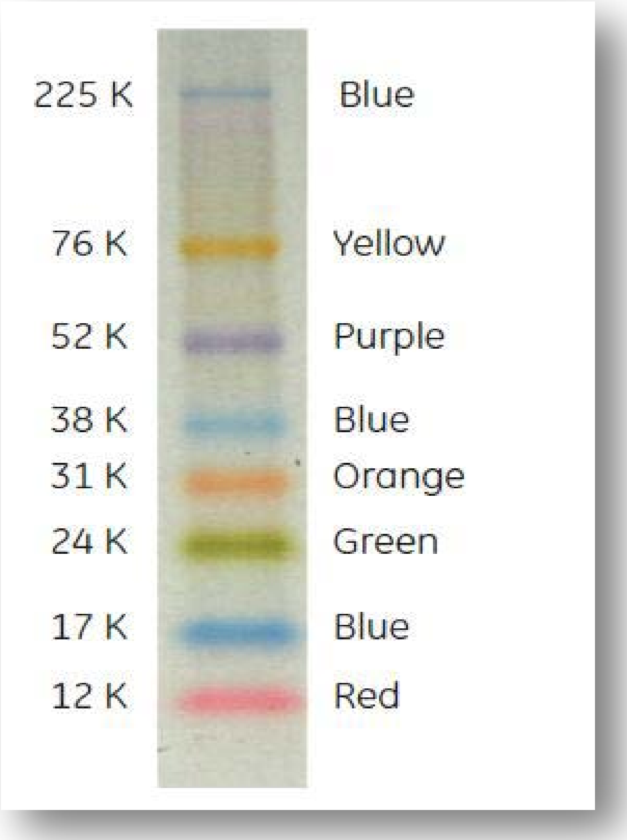
[69] E. D. Strange, D. L. Van Hekken, and V. H. Holsinger, "Effect of Sodium Chloride on the Solubility of Caseins", *Journal of Dairy Science*, vol. 77, pp. 1216–1222, 1994.

[70] D. M. Ragab, E. E. Babiker, and A. H. Eltinay, "Fractionation, solubility and functional properties of cowpea (Vigna unguiculata) proteins as affected by pH and/or salt concentration", *Food Chemistry*, vol. 84, pp. 207–212, 2004.

Chapter VII

Appendices

Appendice I – High Range rainbow molecular weight marker from GEHealthcare.



Appendice II – Paper Submission

Highlights

1- Detergent application in the mobile phase approaches chromatographic profiles of both COMT isoforms.

2- Recovery of COMT isoforms from a Q-Sepharose support in a biological active form.

***Manuscript**
Click here to view linked References

Recovery of biological active catechol-O-methyltransferase isoforms from Q-sepharose

Correia FF[1†], Santos FM[1†], Pedro AQ[1], Bonifácio MJ[2], Queiroz JA[1], Passarinha LA[1]

[1]CICS-UBI- Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, 6201-506 Covilhã, Portugal

[2] Department of Research and Development, BIAL, 4745-457 S. Mamede do Coronado, Portugal

*Corresponding Author:
Luís António Passarinha
Health Sciences Research Centre (CICS-UBI)
Faculty of Health Sciences, University of Beira Interior
Av. Infante D. Henrique, 6200-506, Covilhã, Portugal
Phone: +351 275 329 069
Fax: +351 275 329 099
Email address: lpassarinha@fcsaude.ubi.pt

†These authors contributed equally to this work.

ABSTRACT

The development of new COMT inhibitors led to an improvement of Parkinson disease treatment. However, despite the fact that human SCOMT has been extensively studied, a few studies have been published concerning MBCOMT chromatographic recovery and bioactivity levels. In this work we compare chromatographic profiles of hSCOMT and hMBCOMT, using Q-Sepharose as ligand, in order to evaluate activity levels recuperation and COMT recovery rates. Results show that both isoforms required different conditions for its adsorption; hSCOMT´s adsorption was performed at low ionic strength while MBCOMT required increasing linear salt gradient. However, the application of 0,5% Triton X-100 promoted hMBCOMT's adsorption at low ionic strength. Apparently, the use of detergents approaches both isoforms behavior in terms of chromatographic conditions. The developed methods also appear to be highly effective in bioactivity recovery, presenting rates of 107% for hSCOMT and 67% and 91% for hMBCOMT, relatively to a strategy without and with detergents. The chromatographic strategies with and without detergents resulted in a 4,3 and 7-fold purification respectively, corresponding to specific activity values of 331 nmol/h/mg and 496 nmol/h/mg. Thus, the use of Q-Sepharose as anion exchanger shown to be effective in recovery of both COMT isoforms, which is a requirement for further kinetic and pharmacological trials.

Keywords

Human COMT isoforms; Soluble COMT, Membrane bound COMT; Purification; Anion exchange chromatography.

Abbreviations:

COMT – Catechol-*O*-Methyltransferase; AEC – Anion Exchange Chromatography; MBCOMT - Membrane bound COMT; Membrane Proteins – MPs; SCOMT – Soluble COMT.

1. INTRODUCTION

Catechol-O-methyltransferase (COMT, EC 2.1.1.6.) is a monomeric magnesium-dependent enzyme which catalyzes a transference reaction of a methyl group from their cofactor, S-adenosyl-*l*-methione, to hydroxyl group on catechol substrates [1–3]. In human tissues, two distinct COMT isoforms can be found, a soluble form (SCOMT) present in cytoplasm and a membrane bound form associated to rough-endoplasmatic reticulum (MBCOMT) [1,2,4]. While SCOMT (24.7 kDa) contains 221 amino acid residues, MBCOMT (30 kDa) possess 50 additional amino acid residues, of which around 20 constitutes the membrane anchor region domain [1–3]. Kinetic behavior data suggests that MBCOMT isoform may be the main responsible for catecholamines metabolism in brain [4,5] since it presents a higher affinity for substrates but lower reaction velocity [3].

COMT has become an interesting biopharmaceutical target due to its possible association to some human disorders, particularly in Parkinson disease where the development of new COMT inhibitors lead to an improvement in the disease treatment [1,2,6,7]. Indeed, dopamine replacement therapy with levodopa combined with COMT and aromatic amino acid decarboxylase inhibitors remains one of the most effective treatments for Parkinson disease [1].

Since 1960s human SCOMT has been extensively studied and subjected to numerous purification procedures [8–15]. Anion exchange chromatography (AEC), a well-established technique widely used in isolation of soluble and membrane proteins (MPs) [16–18], has been one of the most usual chromatographic techniques applied to SCOMT isolation [8, 12, 13]. Despite of some of this procedures led to the successful resolving of SCOMT atomic structure, some presented as a drawback significant COMT activity and yield losses [9]. Additionally, usual combination of AEC with others purification steps contributed to reinforce these issues [9,10].

Concerning to MBCOMT, kinetic and biochemical characterizations have been carried out [1,19], but few studies have been published concerning its isolation and so its structure remain unknown [1–3,5,20]. Recently, our research group developed a new purification strategy for recombinant human COMT (hMBCOMT) isolation from crude *Brevibacillus choshinensis* cell lysates, comparing different hydrophobic ligands [20]. Also, some work was developed on MBCOMT purification by AEC, where a partial MBCOMT recovery from microssomal fraction of rat liver homogenates was described [3].

In the design of a suitable purification protocol we exploit progressively biochemical properties of target biomolecule to promote its recovery from a complex protein mixture in an active form [9,16]. These properties will be reflected in specific interactions between the target biomolecule and ligand, which can be largely affected by mobile phase's conditions. So, structural and functional variances between isoforms can be reflected in the protein-ligand interactions established and, as a result, in the chromatographic conditions applied for each isoform recovery. In fact, our previous results show that with same hydrophobic resins, lower ionic strength is required for MBCOMT's adsorption than for

SCOMT [8,20]. Moreover, while SCOMT's elution is only promoted with a decrease in ionic strength, MBCOMT's elution required application of detergents in mobile phase [8,20]. Similarly to this hMBCOMT's behavior on chromatographic hydrophobic adsorbents [20], it's likely that the additional amino acid residues in membrane anchor region domain may contribute for the reinforcement of electrostatic interactions established between MBCOMT and anion exchangers when compared with soluble isoform. In fact, these additional amino acids may contribute for differences in isoelectric point value of both isoforms, respectively, 6.2 and 5.2 for MBCOMT and SCOMT [19].

Therefore, in this work we study, compare and assess the chromatographic behavior of both COMT isoforms using quaternary ammonium (Q-sepharose) as anion exchanger. Overall, the procedure comprises the heterologous expression of the recombinant human SCOMT (hSCOMT) on *E. coli* BL21 (DE3) strain [10,21] and recombinant human MBCOMT (hMBCOMT) on *Brevibacillus* expression system [5], its recovery and isolation from crude cell lysates using Q-Sepharose as chromatographic support. In order to characterize each chromatographic procedure, studies were carried out to (i) analyze Q-sepharose performance in terms of binding and elution conditions for hSCOMT and hMBCOMT recovery and (ii) determine enzyme recovery and activity and procedure yield using COMT activity assays by HPLC coupled with coulometric detector, SDS-PAGE and western blotting.

2. MATERIALS AND METHODS

2.1. Materials

Ultrapure reagent-grade water for fast performance liquid chromatography (FPLC) system was obtained with a Mili-Q system (Milipore/Waters). Neomycin (trisulphate salt hydrate), Carbenicillin disodium salt, isopropylthiogalactosidase (IPTG), tryptone, Bacto yeast extract, Glucose, Calcium chloride dehydrate, lysozyme, Deoxyribonuclease (DNase), dithiothreitol (DTT), Cysteine (L-), Sucrose, DL-Metanephrine Hydrochloride (MN), citric acid monohydrate, 1-Octanesulfonic acid and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium chloride and sodium acetate (anhydrous) were supplied by Fluka (Buchs, Switzerland). Bacto soytone and polypeptone were obtained from Becton Dickinson (NJ, USA). Tris(hydroxymethyl)aminomethane (Tris) and Acetonitrile (HPLC grade) was obtained from Fisher scientific (Epson, United Kingdom). Sodium dihydrogen phosphate (NaH_2PO_4) and Glycerol were obtained from Riedel-de-Haën (Hanover, Germany) and Himedia (Mumbai, India), respectively. Sodium chloride (NaCl) and perchloric acid was purchased from Panreac (Barcelona, Spain). NZYcolour Protein Marker II used for estimation of subunit molecular weight was purchased in NZYTech (Lisboa, Portugal). Anti-rabbit IgG alkaline phosphatase secondary antibody were purchased on GE Healthcare Biosciences (Uppsalla, Sweden). Monoclonal rabbit anti-COMT antibody was produced in BIAL (S. Mamede do Coronado, Portugal) using purified recombinant rat COMT. All chemicals used were of analytical grade commercially available and used without further purification.

2.2. Plasmids, bacterial strains and media

The Champion pET101 Directional TOPO expression kit (Invitrogen Corporation, Carlsbad, CA, U.S.A.) was used for the expression of hSCOMT on *E. coli* BL21 (DE3) strain according to manufacturer's instructions and as previously described [10]. SOB medium [20 g/L triptone, 5 g/L yeast extract, 0.5 g/L NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ with 50 µg/mL cabenicillin] was used for *E. coli* growth.

Plasmid pNCMO2-hMBCOMT was used for hMBCOMT expression on Brevibacillus expression system (Takara Bio Inc., Japan) according to manufacturer's instructions and as previously described [5]. Typically, *B. choshinensis* cells were grown in 2SYNM medium (20.0 g/L glucose, 40.0 g/L Bacto Soytone, 5.0 g/L Bacto Yeast Extract, 0.15 g/L $CaCl_2.2H_2O$ and 50.0 µg/mL Neomycin) and MTNm plates (10.0 g/L glucose, 10.0 g/L polypeptone, 5.0 g/L Bacto Yeast extract, 10.0 mg/L $FeSO_4.7H_2O$, 10.0 mg/L $MnSO_4.H_2O$, 1.0 mg/L $ZnSO_4.7H_2O$, 4.1 g/L $MgCl_2$ and 50 µg/mL Neomycin for MTNm liquid medium; 3.75 g/L Agar and 10.0 µg/mL Neomycin).

2.3. Recombinant COMT isoforms biosynthesis and recuperation

E. coli cells transformed with the expression construct were grown overnight at 37°C in LB medium plates containing 50 µg/mL cabenicillin. A single colony was precultivated in 62.5 mL of SOB medium in 250 mL shake flasks. Cells growth were carried out as previously described [10,21]. The cell pellets were resuspended in a suitable buffer (150 mM NaCl, 10 mM DTT, 50 mM Tris pH 8,0) with protease inhibitors (5 µg/mL leupeptin and 0.7 µg/mL pepstatin) and COMT stabilizers (75 mM DTT, 250 mM sucrose, 150 mM cysteine and 20% glycerol) and lysis were carried as previously described [10,21].

B. choshinensis cells transformed with the expression construct were grown overnight at 37°C in MTNm plates. A single colony of transformed cells was precultivated in 62.5 mL of 2SYNM medium in 250 mL shake flasks. Cells growth and lysis were carried out as previously described [5]. The pellet obtained was incubated with 3.5 mL of lysis buffer (150 mM NaCl, 10 mM DTT, 50 mM Tris, 1 mM $MgCl_2$, pH 8.0) supplemented with protease inhibitors (5.0 µg/mL leupeptin and 0.7 µg/mL pepstatin) and COMT stabilizers (75 mM DTT, 250 mM sucrose, 150 mM cysteine and 20% glycerol) at $4^{o}C$, overnight and until full solubilization of the pellet.

2.4. Anionic Chromatography

Chromatographic assays were performed at room temperature (25 ^{o}C) on a FPLC system (GE Healthcare Biosciences, Uppsalla, Sweden) consisting of a double-piston pumps P500, an MV 7 injection valve equipped with a 2 mL injection loop, a fraction collector FRAC 100, a dual path monitor UV-1 and a data processor 112, controlled by a LCC 501 Plus controller. All buffers pumped into 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 guidelines (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 with different salt concentrations in order to assess sodium chloride concentration required for hSCOMT and hMBCOMT's retention. Unless otherwise stated, column was initially equilibrated with 10 mM Tris HCl, pH 7.8. Aliquots of 2 ml of supernatant containing recombinant hSCOMT were injected into column at 1 ml/min with 10 mM Tris-HCl. After elution of unretained species, sodium chloride concentration was increased to 350 mM in a step mode for 2.5 column volume (CV). Subsequently, sodium chloride concentration in mobile phase was increased to 1 M and this condition was maintained for 2.5 CV.

Solubilized pellet containing hMBCOMT was injected into the column at 1 ml/min with 10 mM Tris-HCl. The elution of unretained species occurred with an increasing sodium chloride gradient from 0 mM to 100 mM (2.5 CV). After elution of unretained species, sodium chloride concentration was increased to 150 mM in a gradient mode until 300 mM NaCl (2.5 CV). Subsequently, strongly bound species were eluted by a linear salt gradient from 350 mM to 550 mM of sodium chloride in 10 mM Tris-HCl buffer (2.5 CV). Finally, a washing step was applied with 1M of sodium chloride in Tris buffer 10 mM (1 CV).

In all chromatographic runs, conductivity was continuously monitored as well as absorbance at 280 nm. Fractions volumes of 3 mL were collected and pooled according to the chromatogram profile obtained and stabilized in a suitable solution composed by 75 mM DTT, 150 mM of cysteine, 250 mM sucrose and 20% of glycerol. Finally, samples were concentrated and desalted with Macrosep® Advance centrifugal devices with Omega™ membrane from VWR (Carnaxide, Portugal) and conserved at 4^{o}C until further analysis.

2.5. Total protein quantification

Protein contents in samples were measured by Pierce BCA Protein Assay Kit (Thermo Scientific, USA) using BSA as standard (25–2000 µg/mL) and calibration control samples according to the manufacturer's instructions.

2.6. SDS-Page and Western Blotting

Reducing Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) and western blot trials were performed, respectively, according to the method of Laemmli [8,22].

2.7. COMT isoforms enzymatic assays

The hMBCOMT and hSCOMT activity levels were evaluated by enzyme capacity to convert epinephrine into metanephrine (MN). COMT isoforms lysates and purified extracts were incubated at 37ºC, using epinephrine as substrate, during 15 min and 10 min, respectively. MN contents in samples were measured by HPLC analysis using MN standards (1-15 nmol/mL) as calibration control. Chromatographic analysis was performed using a HPLC model Agilent 1260 system (Agilent, Santa Clara, CA, USA) equipped with an autosampler and quaternary pump coupled to an ESA Coulochem III detector (Milford, MA, USA). Chromatographic method was developed using as mobile phase (0.1M NaH_2PO_4, 0.024M citric acid monohydrate, 0.5 mM sodium octyl sulphate and 9% acetonitrile, v/v), pH 2.9 on Zorbax 300SB C18 reverse phase analytical column (250 x 4.6 mm i.d. 5 µm) (Agilent, Santa Clara, CA, USA) with a column temperature of 30ºC and flow rate of 1 mL/min. Column effluent was monitored with an electrochemical detector by a coulometric mode using a procedure of oxidation/reduction (analytical cell #1: +410 mV; analytical cell #2: -350 mV).

3. RESULTS AND DISCUSSION

Over the past years, SCOMT 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 [9,11]. Unfortunately, protocols used for purification of MPs are more demanding than for soluble proteins and, as a result, few studies have been published concerning the MBCOMT isolation [3,20].

In this work we established new chromatographic strategies in order to recover both COMT isoforms from crude cell lysates and compare their chromatographic profiles using Q-sepharose as the main isolation step. For this purpose, we performed COMT isoforms biosynthesis on recombinant expression systems developed previously by our group – hSCOMT on *E. coli* BL21 (DE3) strain [10,21] and hMBCOMT on *Brevibacillus* expression system [5] and cell lysates containing each isoform were loaded into Q-Sepharose under specific chromatographic conditions.

3.1. hSCOMT recovery by Q-Sepharose

The recombinant hSCOMT production and extraction from *E. coli* lysates were previously optimized by our research group in order to enhance COMT enzymatic activity recovery [8,10]. In this work we maintain all the reviewed production conditions and the freeze/thaw lysis as extraction method [8,10].

After hSCOMT extraction, an ammonium sulphate (AS) precipitation was included in optimized procedure [8,10]. Ammonium sulphate is a salt that promotes hydrophobic interactions due to its high 'salting- out' power [8,23]. This effect promotes protein aggregation and, as result, can lead to activity and structural losses. Indeed, previous data showed that AS precipitation step may lead to aggregates formation and promoting a decrease on hSCOMT activity levels [8,10]. Furthermore, species adsorption on Q-sepharose is promoted when low salt concentrations are used [17]. So, application of AS precipitation step implies its subsequent removal before purification step, which may contribute for a significant decrease in yield. Concerning this, our strategy included a direct injection of supernatant resultant from crude *E. coli* lysates on Q-sepharose, without the AS precipitation step.

Initially, control experiments were conducted to establish mobile phase's conditions, improving selectivity of chromatographic process and analyze their effects on hSCOMT's retention and elution behavior (data not shown). A complete retention of the target protein was found at 10 mM Tris HCl at pH 7.8, and its elution was optimized using both a sodium chloride increasing gradient strategy and a stepwise strategy. Overall optimized hSCOMT recovery strategy in Q-sepharose included loading of crude *E. coli* lysates 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, as depicted in figure 1.

By analysis of elution profile by SDS–PAGE gel and western blot (Figure 1), can be observed that hSCOMT was found to elute essentially at two different fractions at distinct NaCl concentrations. The major fraction is eluted on peak IV at 350 mM NaCl, resulting on an immunological active strong band and a basal fraction on peak V at 1 M. Despite of most of impurities were immediately removed from column in first peaks (I and II), many protein contaminants were retained with hSCOMT and eluted together in both peaks as we can see in SDS-PAGE results (Figure 1, Peak IV and V). Thus, a considerable amount of contaminant proteins is still present in the enzyme recovery fractions. So, if highly pure COMT fractions are desired for further applications, a final polishment step is required after this strategy.

Another concern is the presence of nucleic acids contaminants in the target recovered fractions. Despite of method disadvantages, previous data showed that AS precipitation step allowed host cell impurities removal, including nucleic acids [8,10]. However, since we removed AS precipitation from our strategy, nucleic acid impurities could be present in lysis supernatant injected on Q-Sepharose. Therefore, we performed an agarose electrophoresis to assess quality of recovered COMT fractions in terms of nucleic acid presence. Agarose electrophoresis results demonstrated that RNA presence was only detected in peak V (Figure 2, lane V). Since RNA global charge is negative it’s not surprising that strong interactions were established with positive ligands such as Q-sepharose. On other hand, the absence of genomic DNA and plasmid DNA was due to DNase enzymatic action during lysis procedure. So, even without AS precipitation step, it was possible acid nucleic removal from the target fractions.

According to several studies, the purified COMT is highly unstable during isolation process and may lose 50-70% of its activity even at 4°C [8,9]. However, the use of stabilizers such as $MgCl_2$, glycerol, DTT, cysteine and other reducing agents stabilized the enzyme and allowed its purification and characterization [8–11,24–28]. So, for activity assays the mobile phase’s buffers were supplemented with DTT (10 mM) and the collected fractions were stabilized with a suitable solution composed by 75 mM DTT, 150 mM of cysteine, 250 mM sucrose and 20% of glycerol in an adequate buffer. Activity assays results show that chromatographic procedure allows a bioactivity recovery of about 107%, whereas the total activity increase from 254 to 271 nmol/h, as seen in Table 1. Additionally and despite of the significant amount of contaminants present in COMT active fractions, it was verified a significant increase in purification levels (3.6-fold) and an increase in specific activity from 70 nmol/h/mg to 250 nmol/h/mg (Table 1). Our previous activity results in hSCOMT partial purification by butyl-sepharose shows a purification degree of 3.9-fold, a value quite similar to the purification levels obtained after application of this strategy. Concerning bioactivity recovery, the applied strategy allow to recover 107% of total activity of injected lysates, while in hSCOMT purification on butyl there was total activity losses.

So, the activity results obtained suggest that the applied AIC strategy are suitable for recombinant hSCOMT recovery, achieving satisfactory bioactivity recovery and purification folds.

3.2. hMBCOMT recovery assays on Q-Sepharose

The manipulation and isolation of integral membrane proteins (MPs) relatively to soluble proteins are generally more difficult [5]. Thus, it becomes essential to develop an adequate protocol for hMBCOMT recovery from a complex mixture of proteins and other contaminants in order to avoid irreversible structural losses and to preserve its biological activity. Indeed, there are no studies proving hMBCOMT catalytic activity recovery after a chromatographic procedure.

Recently, our research group developed a new expression system for recombinant hMBCOMT biosynthesis in its biological and immunological active form [5]. Therefore, in this work we intended to design a suitable downstream process in order to reduce main protein interferences in hMBCOMT active fractions recovered in primary isolation from recombinant host and analyze its bioactivity levels after an ionic chromatographic step.

In anionic exchange chromatography, biomolecules are generally retained at low ionic strength and NaCl is the salt typically used on elution buffers [29,30]. Thus, in an initial strategy 10 mM Tris-HCl buffer at pH 7.8 was tested as adsorption buffer, followed by an increasing linear salt gradient from 0 to 2 M NaCl and a final step at 2 M NaCl. Moreover, a stepwise strategy was also exploited using the same adsorption strategy followed by three different steps at 1 M, 1.2 M and 2 M NaCl in 10 mM Tris-HCl buffer at pH 7.8. High salt concentrations used as elution strategy allow us to verify the lowest ionic strength that lead to removal of all the compounds retained on the column. Then, it was found that after 1 M NaCl step no components were eluted from the column (data not shown).

Concerning hMBCOMT recovery from Q-sepharose with low levels of impurities, several trials were performed using a 10 mM Tris HCl at pH 7.8. Despite of recommend adsorption of biomolecules at low ionic strength, dot blot and SDS-PAGE analysis show that total hMBCOMT adsorption it is not observed and significant levels are immediately eluted. So, we suggest that this effect can be promoted by a decrease in protein solubility or by aggregation. When removed from lipid bilayer, MPs can lose their structural integrity and become more flexible and unstable, which may contribute to the decrease of its solubility and lead to aggregation, reducing separation efficiency [17,20,31]. Indeed, our previous results has demonstrated, by SDS-PAGE and Western analysis, that hMBCOMT has a tendency to form aggregates during purification [20].

In general, use of NaCl increases the solubility of proteins [32,33] which occurs due to a salting-in effect, i.e., a decrease in the electrostatic interactions in a protein [32]. In literature are also described the importance of ionic strength maintenance in MPs solubility [17]. It's common to include low or median NaCl concentrations to improve levels of total protein extracted and to improve its solubility and to avoid protein aggregation in MPs solutions [17]. Considering that, we applied an initial NaCl gradient as adsorption strategy to increase MBCOMT solubility and thus to allow its adsorption on Q-sepharose [17].

In order to study the effect of salt on MBCOMT adsorption, trials were performed using several increasing salt linear gradients as adsorption strategy, from 10 mM of Tris HCl to a specific NaCl concentration. So, the used salt gradients were 0 - 50 mM NaCl, 0 - 74 mM NaCl , 0 - 80 mM NaCl, 0 - 100 mM NaCl and 0 – 150 mM NaCl. As a result of application of these gradients, we improve MBCOMT adsorption relatively to the application of 10 mM Tris HCl in the adsorption buffer (Figure 3-I). Dot blot analysis shows that it was obtained a greater retention when applied a linear gradient from 0 to 100 mM NaCl (Figure 3 –V), while a lower retention was noted in the linear gradient from 0 to 50 mM of NaCl (Figure 3 –II). Then, as it is applied a higher salt concentration in linear gradient, most of the MBCOMT is retained, as seen in Figure 3. However, this effect only arises until the application of gradient from 0 to 100 mM of NaCl. Curiously, when we applied a linear gradient 0 to 150 mM as adsorption strategy, a reverse effect occurs, i.e., hMBCOMT adsorption decrease and its elution occurs in the first peak at significant levels (Figure 3-VI). Perhaps the salting-in effect reinforced by NaCl addition was beneficial for hMBCOMT solubility and its adsorption on Q-Sepharose until 0-100 mM NaCl gradient and thereafter it leads to a significant decrease in electrostatic protein-ligand interactions, leading to the enzyme elution.

After the screening of an adequate hMBCOMT adsorption strategy, we tried to optimize a suitable chromatographic strategy for its isolation. So, overall optimized hMBCOMT recovery assay was initially applied an increasing linear gradient from 0 to 100 mM NaCl, followed by an increasing NaCl gradient from 150 mM to 300 mM NaCl and a final step at 1M NaCl in 10 mM Tris buffer. Dot blot analysis (data not shown) shows that hMBCOMT elution occurs almost completely in salt concentrations higher than 300 mM NaCl.

The new chromatographic strategy (Figure 4-A) consisted in a linear 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 1M NaCl in 10 mM Tris buffer, in which the hMBCOMT should be eluted without significant levels of contaminants. The presence of COMT in collected fractions was assessed by activity assays as described previously.

The fraction with the most significant levels of activity, as seen in Table 2, was collected in the first peak with 133 nmol/h of total activity, representing a loss of 33% of bioactivity in relation to injected lysates. Moreover, it was verified a great increase in specific activity from 73 nmol/h/mg to 496 nmol/h/mg, corresponding to purification levels of 7-fold (Table 1). Residual activity levels were found in retained fractions. This indicates that enzyme was not significantly retained in the column with application of initial salt gradient from 0 to 100 mM and elute mostly in the first peak.

Taking into account previous results, probably the use of detergents during chromatographic process could lead to an improvement of isolation in terms of hMBCOMT solubility, adsorption and bioactivity recovery. Since different MPs may have a distinct behavior in the presence of several detergents, it's essential to identify the type of detergent and its optimal concentration [5]. Thus, we selected Triton X-100 considering hMBCOMT previous studies focusing the influence of detergent selection in its biological activity [5] and in its isolation by HIC [20]. Moreover, in the partial purification of rat MBCOMT from a microssomal fraction of rat liver homogenates by AEC is referred the use of 0.5% in the buffers [3].

In an attempt to improve the previously strategy, we performed cell lysis and hMBCOMT recovery with 0.5% Triton X-100 and mobile phase's buffers were also supplemented with same percentage of detergent and 10 mM of DTT. Concerning the chromatographic strategy (Figure 4-B) we applied 0.5 % Triton X-100 in 10 mM Tris HCl as adsorption strategy, followed by a step at 300 mM NaCl in 0.5% of detergent and a final salt increasing gradient from 350 mM to 1M NaCl and 0.5% Triton X-100 in 10 mM Tris buffer.

By analysis of activity values from Table 2, there was an increase in enzyme retention since there is a decreasing in MBCOMT total activity of non-retained species collected fractions. Moreover, MBCOMT elution occurs mainly in second peak when the 300 mM NaCl step was applied and without significant losses of total activity relatively to injected lysate (from 198 nmol/h to 181 nmol/h). Regarding specific activity, there was a considerable increase in values from 77 nmol/h/mg to 331 nmol/h/mg, corresponding to purification levels of 4,3 fold. Activity values of other collected fractions shows that COMT elute also in salt increasing gradient from 350 mM to 1M NaCl but in minor amounts in peak III (total activity and specific activity values of 32 nmol/h and 185 nmol/h/mg) and in residual amounts in peak IV (total activity and specific activity values of 6 nmol/h and 83 nmol/h/mg).

Comparing both hMBCOMT recovery strategies, with the use of detergent we are able to recover total activity from 133 nmol/h to 181 nmol/h. Despite this, losses in specific activity and purity levels from 496 nmol/h/mg to 331 nmol/h/mg and from 7 to 4,3 fold, respectively, were achieved. Thus, although the application of detergent in chromatographic step has contributed to bioactivity recovery, there was a significant loss in purity levels.

3.3. Comparison of hSCOMT and hMBCOMT assays

The strategies described allowed both isoforms to maintain its biological activity after an anionic exchange chromatography strategy, with considerable recovery rates. Nevertheless, these chromatographic strategies indicate that structural dissimilarities between COMT isoforms were reflected in chromatographic behavior of each isoform.

Despite innumerous similarities between COMT isoforms, hSCOMT and hMBCOMT have some differences at the structural level, which are reflected in their kinetic behavior and their chromatographic profile, as seen in Table 3. In chromatographic techniques used in this study, each isoform behavior was reached with different adsorption steps. In case of soluble isoform, retention occurs with the use of a stepwise of 10 mM Tris HCl at pH 7.8 and the followed steps at 350 mM and 1M of NaCl were used for the elution of all the retained species, including main hSCOMT elution at 350 mM. On the other hand, membrane isoform retention needed a higher ionic strength in the adsorption stage.

These differences in chromatographic profiles are due to hMBCOMT tendency to aggregates formation, since it is more unstable outside of membrane environment. Moreover, COMT isoforms structural differences are also reflected in their isoelectric point. In fact, membrane form exhibits an isoelectric point of 6.2, while SCOMT have an isoelectric point 5.2. So, at pH of chromatographic buffers, SCOMT surface charge may be more positive than MBCOMT superficial charge. Then, as Q-sepharose is an anion exchanger, hSCOMT has more tendency to be absorbed than hMBCOMT.

On other hand, hMBCOMT adsorption at low ionic strength required the use of detergents. Then it was possible to retain hMBCOMT in column at 10 mM Tris HCl accomplished by 0.5% Triton X-100 in adsorption buffer. Indeed, if we observe the hSCOMT chromatographic profile (Figure 1) and the hMBCOMT chromatographic profile with Triton X-100 (Figure 4-B), some similarities can be noted such as the NaCl concentration required for retained species elution.

Concerning the biological activity assays of the two isoforms, the developed chromatographic strategies allowed slightly higher bioactivity recovery rates in case of soluble isoform. While in hSCOMT recovery assays the recovery rate was superior to 100%, in hMBCOMT isoform case the recovery rate never reached 100%, even using detergents in mobile phase. Despite this, the use of 0,5% Triton X-100 allowed total activity recovery levels close to 100% of bioactivity recovery, comparing to chromatographic strategy without detergents where a bioactivity recovery of 67% was obtained.

Nevertheless, COMT presents a tendency to elute in a multiple peak pattern as previously described by our research group [8,20]. In hSCOMT chromatographic strategy its elution occurs mainly in a single peak (peak IV) and only in residual levels in peak V (Table 1). However, in hMBCOMT chromatographic assays all recovered fractions, beyond the target fractions, present residual COMT activity levels as we can see in Table 2. Regarding this, apparently no significant bioactivity losses were observed in hMBCOMT recovery procedure despite of difficulties in manipulation of MPs. As matter of fact, regarding the sum of total activity values of all collected fractions shows there were no significant bioactivity losses. So, the total activity of all hMBCOMT active collected fractions was 187 nmol/h relatively to strategy without detergents and 272 nmol/h in strategy with 0.5% Triton x-100, corresponding to a respective bioactivity recovery of 95% and 137% relatively to injected lysate.

Concerning specific activity values, the developed chromatographic strategies were more effective for hMBCOMT than for SCOMT. While SCOMT presents a specific activity value of 250 nmol/h/mg (Table 1), MBCOMT has values of 331 and 496 nmol/h/mg, relatively to assays with and without detergents (Table

2). Moreover, purification levels are more significant in the case of hMBCOMT with values of 4,7 and 7 fold in contrast of 3,6 of SCOMT.

The chromatographic behavior described for both isoforms is affected by its structural and biochemical characteristics and the use of detergents appears to minimize the experimental discrepancies between hSCOMT and hMBCOMT.

4. CONCLUSIONS

Protocols used for purification of MPs are more demanding than for soluble proteins and, as a result, a few studies have been published concerning the hMBCOMT. Thus, for the first time, we established new chromatographic strategies for recovery of both COMT isoforms from crude cell lysates in order to compare their chromatographic profiles using Q-sepharose as anion exchanger, assessing its bioactivity.

In general, the recovery of COMT isoforms was performed by ionic strength manipulation, specifically in the NaCl concentration in the mobile phase. Then, hSCOMT complete retention was found at lower ionic strength, while hMBCOMT required the application of a salt linear gradient for its adsorption. Also, hMBCOMT adsorption can be promoted with application of 0.5 % Triton X-100 in 10 mM Tris HCl. The elution of both isoforms elution was performed by an increase in ionic strength.

Also, the performed chromatographic strategies allowed to both isoforms maintaining its biological activity after an anionic exchange chromatography, with considerable recovery rates. For SCOMT, we are able to recover about 107% of bioactivity and to increase 3.6-fold the purification levels. Regarding hMBCOMT, bioactivity recovery rates were more significant with the application of detergents, 91% against 67% obtained in the strategy without detergents. Otherwise, the strategy without detergents allows high recoveries in specific activity and purity levels when compared with the strategy using Triton X-100. Specifically, the optimized procedures resulted in a 4,3 and 7-fold purification respectively in strategies with and without detergents, corresponding to a specific activity values of 331 nmol/h/mg and 496 nmol/h/mg.

Comparing both isoforms studies, for its recovery different chromatographic conditions are required. However, hMBCOMT chromatographic profile obtained with Triton X-100 share some similarities with hSCOMT chromatographic profile, suggesting that the use of detergents to minimize the effect of the structural and functional differences of both COMT isoforms in its recovery strategy. In conclusion, the comparative results shows that structural and functional variances between COMT isoforms can be reflected in established ionic interactions and in chromatographic conditions required for recovery of each isoform in an active form.

Also, although Q-Sepharose has not shown a high selectivity in hSCOMT and hMBCOMT isolation it was possible that to maintain its biological activity with significant bioactivity recovery rates after an anionic exchange chromatography strategy.

5. Acknowledgements

This research was partially supported by University of Beira Interior − Health Sciences Research Centre (CICS) and the Portuguese Foundation for Science and Technology (FCT) by COMPETE: FCOMP-01-0124-FEDER-027563 with the project EXPL/BBB478/BQB/0960/2012. F.M. Santos also acknowledges a fellowship from the project “Technologies for Purification and controlled release of biopharmaceuticals to be applied in age-related diseases” (CENTRO-07-ST24-FEDER-002014). A.Q. Pedro also acknowledges a fellowship (SFRH/BD/81222/2011) from FCT. The authors also acknowledges the program COMPETE and the FCT project (Pest-C/SAU/UI0709/2011).

6. REFERENCES

[1] M.J. Bonifácio, P.N. Palma, L. Almeida, P. Soares-da-Silva, CNS Drug Rev., 13 (2007) 352.

[2] P.T. Männistö, S. Kaakkola, Pharmacol. Rev., 51 (1999) 593.

[3] M.J. Bonifácio, P. Soares-da-Silva, Meth. Mol. Biol., 228 (2003) 231.

[4] M.A. Vieira-Coelho, P. Soares-da-Silva, Brain Res., 821 (1999) 69.

[5] A.Q. Pedro, M.J. Bonifácio, J.A. Queiroz, C.J. Maia, L.A. Passarinha, J. Biotechnol., 156 (2011) 141.

[6] V. Bonifati, G. Meco, Pharmacol. Ther., 81 (1999) 1.

[7] L. Almeida, J.F. Rocha, A. Falcão, P.N. Palma, A.I. Loureiro, R. Pinto, et al., Clin. Pharmacokinet., 52 (2013) 139.

[8] L.A. Passarinha, M.J. Bonifácio, J.A. Queiroz, Biomed. Chromat., 438 (2007) 430.

[9] C. Tilgmann, I. Ulmanen, J. Chromat. B: Biomed. Sci. Appl., 684 (1996) 147.

[10] L. A. Passarinha, M.J. Bonifácio, P. Soares-da-Silva, J.A. Queiroz, J. Chromat. A., 1177 (2008) 287.

[11] V.S. Nunes, M.J. Bonifácio, J.A. Queiroz, L.A. Passarinha, Biomed. Chromat., 24 (2010) 858.

[12] H.L. White, J.C. Wu, Biochem. J., 145 (1975) 135.

[13] K. Lundström, C. Tilgmann, J. Peränen, N. Kalkkinen, I. Ulmanen, Biochim. Biophys. Acta, 1129 (1992) 149.

[14] C. Tilgmann, N. Kalkkinen, Biochem. Biophys. Res. Commun., 174 (1991) 995.

[15] S.R. Costa, M.J. Bonifácio, J.A. Queiroz, L.A. Passarinha, J. Chromat. B, Analyt. Technol. Biomed. Life Sci., 879 (2011) 1704.

[16] R. Prasad, in: B.S. Selinsky, Membrane Protein Protocols, Humana Press, New Jersey, 2003, pp. 195.

[17] C. Hunte, G. von Jagow, H. Schagger, Membrane Protein Purification and Crystallization, Academic Press, California, 2nd ed., 2003.

[18] A. Porat, D. Winters, L. Cai, S. Smith, F. Abroson, L.-T.T. Tam, et al., Prep. Biochem. Biotech., 42 (2012) 304.

[19] J. Roth, Rev. Physiol. Biochem. Pharmacol., 120 (1992) 1.

[20] F.M. Santos, A.Q. Pedro, R.F. Soares, R. Martins, M.J. Bonifácio, J.A. Queiroz, et al., J. Sep. Sci., (2013) 1693.

[21] R. Silva, S. Ferreira, M.J. Bonifácio, J.M.L. Dias, J.A. Queiroz, L.A. Passarinha, J. Biotechnol., 160 (2012) 161.

[22] U.K. LAEMMLI, Nature. 227 (1970) 680.

[23] J.A. Queiroz, C.T. Tomaz, J.M.S. Cabral , J. Biotechnol., 87 (2001) 143.

[24] N.J.H. Cotton, B. Stoddard, W.W. Parson, J. Biol. Chem., 279 (2004) 23710.

[25] J.D. Batchelor, A. Olteanu, A. Tripathy, G.J. Pielak, J. Am. Chem. Soc. 126 (2004) 1958.

[26] R.L. Letsinger, R. Elghanian, G. Viswanadham, C.A. Mirkin, Use of a Steroid Cyclic Disulfide Anchor in Constructing Gold Nanoparticle-Oligonucleotide Conjugates, Bioconjugate Chem. 11 (2000) 289.

[27] A.K. Bera, A. Grinberg, R. Bernhardt, Arch. Biochem. Biophys., 361 (1999) 315.

[28] M. Li, H. Zhou, H. Zhang, P. Sun, K. Yi, M. Wang, et al., Preparation and purification of L-cysteine capped CdTe quantum dots and its self-recovery of degenerate fluorescenc, J. Lumin. 130 (2010) 1935.

[29] R. Monjezia, B.T. Tey, C.C. Sieo, W.S. Tan, J. Chromat. B, 878 (2010) 1855.

[30] P.R. Levison, J. Chromat. B, (2003) 17.

[31] E. Carpenter, K. Beis, A. Cameron, Curr. Opinion Struct. Biol., 18 (2008) 581.

[32] E.D. Strange, D.L. Van Hekken, V.H. Holsinger, J. Dairy Sci., 77 (1994) 1216.

[33] D.M. Ragab, E.E. Babiker, A.H. Eltinay, Food Chem., 84 (2004) 207.

Legend Figures:

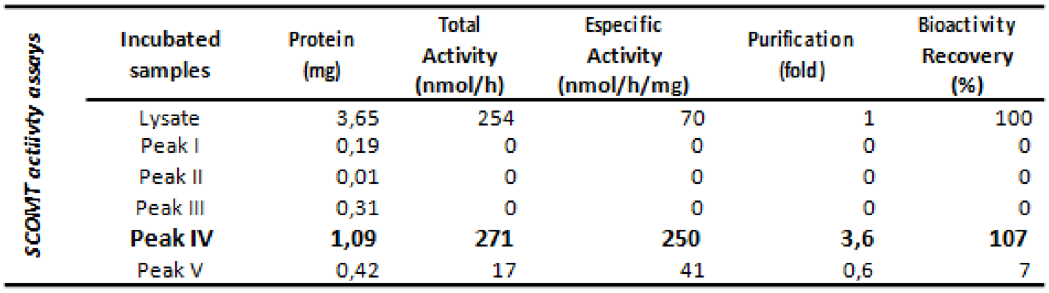
Figure 1 – The soluble COMT chromatographic profile on Q-Sepharose and respective SDS-PAGE/Western blot analysis of collected fractions. Adsorption was performed at 10 mM Tris HCl buffer at pH 7.8, followed by an intermediate step at 350 mM NaCl in 10 mM Tris HCl buffer and a final step at 1 M NaCl. Black line represents absorbance at 280 nm, black dashed line the NaCl concentrations in mobile phase. hSCOMT position in active fractions is represented by an arrow.

Figure 2 – Agarose electrophoresis analysis of the recovered fractions from the SCOMT chromatographic assay on Q-sepharose. Lane — lysis supernatant injected on Q-sepharose; Lanes I, II and III— Fractions I, II and II obtained at 10 mM Tris HCl buffer at pH 7.8 from peaks I, II and III, respectively; Lane IV— Fraction IV obtained at 0,35M NaCl (peak IV) and Lane V — Fraction V obtained at 1M NaCl (peak V).

Figure 3 – Dot blot analysis of colleted fractions in flow through fractions on Q- Sepharose using several adsorption strategies. (I) Adsorption with 10 mM Tris HCl, (II) Adsorption with linear gradient 0 – 50 mM NaCl, (III) Adsorption with linear gradient 0 – 74 mM NaCl, (IV) Adsorption with linear gradient 0 – 80 mM, (V) Adsorption with linear gradient 0 – 100 mM NaCl and (VI) adsorption with linear gradient 0 – 150 mM NaCl.

Figure 4 - hMBCOMT chromatographic profile on Q-Sepharose, (A) without and (B) with detergents application. (A) Adsorption was performed at an increasing linear gradient from 0 to 100 mM of NaCl. Desorption of retained species was performed at 300 mM NaCl and with an increasing linear gradient from 350 mM to 1M NaCl. (B) Adsorption was performed at 0,5% of Triton X-100 in 10mM Tris HCl at pH 7.8. Desorption of retained species was performed at 300 mM NaCl and with an increasing linear gradient from 350 mM to 1M NaCl, including 0,5% Triton in both elution buffers.

Table 1 - Recombinant hSCOMT activity levels after recovery by AEC, using Q-Sepharose as anion exchanger.

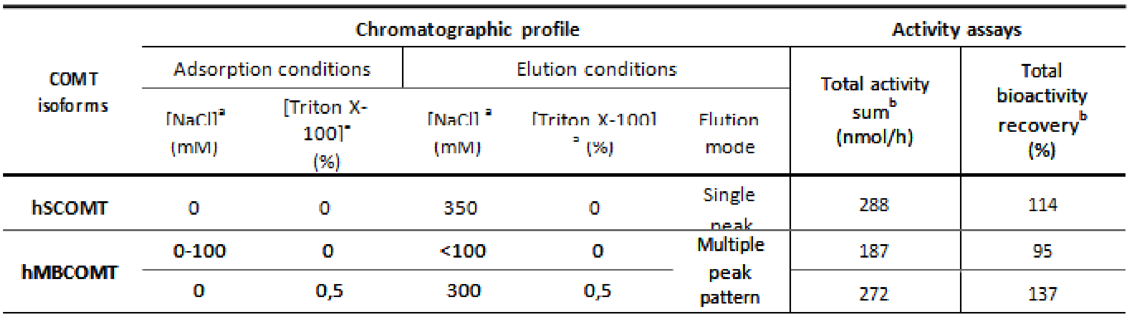


	Incubated samples	Protein (mg)	Total Activity (nmol/h)	Especific Activity (nmol/h/mg)	Purification (fold)	Bioactivity Recovery (%)
SCOMT actiivty assays	Lysate	3,65	254	70	1	100
	Peak I	0,19	0	0	0	0
	Peak II	0,01	0	0	0	0
	Peak III	0,31	0	0	0	0
	Peak IV	**1,09**	**271**	**250**	**3,6**	**107**
	Peak V	0,42	17	41	0,6	7

Table 2 - Recombinant hMBCOMT activity levels after recovery by AEC, using Q-Sepharose as anion exchanger.

	Triton X-100 percentage (%)	Incubated samples	Total Activity (nmol/h)	Specific Activity (nmol/h/mg)	Purification (fold)	Bioactivity Recovery (%)
MBCOMT activity assays	0	lysate	201	73	1	100
		Peak I	**133**	**496**	**7**	**67**
		Peak II	6	11	0	3
		Peak III	5	21	0,3	2
		Peak IV	17	83	1	9
		Peak V	18	54	0,7	9
		Peak VI	8	65	0,9	4
	0,5	lysate	198	77	1	100
		Peak I	53	66	0,9	27
		Peak II	**181**	**331**	**4,3**	**91**
		Peak III	32	185	2,4	16
		Peak IV	6	83	1,1	3

Table 3 - Outline of salt and Triton X-100 concentrations used for both COMT isoforms recovery from Q-sepharose and respective activity values.



COMT isoforms	Chromatographic profile					Activity assays	
	Adsorption conditions		Elution conditions			Total activity sum[b] (nmol/h)	Total bioactivity recovery[b] (%)
	[NaCl][a] (mM)	[Triton X-100][a] (%)	[NaCl] [a] (mM)	[Triton X-100] [a] (%)	Elution mode		
hSCOMT	0	0	350	0	Single peak	288	114
hMBCOMT	0-100	0	<100	0	Multiple peak pattern	187	95
	0	0,5	300	0,5		272	137

[a] All components were prepared in 10 mM Tris HCl

[b] These values correspond to the sum of activity data of all recovered fractions

Figure 1
Click here to download high resolution image

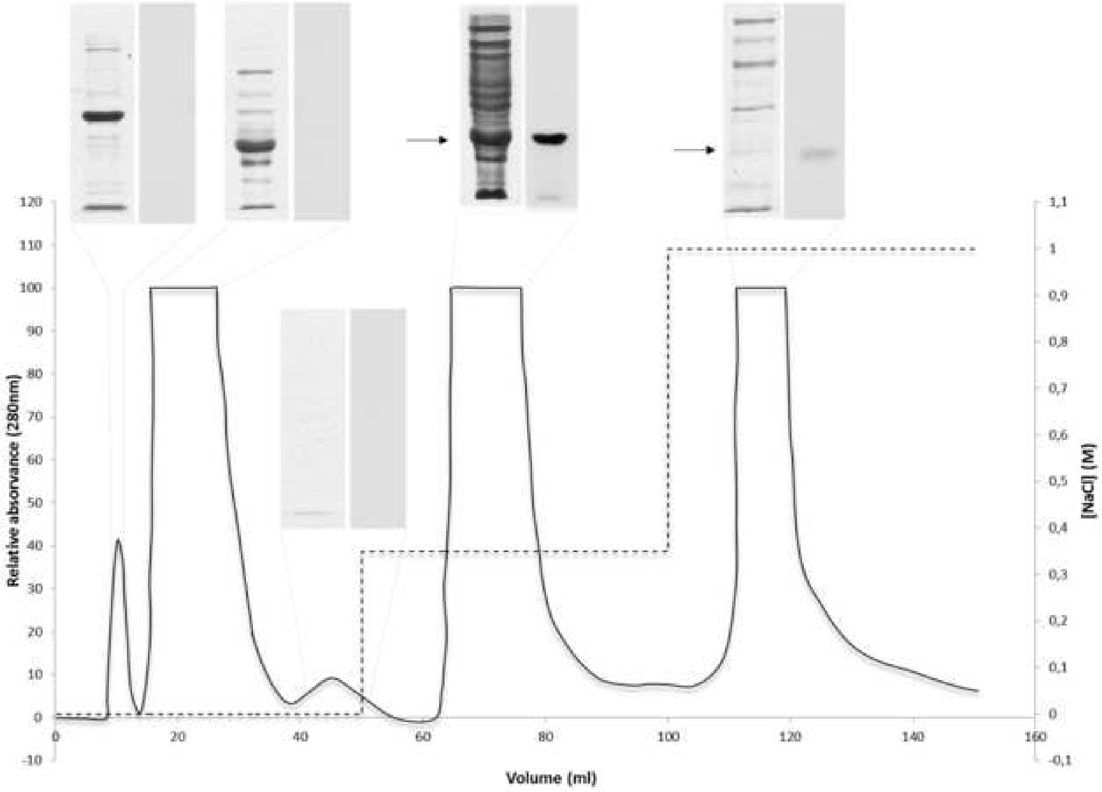


Figure 2
Click here to download high resolution image

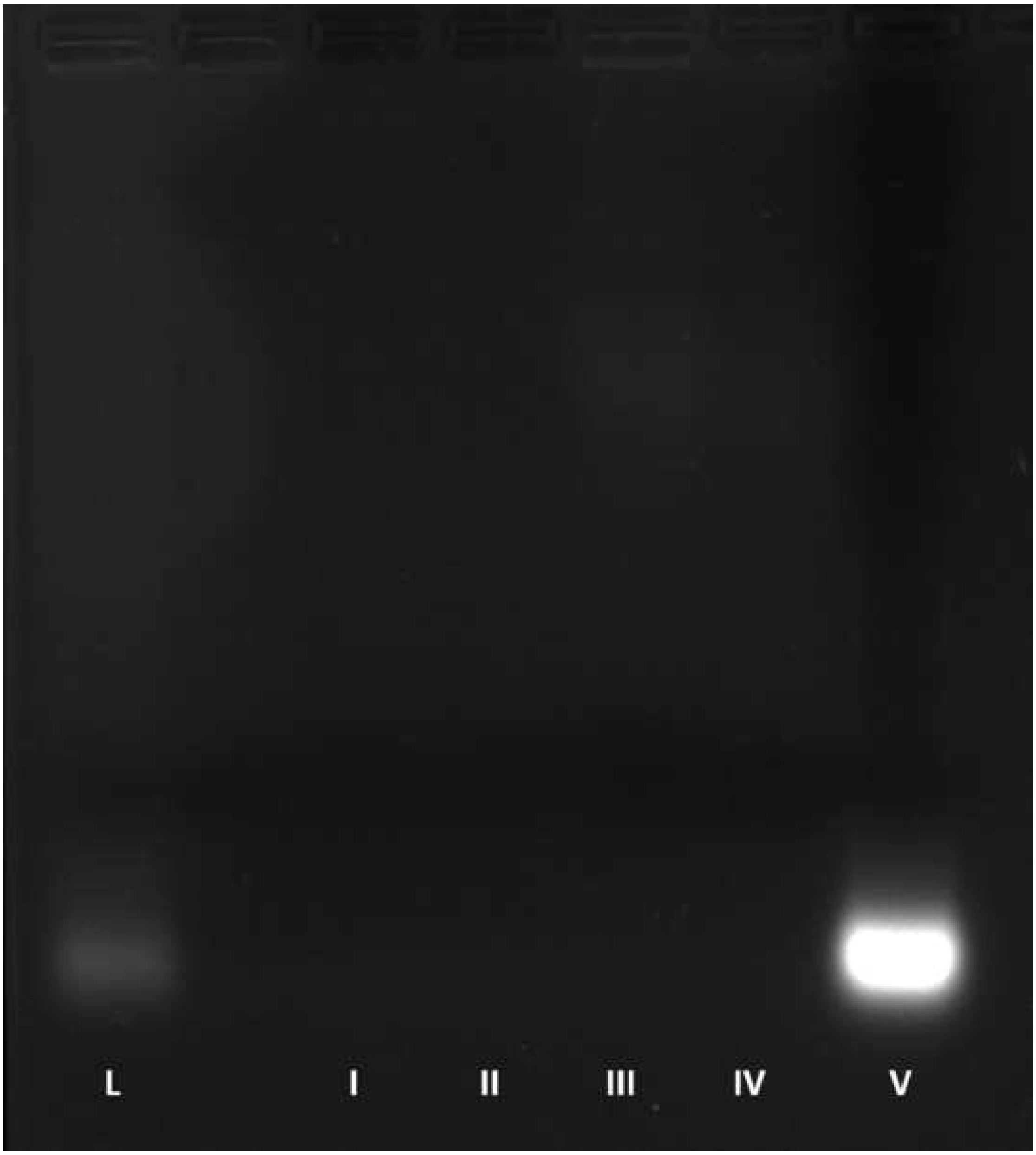


Figure 3
Click here to download high resolution image

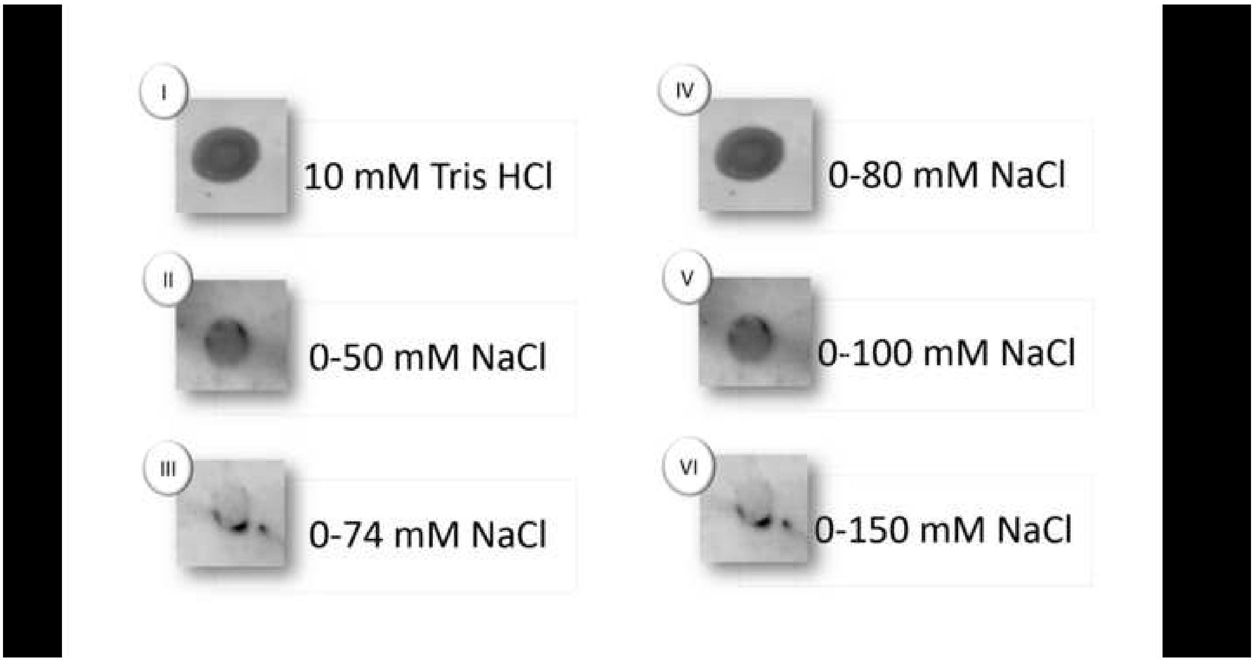


Figure 4
Click here to download high resolution image