UNIVERSIDADE DA BEIRA INTERIOR
Sciences

Chromatographic Design for the Purification of Recombinant Human Membrane COMT

Fátima Raquel Milhano dos Santos

Thesis for the obtention of a Master degree in

Biochemistry

(2nd Cycle of Studies)

Supervisor: Prof. Dr. Luís António Paulino Passarinha

Covilhã, June 2012

Para o meu pai
Sinto-te presente todos os dias

Acknowledgments

Em primeiro lugar gostaria de agradecer ao professor Doutor Luís Passarinha pela excelente orientação, pela partilha dos seus conhecimentos e pela sua inesgotável amizade e apoio. Nunca vou esquecer a confiança depositada em mim e no meu trabalho e pela motivação que me deu para nunca desistir das minhas aspirações.

Ao professor Doutor João Queiroz por todo o apoio científico no decorrer do meu trabalho.

À minha querida família que amo muito quero agradecer todo o carinho, todo o amor e por estarem presentes em todos os momentos da minha vida. À minha mãe por ser um exemplo de força e coragem e a quem devo tudo. Às minhas queridas e lindas irmãs Xana e Inês pela amizade e alegria que me dão. À Inês pelo carinho que me dás principalmente naqueles dias de fragilidade e à Xana pelo altruísmo, pela pessoa alegre que és e por seres a âncora desta família. Ao meu pai que é um exemplo de vida para mim dedico a minha dissertação. Estás e sempre estarás no meu coração.

Ao Diogo Maio, por todo o amor e carinho que me tem dado e por seres o meu abrigo nos últimos meses. Nunca esquecerei o teu apoio e paciência, sem ti não teria sido possível. Obrigado por me fazeres feliz todos os dias.

A todos os meus colegas e amigos que contribuíram para o meu percurso escolar. Ao "meu quarteto" – Ângela Sofia, Inês Pires e Tatiana Saraiva – que me acompanharam desde o meu primeiro dia de vida académica, um agradecimento especial porque se "vierem às quatro horas às três já eu sou feliz". À Joana Feiteiro que é a prova viva que não é o tempo que define uma amizade e ao Paulo Saldanha pela amizade.

À Rita Martins, a minha "co-orientadora", pela partilha dos seus preciosos conhecimentos cromatográficos, pelo apoio e pela amizade. À Filomena Silva por ser a pessoa fofinha que é e igualmente por toda a sua amizade e apoio.

A todos os membros do CICS que contribuem para o bom ambiente de trabalho o que motiva todo o trabalho científico que ali se realiza. Um agradecimento especial aos membros do grupo de Biotechnology and Biomolecular Sciences. Ao Augusto, Rui, Luís e Margarida pela entreajuda, companheirismo e amizade.

Aos meus queridos amigos que ajudaram a tornam-me na pessoa que sou hoje agradeço toda a amizade. Vocês sabem que terão sempre a minha amizade.

Resumo

Estudos sugerem que a forma membranar da catecol-*O*-metiltransferase (MB-COMT) é a principal responsável pela *O*-metilação de catecolaminas a concentrações fisiológicas. Apesar da sua relevância na área farmacológica e clinica, até à data nenhum estudo permitiu o isolamento total da proteína MB-COMT. Assim, torna-se necessário o desenvolvimento de um processo cromatográfico sustentável de forma a obter quantidades significativas de enzima na sua forma activa e pura para posterior aplicação em estudos bioquímicos, estruturais e cinéticos. Pela primeira vez, pretende-se comparar a performance de três matrizes hidrofóbicas (Butil-, Epóxi- and Octil-sepharose) na purificação da humana MB-COMT a partir de lisados de células *Brevibacillus choshinensis*. Deste modo, as matrizes hidrofóbicas foram avaliadas em termos de selectividade e condições de ligação e eluição. Os resultados cromatográficos demonstram que o isolamento da MB-COMT é alcançado utilizando 375 mM fosfato monossódico na sua adsorção. A MB-COMT, tal como a forma solúvel, parece eluir em quatro fracções distintas com concentrações de 0,25; 0,7 e 1% de Triton X-100. Adicionalmente, estudos cromatográficos adicionais indicam que a enzima hMB-COMT é isolada na matriz octyl- em condições de operação suaves mas na epoxy- necessita-se de concentrações de sal superiores para a adsorção completa da enzima. Em conclusão, neste trabalho pela primeira vez foi possível o isolamento total de quantidades significativas de MB-COMT num único passo cromatográfico e com elevada seletividade. Embora as aplicações bem-sucedidas da cromatografia de interacção hidrofóbica (HIC) na purificação de proteínas membranares sejam raras, neste trabalho prova-se que as matrizes hidrofóbicas tradicionais podem oferecer vantagens adicionais de modo a cumprir os requisitos específicos necessários para ensaios cinéticos e farmacológicos.

Palavras-chave

Proteínas membranares; MBCOMT humana; Purificação; Cromatografia de interacção hidrofóbica; Doença de Parkinson

Abstract

Several studies suggest that membrane form of catechol-*O*-methyltransferase OMT (MB-COMT) is the main responsible for *O*-methylation at physiologically low concentrations of catecholamines. Despite this, until now no studies have been allowed the total isolation of MB-COMT. Then, a sustainable chromatographic step should be developed in order to obtain significant quantities of active and pure enzyme for posterior application on biochemical, kinetic and structural studies. For the first time, we intend to compare the performance of three hydrophobic adsorbents (Butyl-, Epoxy- and Octyl-sepharose) in the purification of human membrane-bound COMT (hMBCOMT) from crude *Brevibacillus choshinensis* cell lysates. The hydrophobic matrices were evaluated in terms of selectivity, binding and elution conditions. Results show that the isolation of MB-COMT was possible using 375 mM monosodium phosphate concentrations for its adsorption. The hMB-COMT, as the soluble form, was found to elute at four different fractions at 0.25; 0.7 and 1% Triton X-100. Preliminary chromatographic trials indicate that hMBCOMT may be isolated on octyl- with mild salt conditions but on epoxy- were required high salt concentrations to complete enzyme adsorption. Thereby and in conclusion, in this work and for the first time was possible the total isolation of significant amounts of MB-COMT on a single chromatography step with high selectivity. Although successful applications of Hydrophobic Interaction Chromatography (HIC) in the purification of membrane proteins are uncommon, in this work we prove that traditional hydrophobic matrices can open a promising unexplored field in order to fulfill specific requirements for kinetic and pharmacological trials.

Keywords

Table of Contents

List of Figures

List of Tables

List of Acronyms

B. chosinensis	*Brevibacillus choshinensis*
BSA	Bovine serum albumin
CMC	Critic micellar concentration
COMT	Catechol-*O*-Methyltransferase
DNase	Deoxyribonuclease
DTT	Dithiotreitol
EDTA	Ethylenediamine tetraacetic acid
HIC	Hydrophobic Interaction Chromatography
hMB-COMT	Human Membrane bound Catechol-*O*-Methyltransferase
HPLC	High Performance Liquid Chromatography
MB-COMT	Membrane bound Catechol-*O*-Methyltransferase
MPs	Membrane proteins
MgCl$_2$	Magnesium chloride
NaCl	Sodium chloride
OD$_{660}$	Cell density at 660 nm
PD	Parkinson disease
PVDF	Polyvinyl difluoride
SAH	S-adenosyl-*l*-homocysteine
SAM	S-adenosyl-*l*-methionine
SCOMT	Soluble Catechol-*O*-Methyltransferase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Tris	Tris(hydroxymethyl)aminomethane

Chapter I

Introduction

1.1 Membrane proteins:

The membrane proteins (MPs) play a crucial key role in the survival of cells taking part in processes such signal transduction, transport of matter and information, recognition of changes in cellular environment, energy production and maintenance of cellular structures [1–6]. Furthermore, membrane proteins represent 20 to 30 per cent of the proteomes of many organisms and more than 40 per cent of small drug targets and they have been implicated in many pathologies [1,4–8]. Thus, the determination of the structure of this class of proteins becomes essential to understand dynamics events as catalytic mechanisms, conformational changes and ligand-protein interactions. Such knowledge could offer some lights for the design of new pharmacological drugs and fulfill the gap of information about the correlation between MPs conformational/ activity changes and some diseases [2,4,5].

Nowadays, membrane proteins remain one of the challenging areas of proteomics perhaps due to obstacles encountered on handling of this class of proteins, which don't occur on soluble proteins manipulation [5,7–9]. Until 1985, date of publication of the first high resolution membrane protein structure, was impossible to analyze the structure of membrane proteins [1,6]. Since the photosynthetic reaction center of *Rhodopseudomonas viridi* structure discover, despite the efforts of many research groups only a few three-dimensional (3D) structures of integral MPs have been solved as compared with soluble form proteins [1,2,4–8]. Moreover, only a small part of these structures correspond to mammalian MPs and the most belong to prokaryotic organisms [1,2,10]. This fact has some repercussions in drug design for human diseases since few mammalian MPs structures are available [2].

Overall, membrane proteins expression, purification and analysis represent considerable challenges in MPs structure resolution [3]. Nevertheless, extensive optimization and selection of the most important parameters and technical advances in field have become the process of analysis and structure determination of MPs quicker [1,3,4]. Therefore, although MPs might remain an undeveloped field for another decade it is expected that the development of novel methodologies accelerate the progress in structural biology of this important class of proteins [3,5].

1.1.1 Membrane proteins environment mimetization:

Membrane proteins are generally encountered attached to cell membrane and its lipid bilayer composition must therefore be a support at least to allow the optimal functioning for the proteins in this lipid environment [1,6,12,13]. In particularly, protein stability, assembly and function within cell's membrane are maintained through protein-lipid interactions, such as, Van der Waals interactions, hydrogen bonding and salt bridges, and due to physical properties of the lipid bilayer such as lipid fluidity, membrane tension and others [1,3,6,12]. Thus, membrane proteins are classified as peripheral or integral according to their interaction with the membrane [10,14]. While, peripheral MPs are poorly associated with the membrane, integral MPs are partially or totally embedded in the membrane bilayer [6,9,10,14]. These different interactions are reflected in the methodological challenges presented when handling these two classes of MPs. The peripheral MPs are easily solubilized in aqueous solutions but for integral MPs solubilization is necessary a suitable environment in order to satisfy their high hydrophobicity requirements. Therefore, the main difficulties in handling with MPs are found with integral MPs rather than in peripheral MPs as described in the literature [9,10,14].

The complex behavior of cell membrane requires that the extraction and solubilization of MPs are crucial for the purification success and biochemical, biophysical and structural characterization [9,15–17]. Over the last years, MPs have been partially or totally extracted using several methods, such as, the use of chelating agents, ionic strength or pH manipulations, sonication, organic solvents, bicelles and amphipols, enzymatic digestions and others [9,16].
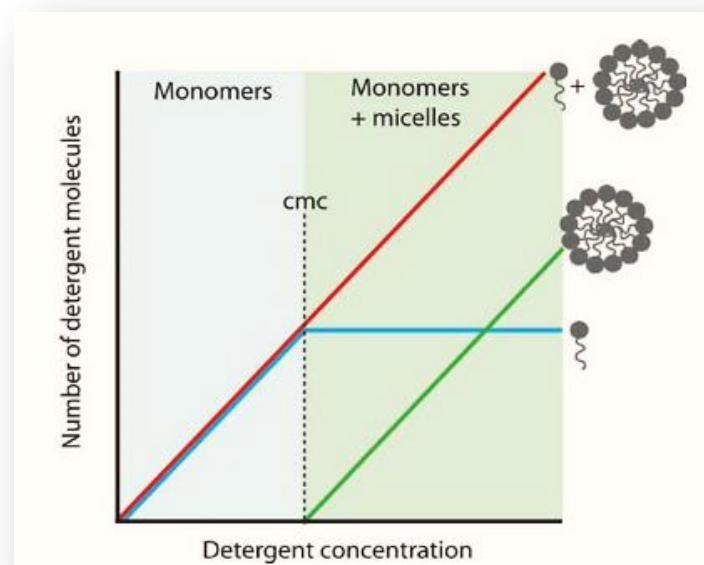

Figure 1 - Detergent molecules behaviour in solution. At low concentrations, detergents are monomeric (blue area and bellow the blue line) but above the CMC (dashed line) micelles are formed. Membrane protein solubilization and purification requires concentrations above the CMC (green area) (adapted from [17]).

Typically, the experimental approach for MPs solubilization is the use of detergents, with the capacity of mimics the cell membrane [9,16,17]. Detergents are a group of molecules structurally dissimilar but share a common amphipathic character, while, they are composed by a polar head group and a hydrophobic tail [17,9]. These molecules exhibit peculiar properties in aqueous solutions: they are able to behave as solubilized monomers or as micelles at higher concentrations (Fig. 1). The detergent concentration at beginning of micelles formation is called the critical micellar concentration (CMC) and it is a characteristic parameter of each detergent. Besides the type of detergent, the CMC is dependent of temperature, micelle size, interaction between the polar groups, pH and ionic strength in case of charged detergents, and presence of protein, lipid or other detergent molecules [9,17,16].

Generally, the CMC decreases with the length of the alkyl chain detergent and increases due to introduction of double bonds and branch points [9]. The CMC is a very important concept in MPs solubilization since only above this concentration the detergents gain the ability to mimic the natural lipid bilayer environment and keep the membrane protein in solution for a physiologically relevant state [9,17].

For any membrane protein research study, the choice of a suitable detergent is essential for a right solubilization and manipulation of MPs [5,8–10,15]. There are several detergents available commercially and sometimes one of the reasons that makes the study of membrane proteins so hard it is the lack of strategies for detergents screening [8,15,17].
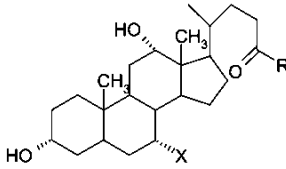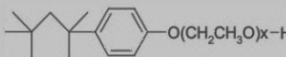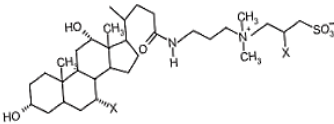
In spite of the detergents structure varies extensively, the main goal is to promote a properly interaction with the protein, maintaining its structure and structural properties [5,15–17]. Detergents are classified according to their structure into four major categories (Table 1): ionic detergents, bile acid salts, nonionic detergents and zwitterion detergents [9,17]. Also, detergents are classified as harsh or middle depending on the structure and the ability to preserve the activity and physiological structure of the target MPs. The harsh detergents maintain hardly the structure of proteins but the probability of denaturation is high. While with the mild detergents there is a good chance that a physiological relevant structure be maintain [17].

In general, the harshness of the detergent increases as the head group becomes charged and as the size of head group and hydrophobic tail rise [17]. For example, few membrane proteins are stable in SDS (ionic detergent with a small and charged head group). But nonionic detergents, as Triton X-100 (possess an uncharged hydrophilic head) are able to preserve MPs structure [9,17]. As described in Table 1 the nonionic detergents with a short hydrophobic chain can be deactivated unlike other detergents with a longer hydrophobic tail. Then, knowing the characteristics of all types of detergents we can predict the behavior of the MP in a specific detergent solution [9,17].

Unfortunately, the most important conditions for detergent selection are difficult to predict [15]. Despite the range of detergents disposable, no single detergent has emerged as the

choice as judge by several studies published in literature [5,10]. Although solubilization procedures are vast on literature, the use of detergents is still largely empirical [2,16].

In first place, the stability of many membrane proteins is difficult to achieve in detergent solution, being sometimes the presence of lipids a major requirement [1,5,15]. Furthermore, there is no guarantee that exist a suitable detergent in which interest protein is stable [15].

Table 1 – Types of detergents and their main characteristics (adapted from [9])

Type of detergent Structure	Characteristics	Strengths/ Weakness	Structures solved	Examples
Ionic detergents $CH_3(CH_2)_{10}CH_2O-\overset{O}{\underset{O}{S}}-O^-$	• Charged head group (+ or -) • Hydrophobic hydrocarbon chainor steroidal backbone	• Extremely effective in MPs solubilization • Generally denaturing to some extent	bR, LCHII, DAGK,	SDS
Bile acid salts	• Rigid steroidal groups on backbone • They have a polar and an apolar face, instead of a defined head group • They form small kidney-shaped aggregates unlike the spherical micelles	• Mild and not generally deactivating	Ca^{2+}-ATPase	Sodium deoxycholate Sodium cholate
Nonionic detergents $RO(CH_2)x\text{-}CH_3$ $O(CH_2CH_3O)x\text{-}H$	• Uncharged hydrophilic head • They break lipid-lipid interactions and lipid-protein interactions rather than protein-protein interactions	• Mild and non-denaturing able of isolate MPs in its biologically active form • Short chain forms can be deactivating	Ca^{2+}-ATPase	n-octyl-ß-D-glucopyranoside Triton X-100 Triton X-114
Zwitterionic detergents	• Combines the properties of ionic and nonionic detergents	• • Uses in structural studies • Generally more deactivating than nonionic detergents	Rhodopseudomonas sphaeroides reaction centre, rhodopsin	CHAPS CHAPSO

Finally, the discrepancy of detergent's effects on distinct proteins and the complexity and specificity of protein-lipid interactions difficult the construction of a general solubilization and reconstitution strategy [10].

Thus, the present successes in MPs structure resolution are largely dependent on the development of novel detergents or detergent mimetization [17]. A better understanding of parameters that influence the solubility and functionality of MPs in detergent solutions will be critical to new advances in this area [9].

1.1.2 A challenging study – troubleshooting in hands-on of membrane proteins:

Integral membrane proteins presents a problematic experimental behavior that turns its manipulation harder than the study of their soluble counterparts [2,8–10]. There are several reasons that become this area one of the most challenging in proteins chemistry and these challenges remain the same since the resolution of the first membrane protein's structure [1,2,7].

A major challenge in MPs area is to work outside the natural lipid environment [9] . In a natural environment, membrane proteins maintain their structure and function but when removed from their lipidic environment they lose their structural integrity and become more unstable and flexible. This can lead to aggregation, which reduces the efficiency of all separation techniques and a decrease or loss of catalytic activity [1,5,9].

 All biophysical methods to determine structure and function such as NMR, X-ray crystallography or circular dichroism are impossible to be perform in its native environment [9]. Then, is required the interest protein extraction from their lipid environment and their consequent solubilization [1,9,15]. This leads to additional requirements because detergents may hamper the purification process and restrict the applicability of certain techniques because they interfere with separation processes [1,5,9]. So, the selection of a suitable detergent must preserve the functional state of membrane protein and at the same time be compatible with the isolation step and structural studies performance [1,2,5,9,15]. Moreover, despite these molecules appear to be essentials for catalytic activity retention, if the choice of detergents or concentrations is not adequate, irreversible inactivation of the protein by detergents could occur [5].

On the other hand it is necessary to obtain sufficient amounts of the interest protein for structural studies [2,4,7,9] The natural expression of these proteins is generally weak and the most of pharmaceutically relevant MPs are present in low concentrations in cell membrane [2,4,9,13]. The first MPs structures described in the literature were resolved due to their natural abundance, but in the majority of the cases is necessary a recombinant MP overexpression in a heterologous system [2,4,9,13].

The heterologous expression of MPs increase the yields, but there are some difficulties associated: protein aggregation into cytoplasm, incorrectly fold and the requirement of post-translational modifications that are unavailable in bacterial hosts [1,13]. New strategies can

reduce some of these troubleshootings, for example, the aggregation of foreign MPs in heterologous systems can be reduced and targeting to the membrane favored by using low-copy-number of plasmids with weak promoters and fusion proteins between the MP of interest and a protein known to target the cell membrane [4]. So, the development of new capable heterologous expression systems and suitable strategies remains essential to obtain enough high-quality recombinant protein for posterior applications [4].

Despite the inherent problems of MPs studies, advances in this field are the major key for increasing the number of resolved MPs structures [1,7,9]. Recently, several progresses has been made on production of MPs applying several host microorganisms and [7,2,4].

1.2 Purification of membrane proteins:

Crystallization and other posterior structural, biochemical and functional assays require significant amounts of relatively pure protein in their native conformation, active, stable and homogeneous form [4,5,13,18]. With larger amounts of protein, there are higher chances to fulfill these conditions with a suitable purification strategy [5,13,18,19]. Typically, large quantities of protein in target samples promotes its retention due to the favorable protein/impurity ratio [13]. These levels of the target MP can't be obtained easily from natural sources, so the development of a suitable overexpression system is crucial [1,2,5,13]. The purification procedure should be fast and efficient in order to increase protein yields and to prevents significant decline of enzymatic activity [4,19]. The design of a functional purification strategy involves a significant number of trials where several approaches can be developed in order to facilitate the purification of membrane proteins and maintenance of active physiological states [4,10].

Preliminary processes such as solubilization may contribute to a partial sample purification [5,20]. Indeed, during the membrane solubilization step many contaminants which may interfere with the isolation procedure can be totally removed. Also the quality and reproducibility of sample preparation may be a critical parameter in solubilization and subsequent purification steps [20].

Unfortunately, the protocols used for purification of MPs are more demanding than for soluble proteins, while   require the use of detergents to ensure an accurate protein solubilization, homogeneity and integrity [1,2,6,15]. But if membrane protein is unstable in detergent solution it is not advantageous apply this kind of strategies. Alternatively, in purification can be used lipid/detergent mixtures, in which hydrophobic regions of the protein are solvated by the nonpolar groups available in a dispersed lipid solution [2,5,9]. Another troubleshooting in the purification of MPs is that a higher degree of delipidation can have serious repercussions on enzymatic activity [5,17]. Although there is a gap in systematic studies, it is well known that often the association of a minimal number of lipids to the membrane associated domain is important to the maintenance of its integrity and stability [2,5,17]. In some cases, despite

the high purification degree achieve, MPs may aggregate during the concentration step [17]. Furthermore, high delipidation time is also a significant factor, since the probability of structural and activity loss of MP increase [5]. Therefore, due to MPs instability and lipid dependence, the design of the purification procedure must be perform in order to decrease the number and cycle time of isolation steps avoiding the delipidation phenomenon [5].

1.2.1 Chromatographic techniques for membrane proteins purification

An appropriate selection of accurate techniques and suitable working conditions are essential for the success of every purification strategy [21]. Normally, the range of separation techniques available is limited and attempts the   full isolation of MPs, however in the majority of the experiments only partial purification of the protein can be achieved [5]. So, it can be advantageous exploit well-known biochemical protein properties to design a successful chromatographic technique [10].

Chromatography has become a preferential technique for separation, perhaps due to its high resolving power and the existence of several chromatographic methods with different selectivity [18,21]. In contrast to other separation procedures that are limited to certain types of substances, chromatography can be applied to a wide spectrum of compounds [10,22].

In general, the components of a complex mixtures are separated according to their physico-chemical properties, and preferably distributed in a stationary phase or a mobile phase [18,23]. Typically, the stationary phase, insoluble in the buffer, is packed into a column and a mobile phase is pumped through this stationary phase [10,23]. There are five main stages in chromatographic process (Fig. 2): equilibration, loading of sample, washing, elution of retain molecules and matrix regeneration [23].

The equilibration of the column ensure that initially all stationary phase is in the same optimal conditions for the target biomolecule binding [14,23]. The sample loading consists in the injection of a specific quantity, depending if the process have an analytical or preparative purpose [23]. After the loading, the sample goes through the column by the continuous addition of mobile phase and their components can stay in the mobile phase or be distributed in the stationary phase by specific interactions [23]. In washing stage impurities that don't interact with the stationary phase are removed from column using the same buffer of column equilibration [14,23]. The elution of retained species is carried out through an elution buffer that decrease interactions between the matrix and target biomolecules [23]. In general, subtle modifications on the mobile phase composition allow the elution of some contaminating proteins or other compounds weakly adsorbed to the matrix [23].

On the other hand, significant alterations of mobile phase are generally applied to promote elution of the target protein [23]. During elution, strongly adsorbed proteins move more slowly than the weakly bound biomolecules, so their elution is slower [23]. Alterations on

ionic strength are the most common mean of eluting adsorbed proteins and this strategy can be applied using gradient or stepwise profiles [23].
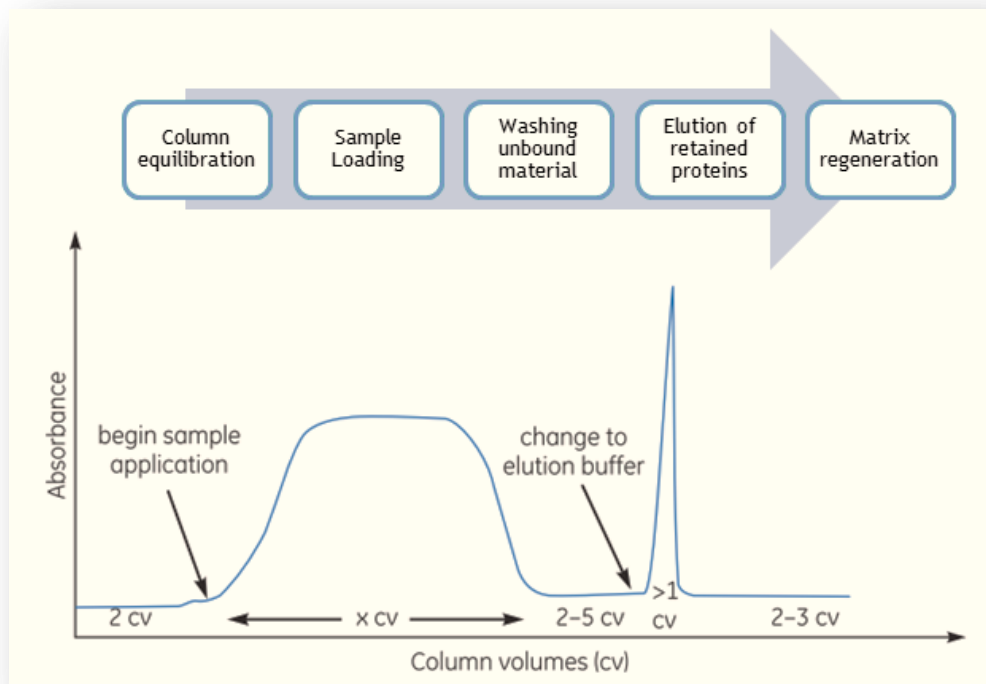
Figure 2 - General approach in a chromatographic procedure (adapted from [14]).

The regeneration step is a very important cleaning procedure that maintains the binding capacity, selectivity and support life-time [23]. After each run, precipitate, denature proteins and other biomolecules can remain in matrix and they have to be removed in order to maintain the native matrix characteristics [23]. This cleaning procedure depends on matrix type but in general is characterized by highly acid or basic environmental conditions, low or high-salt concentrated solutions, organic solvents as 70% ethanol or some detergents [23].

During the last decades, several chromatographic techniques have been used for MPs purification, as single step or in combination with other techniques [5,10]. Among the most widely used separation techniques are the immobilized metal-ion affinity chromatographies (IMAC) or other affinity chromatographic methods, size-exclusion chromatography (SEC) and ionic interaction chromatography (IEC) [1–3].

Affinity purification techniques have become very popular in protein isolation fields because they allow efficient and fast purification with few steps [4,5,13]. These characteristics are ideal to prevent the removal of native lipids and preserve the MP integrity [4]. This chromatographic procedure promote specifics interactions with a recognition Tag, that is expressed together with the target MP, in its amino(N)-terminus or the carboxyl(C)-terminus. So, only the proteins having this affinity tag or a natural affinity for specific ligands on the matrix will be retained [5,15,23]. However, the presence of tags may affect the MP

expression levels, disturb the folding process, increase the protein susceptibility to proteolytic degradation and lead to conformational modifications [4,5]. Additionally, purifications using affinity chromatography in a single-step are unusual and regularly a low-cost technique is used primarily to avoid potential damages into the affinity column [5]. Another current problem for tags applications, is the need of a specific proteolytic cleavage that can result in heterogeneous fragments preparations [5]. Indeed, despite the advantages, only three MPs structures described were produced as fusion proteins and in all cases using Histine tags (His-tag) and IMAC to complete purification, being this strategy the most widely applied affinity strategy [5,13].

Ion-exchange chromatography (IEC) is a suitable separation technique often used in early stages of purification [5,10]. The biomolecules are separated based on electrostatic interactions between protein and the charged ligands, cation or anion exchangers [5,23]. Thus, there are two types of IEC: a cation exchange chromatography where anion exchangers interact to positive charged molecules and a anion exchange chromatography where cation exchangers bind anionic molecules [5,23]. Generally, compounds of the load sample are retained at low salt concentrations and the elution can be achieved by increasing the ionic strength or by changing pH [23]. The many advantages of this method are the support low cost, high flow rates that allows large-scale trials and the well described protein-matrix interactions and binding/elution conditions [5].

Another issue is that detergents must be used with some careful during protein purification by IEC. Almost all uncharged detergents can be used, since they are able to solubilizing MPs. However, the ionic detergents might interfere with the ionic chromatographic performance step [5].

1.2.2 Hydrophobic interaction chromatography:

Hydrophobic interaction chromatography (HIC) is a powerful separation technique where protein purification is based in hydrophobic interactions between hydrophobic ligands immobilized on matrix and non-polar regions on proteins surface [5,18,21]. This type of chromatography have been developed to facilitate protein purification, displaying binding characteristics complementary to other chromatographic techniques, and have been applied successfully in early crude fractionation stages [10,18]. The biomolecules with hydrophobic characteristics are adsorbed on stationary phases at high salt concentrations, but below those required for protein precipitation [18,23,24,5]. Therefore, HIC is a typical separation technique apply immediately after a salt precipitation step [5]. The elution is achieved by decreasing the ionic strength or the polarity of mobile phase and by adding detergents or others agents. Namely, urea and chaotropic salts, recognized as displacers of proteins, have been used mainly with proteins that fail to elute at low salt concentrations, which is the case of membrane proteins [24].

The main factors affecting protein chromatographic behavior in HIC are protein hydrophobicity, their surface hydrophobicity distribution and molecular size [18,21,24]. The protein hydrophobicity depends on hydrophobicity of the exposed and buried amino acids as the higher percentage of hydrophobic amino acids on surface more hydrophobic is the protein [18,24]. The number of hydrophobic amino acids, their different distribution and hydrophobicity are characteristic of each protein, so they can improve the selectivity promoting the isolation of a specific protein from a complex proteomic extract by HIC [18,24]. However, the behavior of a protein in HIC is affected by the operating conditions [24]. Specifically, characteristics of the mobile phase (salt type, ionic strength and pH), properties of stationary phase (chemical nature and chain length of the backbone, ligand or matrix type and degree of substitution) and temperature [5,18,21,24].

An ideal salt selection results in significant alterations in protein retention and also in separation selectivity since they are able of handle the protein conformation, hydrophobic interaction forces and number of the water molecules that hydrate the target protein [24,18]. By changing the salt type on the elution buffer takes to substantial variations on protein retention and selectivity [18]. There are two types of salts depending of their effects on hydrophobic interactions: chaotropic and antichaotropic salts.

The Hofmeister series is followed by the influence of different salt types on hydrophobic interactions as seen in figure 3. The antichaotropic salts, at begin of the series, are considered water structuring because they promotes hydrophobic interactions and protein precipitation (salting-out effect). The chaotropic salts, at the end of the series, randomize the structure of the liquid water and thus tend to decrease the strength of hydrophobic interactions (salting-in effect) [24,18].

Increasing salting-out effect

Anions: PO_4^{3-}, SO_4^{2-}, CH_3COO^-, Cl^-, Br^-, NO_3^-, ClO_4^-, I^-, SCN^-

Cations: NH_4^+, Rb^+, K^+, Na^+, Li^+, Mg^{2+}, Ca^{2+}, Ba^{2+}

Increasing salting-in effect

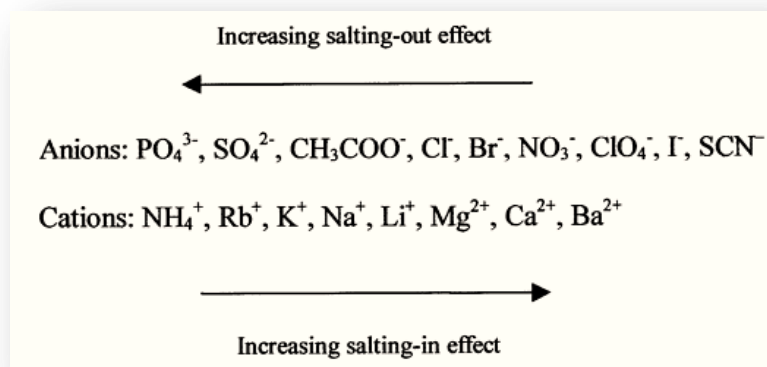**Figure 3** – The Hofmeister series (adapted from [18]).

The matrix selection also greatly influences the hydrophobic protein-ligand interactions. So, sometimes a ligand screening may be necessary. The most used ligands are linear chain alkenes (butyl and octyl) and some aromatic groups (phenyl) [18,24]. Phenyl matrix is recognized due to their mixed hydrophobic and aromatic (π-π) interactions. Tanford and

coworkers (1972) refers that at a constant degree of substitution on the matrix, the n-alkane ligands constitute a homologous series in a hydrophobicity scale (Figure 4) [25,18].
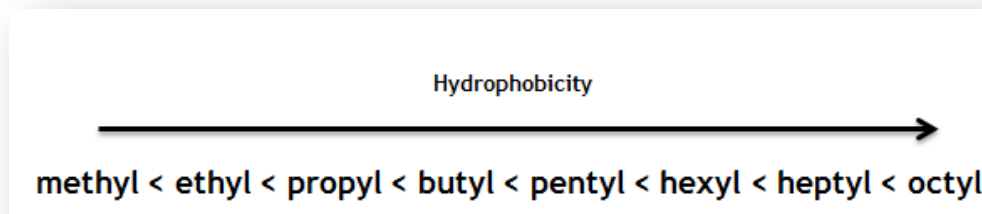

Figure 4 – Hydrophobicity scale of n-alkane ligands (Adapted from [18]).

Generally, the hydrophobicity and the strength of interaction between the protein and the matrix increase with the rise of n-alkyl chains length, however the selectivity and resolution can compromise and decrease [18,24,26]. On the other hand, an increase in the degree of substitution leads to an increase in the protein binding capacity of the stationary phase, due to the higher probability of forming multipoint attachment between the protein and the ligand. The disadvantage is the protein elution without the use of harsh conditions which can leave to biomolecule denaturation [18,24].

Indeed, HIC appears to be a good alternative to reverse-phase chromatography (RPC), since exploits the hydrophobic properties on a more polar and less denaturing environment than RPC, wherein apply non-polar solvents for the protein elution [5,18]. Moreover, the biomolecules damage is slightest than on affinity, ion-exchange or reversed-phase chromatography due to the weaker interactions [18]. The mild conditions allow the maintenance of biological activity and protein structure. Besides this, it was proposed that the Van der Waals forces, referred above as the main responsible for the support of MPs on cell membrane, are also the major factor for hydrophobic interactions and, consequently, to maintenance of biological activity of proteins in HIC [18,23].

Successful applications of HIC to MPs purification are rare due to the strong binding of detergents to matrix. The process involves the use of detergents in order to reduce the major obstacles and three essential steps may be followed. Firstly, most of the detergent must be removed before the HIC step or alternatively inject crude cell lysates directly. Moreover, is recommended the use of these commercial matrices: phenyl-sepharose or octyl-sepharose with middle phosphate concentrations. Finally, a high detergent concentration helps to promote the elution of the target protein [5].

1.3 The catechol-*O*-methyltransferase enzyme

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6.) is a magnesium-dependent enzyme widely distributed in mammals whose highest activity levels are found in liver, kidney and gut wall. This enzyme catalyzes the methylation of catechol compounds transferring a methyl group from S-adenosy-L-methione (SAM) to a hydroxyl group on catechol substrates, producing the *O*-methylated catechol and S-adenosyl-L-homocysteine (SAH) (Figure 5) [27–32].
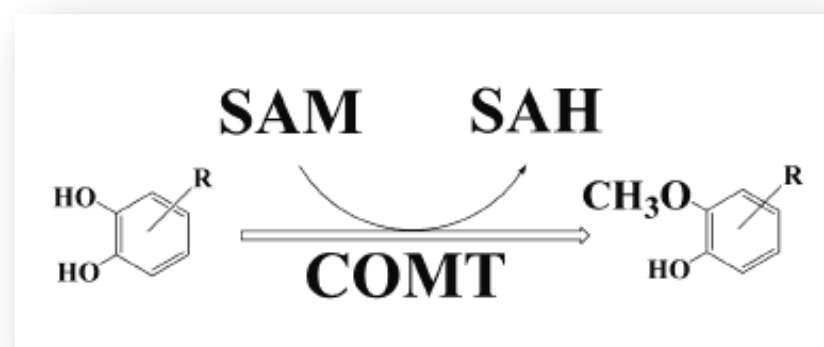
Figure 5 - The *O*-methylation of the catechol substrate catalyzed by COMT (Adapted from [33]).

COMT metabolizes a large range of catechols including catecholamines, as dopamine, norepinephrine, epinephrine, their hydroxylated metabolites, catecholestrogens, ascorbic acid, dietary phytochemicals and medicinal compounds such as triphenols, L-methyldopa or flavonoids [27,28,32].

1.3.1 Physiological functions

The main physiological role of COMT is the inactivation of biological active or toxic catechol compounds and some other hydroxylated metabolites, both endogenous and exogenous [27,28,34,35]. COMT take part on detoxification pathways since it acts as an enzymatic detoxicating barrier between the blood and other tissues, such as intestinal mucosa and the brain, protecting them against the harmful effects of xenobiotics [28,27,31]. Also is suggest that COMT plays an important role in regulation of both dopaminergic and noradrenergic systems in normal brain and in modulation of the dopaminergic tone, directly or indirectly, in the kidney and intestine tract [27,28,32]. Moreover, COMT protects the embryo and the placenta development during the first trimester of pregnancy from activated hydroxylated compounds [28]. In addition to its roles, COMT is important in the metabolism of catechol drugs used in the treatment of hypertension, asthma, and Parkinson disease [36].

1.3.2 COMT Gene and enzyme isoforms

COMT presence was reported in prokaryotes and eukaryotes, namely bacteria, yeasts, plants, invertebrates and vertebrates [27]. In mammalians, COMT protein typically appears in two distinct isoforms, according to their subcellular location: a soluble form present in cytoplasm (S-COMT) and a membrane form associated to rough-endoplasmatic reticulum (MB-COMT) [27,28,35,37,38].

Both COMT isoforms are coded on a single gene localized on chromosome 22, band q11.2. The COMT gene in humans contains six exons, being the first two exons noncoding and the translation initiation codons for both isoforms are located on the third exon. Two separate promoters control the synthesis of two analogous transcripts with different sizes: a 1.5 kb transcript constitutively expressed to MB-COMT translation and a 1.3 kb transcript, that is subject to tissue-specific transcription regulation, to S-COMT translation as seen in figure 6 [27,28,35]. The longer transcript also translates the soluble isoform by the leaky scanning mechanism of translational initiation [27,28,39].
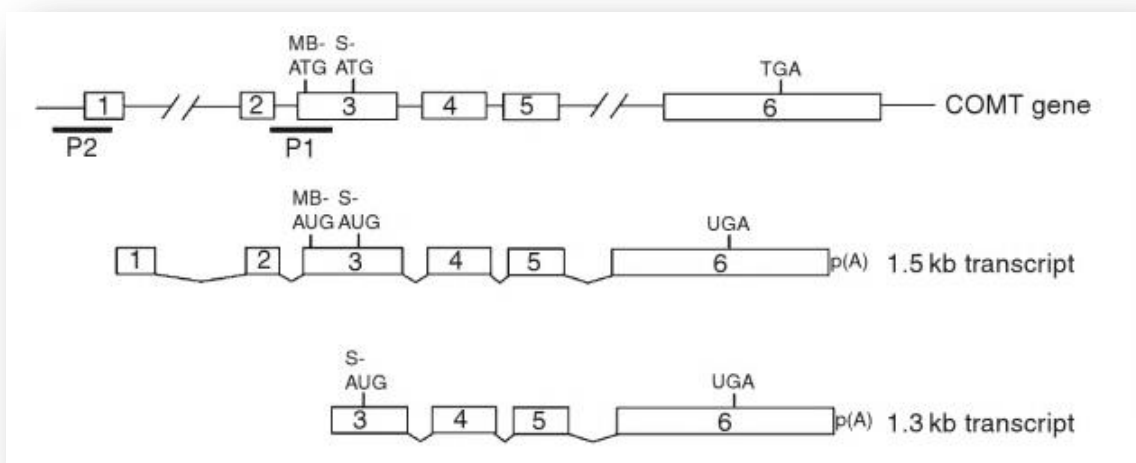
Figure 6 – The COMT gene and transcripts. The gene encodes two primary transcripts of 1.5 and 1.3 kb from the P2 and P1 promoters, respectively (Adapted from [72]).

1.3.3 Genetic polymorphisms

Genetic variability have been reported on human red blood cells (RBCs) and liver [27]. In human tissues, genetic polymorphisms trigger to variations in the COMT enzyme activity levels. Then, COMT enzyme activity presents a trimodal distribution with high (COMT$^{H/H}$), intermediate (COMT$^{H/L}$), and low (COMT$^{L/L}$) activity groups [27,28,36]. These polymorphisms lead to 3- to 4-fold differences in COMT activity in human erythrocytes and liver and, according to segregation analysis of family studies, they are concordant with an autosomal codominant inheritance pattern [28,36]. These functional COMT polymorphisms are

correlated to a Guanine to Adenine transition, at codon 108 in S-COMT case or at codon 158 in MB-COMT, resulting in Valine to Methionine (V158M) substitution in the polypeptide chain. The polymorphism was also related with individual variation in COMT thermal instability. In general, the Met108/158 variant is associated with low enzymatic activity, while the Val108/158 variant is associated with high activity [35,36]. The low activity phenotype are also related with a thermolabile enzyme, even at 37°C, being that the site of the low COMT mutation is presumably on the structural locus [28,36]. This evidence also indicates that the low thermal stability is proportional to low COMT activity [28]. Moreover, seeing that there is only one COMT gene without any known tissue-specific splice variants, it is possible that the codon 108/158 polymorphism is responsible for functional alterations of COMT in all tissues [28,35].

In a study with Korean patients, was found a polymorphism at codon 22/72 (soluble/membrane-bound form) caused by a nonsynonymous SNP (guanine to thymine transition) that leads to alanine to serine substitution (A72S) [36]. Using as reference group Ala/Ala genotype, they identified the combined genotype (Ala/Ser or Ser/Ser), at codon 22/72, as allele risk for schizophrenia. Moreover, they show that the A72S substitution was related with reduced COMT activity levels. These results supports the general idea that COMT haplotypes implicated in schizophrenia are associated with low COMT expression [36].

1.3.4 Disorders associated to COMT

Multiple studies have been done in order to search correlations between COMT genetic variations and some diseases [27,28,36,39–41]. In spite of most of these studies failed to establish significant correlations, some relationships with COMT gene polymorphisms has been detected.

Genetic polymorphisms and variations on enzymatic activity levels of COMT have been associated to several pathologies such as psychiatric disorders [28,32,36,41–43], neurodegenerative diseases [28,32,35,41] estrogen-induced hormonal cancers [28,44,45], cardiovascular diseases [46,47] and an increase in consumption of addictive substances [28,41] (Table 2).

Table 2 – Summary of COMT associated disorders and their genotype and phenotype variations.

Type of disorder	Diseases	Genotype/ Genetic variations	Phenotype	References
Psychiatric disorders	Obsessive compulsive disorder	• Met158 allele	• Low COMT activity	[28,36,41,48–50]
	Schizophrenia	• Val158 allele • Ser22 allele • DNA hypomethylation (MB-COMT promoter)	• High COMT activity • Reduced COMT activity • Low MB-COMT expression	[28,32,41,43,51,52]
	Nervous Anorexia	• Val158 allele	• High COMT activity	[36,49,50]
	Major depression	• Met158 allele	• Low COMT activity	[28,32,41]
Neurodegenerative disorders	Parkinson disease (PD)	• Met158 allele	• Increased risk for PD	[28,35,47]
	Huntington's disease	•	• Decreased COMT activity in the spinal cord	[28]
	Endometrial Cancer Cases	• Elevated CpG methylation of the MB-COMT promoter	• Decreased MB-COMT expression •	[28,53]
Substance Abuse	Polysubstance abuse	• Val158 allele	• High COMT activity	[28,41]
	Late-onset alcoholism (type 1)	• Val158 allele • Met158 allele	• High COMT activity • Low COMT activity	[36,41]

1.3.5 COMT isoforms: S-COMT and MB-COMT

As referred above, COMT occurs in two forms: mainly in an intracellular and soluble (cytoplasmatic) enzyme (S-COMT) and in a less abundant, membrane-bound form (MB-COMT) [27,28,35].

S-COMT is generally located in cells cytoplasm, while MB-COMT is located in the rough endoplasmic reticulum, facing the cytoplasm [28,27]. The S-COMT location in the nucleous

was reported on transfected cells and also of mammary epithelial cells undercertain circumstances, such as increased levels of catecholestrogens [27]. The long transcript of COMT gene, corresponding to MB-COMT, has been found in all tissues, with higher levels in human liver, brain, kidneys, adrenals, and lungs [27,28]. On another other hand, the short transcript, which is related to soluble isoform, is particularly abundant in liver, kidneys, and mammary glands and in very small amounts in the human brain [27,28]. Consequently, the soluble to membrane-bound isoform ratios differ among tissues despite of transcript and protein levels don't have a direct correlation [27]. The S-COMT is much more expressed than MB-COMT in most of human tissues except in human brain where it represent about 30% of total COMT detected [27,28,31,35,37].

Human S-COMT is a nonglycosylated protein containing 221 amino acid residues with a molecular weight of 24.3 kDa [27–29]. The membrane form have a homologue primary sequence but contains an extra peptide in its N-terminal domain and a molecular weight of 30 kDa [27–29]. This extra peptide that contains fifty additional amino acids, 21 corresponding to hydrophobic amino acid residues, constitutes the membrane anchor region of the enzyme. This allows that molecule is suspended in the cytoplasmic side on the intracellular membranes [28,27].

Both isoforms share an identical kinetic mechanism, a similar affinity for co-factor (SAM), an identical requirement of magnesium, an optimal similar pH for maximum activity and same immunological behavior [27,28]. Despite this, S-COMT and MB-COMT are distinct enzymes. Membrane isoform presents a much lower Km and capacity than soluble form [27,28]. This Km value and the specific   distribution of MB-COMT suggest that the two isoforms have a different physiological role [27,28,35].

1.3.6 COMT stability

Numerous purification data suggested that COMT is highly instable and loses its activity rapidly during isolation and storage [19]. Indeed, recombinant hSCOMT can lost 80% of its activity in 30 min at physiological temperature [34]. However, in another study, a stability test showed that the recombinant human S- and MB-COMT proteins were quite stable at 37 °C but their mutant forms lost 20–50% of catalytic activity after preincubation at 37 °C for 2 h [38].

As referred above, a single nucleotide polymorphism (SNP) such as Met158 may decreases the structural stability and thermolability of COMT, even at 37°C. Therefore low thermal stability and the low COMT activity are related [28,36,54]. Also, differences between the apparent stabilities of two protein variants have been seen in a variety of tissues [34].

The COMT enzyme contains some cysteine's in its primary structure and are suggested that their position in secondary structure and hydrogen bonding with the serine hydroxyl group become them susceptible to oxidation [55]. Consequently, the reason for weak stability

behavior of COMT is probably due to the oxidation of the free cysteine-SH (thiol) groups in order to form intra- or intermolecular disulfide bridges [19,55]. Furthermore, studies shows that SAM and magnesium chloride (MgCl$_2$) decreases the cysteine's oxidation protecting COMT against inactivation, which reveals that cysteine's are essential to catalytic activity [55].

Experimental stability studies reveals that ethylenediaminetetraacetic acid (EDTA), MgCl$_2$ and β- mercaptoethanol combined with a reducing agent dithiothreitol (DTT) into buffer has a stabilizing effect on all enzyme preparations [5,19,55]. Frequently, protein stabilization is achieved with the use of protein inhibitors.The protease activity is an issue encountered during the purification of soluble and membrane associated proteins. Proteases inhibitors, such as EDTA, Diisopropyl fluorophosphate (DFP) and 6-Aminohexanoic acid, are currently used in laboratory hands-on [5]. Also sugars, divalent metals, glycerol and some aminoacids appear to have a stabilizing effect on proteins, protecting them against the loss of activity and thermal denaturation [56–58]. Parameters like temperature and pH (7.5 and 8.0 for COMT enzyme) are generally essential to ensure a stable catalytic activity [5,30].

In this work we meticulously select DTT, MgCl$_2$, glycerol and cysteine as COMT stabilizers and also leupeptin and pepstatin as protease inhibitors. Despite of this, further studies should be conducted in order to find the stabilizers with better capacity for maintenance of COMT native folding.

1.4 The membrane associated catechol-*O*-methyltransferase enzyme (MB-COMT)

Initially, due to the MB-COMT activity, was thought that it was only an artifact resulting from nonspecific binding of the soluble form to cellular membrane [30]. But the MB-COMT represent a biochemically distinct molecular entity despite sharing some similarities with the soluble form [30].

In general, MB-COMT presents a much lower Km value and capacity than soluble form, except for the catechol estrogens, which possess similar Km values [27–31]. So, the affinity of MB-COMT for catecholamines appears to be 10 to 100 fold higher than S-COMT and this characteristic appears to be common for different species [27–29]. Also the pI value for both isoforms is different. For the membrane-bound is 6.2 and for the soluble form 5.2 [30].

Concerning about the values of V$_{max}$ for both isoforms, it is visible that they are highly dependent of substrate physiological concentrations [28,30]. Thus, at low concentration of substrate, the activity of MB-COMT (a high-affinity form) is probably more predominant than soluble form activity. The same is confirm for soluble form when a substrate concentration is raised above saturation, which point that S-COMT activity becomes to increase and eventually prevail over MB-COMT activity [30].

In fact, dopamine and noradrenaline levels in striatum and hypothalamus of brain homogenates are low which evidences the MB-COMT importance for *O*-methylation at physiologically low concentrations of catecholamines [28,35,37]. Indeed, MB-COMT is considered the predominant form at dopamine and noradrenaline concentrations bellow to, respectively, 10 µM and 300 µM [28] and it may contribute approximately 40% for the total brain *O*-methylating activity [30].

Despite several studies consider MB-COMT relevance at brain level, a study, containing COMT gene modified mice, indicates that MB-COMT has an important role in peripheral tissues. Also, they detailed that MB-COMT can efficiently compensate the absence of S-COMT [59].

The reasons for these differences between the two isoforms remains unknown but there are some theories establish [27]. Although be poorly characterized, membrane bound portion may interact favorably with subtract rising the affinity of the enzyme [27,28]. Another theory is that no conformational changes in the basic structure or in active site of MB-COMT can increase their affinity to substrate [27,28].

In conclusion, despite the two COMT isoforms have considerable structural similarity it's clearly evident specific differences at physical structure and biochemical properties of both species [30].

1.4.1 MB–COMT expression

As referred above, the development of a suitable overexpression system is crucial to obtain enough quantities of the target membrane protein [2,4,9,13]. However, the overexpression must be criteriously chosen because some systems are not able to express the recombinant protein [60].

Recombinant human MB-COMT (hMB-COMT) was been produced in several eukaryotic and prokaryotic systems such as *E. coli* strains, Sf9 insect cells, transfected human embryonic kidney fibroblast cell lines, human HeLa and hamster BHK-cells [60]. Nevertheless, these systems present as disadvantage the low levels of activity of expressed hMB-COMT [60].

Brevibacillus choshinensis is a gram positive bacterium with an exceptional ability to produce and secrete proteins heterologous proteins [61]. The protein overexpression based on these cells presents innumerous advantages: highly efficiency of biosynthesis and secretion, protein production in an active form, low levels of proteases, host bacterium culture, genetic manipulation facilities and others [60,61].

Recently, this new recombinant expression system was applied for hMB-COMT biosynthesis in its biological and immunological active form [60]. Furthermore, this microsystem allows the production and recuperation, in a single step, of hMB-COMT protein which facilitates the enzyme recuperation and the subsequent purification [60].

1.4.2 MB-COMT Purification

Over the past years, chromatographic separation procedures were used for COMT purification [19,34,62–68]. Nevertheless, only few studies in literature focused in the MB-COMT purification fields.

In literature, there is only a reference to a partial purification of rat MB-COMT from microssomal fraction of rat liver homogenates by anion exchange chromatography [29]. The purification strategy involves the protein solubilization with Triton X-100, injection of solubilized membrane preparation at 0.5% Triton X-100 onto a Resource Q column, prepacked with Source 15Q and preequilibrated with 0,5% Triton X-100 in Tris buffer and the elution of retained species using an increased sodium chloride concentration gradient [29]. Figure 7 represents a characteristic chromatogram obtains in this purification procedure.

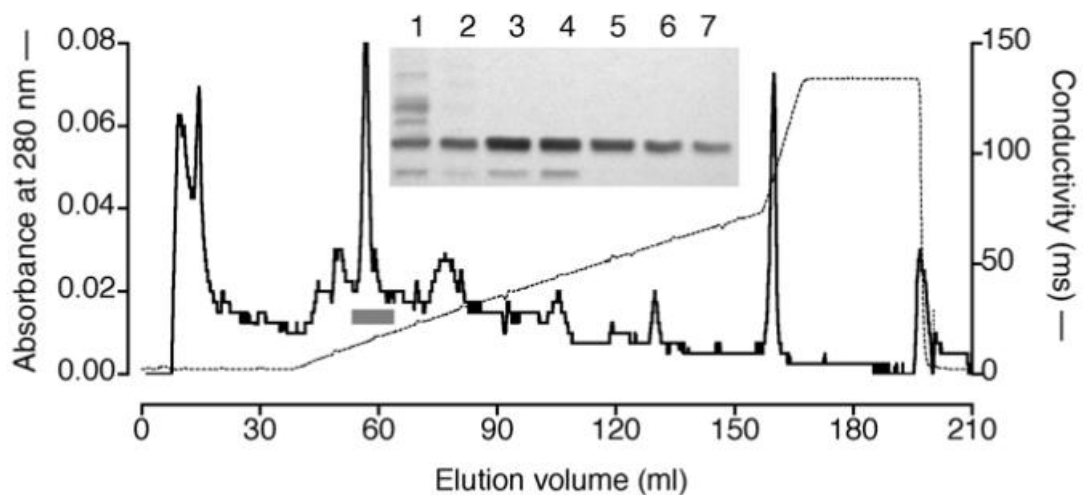
Figure 7 – Typical chromatogram obtain in partial MB-COMT purification from a Resource Q column at pH 7.8 by a NaCl gradient. (Adapted from [29])

Recently, hydrophobic adsorbents were successfully applied on S-COMT purification with excellent results [19,34,63]. So, HIC may be a suitable method for the hMB-COMT isolation due to strong hydrophobic characteristics of membrane anchor region.

Chapter II

Materials and Methods

2.1. Materials:

Ultrapure reagent-grade water for ÄKTA™ avant was obtained with a Mili-Q system (Milipore/Waters). Neomycin (trisulphate salt hydrate), Agar, Bacto yeast extract, glucose, calcium chloride dihydrate, ferrous sulfate heptahydrate, manganese sulfate monohydrate, zinc sulfate heptahydrate, magnesium chloride anhydrous, Ethylenediamine tetraacetic acid (EDTA), lysozyme, Deoxyribonuclease (DNase), dithiothreitol (DTT), Cysteine (L-), Bovine serum albumin (BSA), S-(5'-adenosyl)-L-methionine chloride (SAM) and epinephrine (bitartrate salt) were obtained from Sigma Chemical Co. (St. Louis, MO). Bacto soytone and polypeptone were obtained from Becton Dickinson (NJ, USA). Acrylamide 30%/Bis solution was obtained from BioRad (Hercules, CA). Tris(hydroxymethyl)aminomethane (Tris) and CAPS was obtained from Fisher scientific (Epson, United Kingdom). Glycerol and sodium chloride was obtained from Himedia (Mumbai, India) and from Panreac (Barcelona, Spain), respectively. The full range rainbow protein standards used for estimation of subunit molecular weight and the anti-rabbit IgG alkaline phosphatase secondary antibody were purchased by GE Healthcare Biosciences (Uppsalla, Sweden). Polyclonal rabbit anti-COMT antibody was produced in BIAL using purified recombinant rat COMT [51]. All chemicals used were of analytical grade commercially available and used without further purification.

2.2. Plasmid, bacterial strain and media:

The plasmid pNCMO2-hMBCOMT was the expression construct applied and *Brevibacillus* Expression system (Takara Bio Inc) the system for human MB-COMT expression. *Brevibacillus choshinensis* (*B. choshinensis*) cells growth occurs 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 hMBCOMT production, recuperation and solubilization

Unless otherwise stated, recombinant hMB-COMT was carried out according to the following protocol. The *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 were grown at 30°C and 120 rpm until cell density at 660 nm (OD_{660}) reached 2.6. The next step was the addition of pre-inoculum in 125 ml of 2SYNm medium on 500 mL shake flasks, since the inoculation volume was fixed to achieve an initial OD_{660} of 0.2 units. After a 48h growth at 30°C and 120 rpm, cells were harvested by centrifugation (5000xg, 25 min, 4°C) and stored frozen at -20.0°C until use [60]. The bacterial cell pellet obtained was incubated with 5 mL of lysis buffer (150 mM NaCl, 10 mM DTT, 50 mM Tris, 1 mM $MgCl_2$, pH 8.0) complemented with protease inhibitors (5.0 μg/ml leupeptin and 0.7 μg/ml pepstatin), disrupted by lysozyme treatment (10 mg/ml) for 15 minutes at room temperature and followed by six freeze (-196 °C in liquid nitrogen)/thaw (42°C) cycles. Then, desoxyrribonuclease (1,0 mg/ml) was added to the lysate and the soluble material removed by centrifugation (16000xg, 20 min, 4°C). Full solubilization was carried out with pellet incubation with same buffer used in lysis, at 4°C overnight.

2.4. Hydrophobic interaction chromatography

The chromatographic assays were performed at room temperature in an ÄKTA Avant system with UNICORN 6 software (GE Healthcare, Uppsala, Sweden) equipped with a 2 mL injection loop. All buffers pumped in system were prepared with Mili-Q system water, filtered through a 0.20 μm pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically. The HIC stationary phases understudy, butyl-Sepharose 4FF and octyl-Sepharose 6FF, were purchased on GE Healthcare Biosciences. According to the protocol of Sundberg and Porath (1974), epoxy-Sepharose CL-6B was activated by covalent immobilization of 1,4-butanediol diglycidyl ether on Sepharose CL-6B. The three hydrophobic media were packed according to company guide-lines (20 mL of gel volume) into a C16 glass column purchased from GE Healthcare Biosciences. Screening experiments were performed using different resin combinations and salt concentrations in order to determine the monosodium phosphate concentration required for the retention of hMBCOMT in each stationary phase. Unless otherwise stated, the stationary phase's octyl-, butyl- and epoxy-Sepharose were equilibrated respectively with 250 mM, 375 mM and 850 mM NaH_2PO_4 in 10 mM Tris-HCl, pH 7.8 at a flow rate of 1 mL/min. The solubilized pellet containing hMB-COMT (2 mL with a protein concentration near 7.6 mg/mL) were injected onto the column using a 2 mL loop at the same flow rate and salt concentration. After elution of unretained species at the salt

step, ionic strength of the buffer was decreased to monosodium phosphate-free 10 mM Tris-HCl (pH 7.8) buffer. This condition was maintained with 3 column volume (CV) in order to elute the bound and weakly retained species. Subsequently, the strongly bound species were eluted by a linear detergent gradient from zero to 1% of Triton X-100 in 10 mM Tris-HCl buffer with 3 CV. Finally, a wash step was applied with 1% Triton in Tris 10 mM buffer with 1 CV. In all the chromatographic runs, absorbance and conductivity was continuously monitored at 280 nm and 1.8 mL fractions were collected. These fractions were pooled according to the chromatograms profile obtained and stabilized in an suitable solution compose by 75 mM DTT, 250 mM of cysteine and 20% of glycerol in Tris-HCl buffer 10 mM pH 7,8, as previously described [63]. Subsequently, the samples were concentrated and desalted with Macrosep® Advance centrifugal devices with Omega™ membrane (VWR) and conserved at 4°C for 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. MBCOMT enzymatic assay

The hMB-COMT activity levels were evaluated by the enzyme capacity to convert epinephrine to metanephrine. For this assay, aliquots of the solubilized membrane protein (previously optimized for 2.0 mg of total protein per mL) were added to a constant concentration of epinephrine 1mM (maintaining SAM concentration at 250 µM) during 15 min (previously optimized) at 37°C. The reaction was stopped with 2 M of percloric acid and the samples were processed. The incubation samples were injected and analyzed in a HPLC with an electrochemical amperometric system [69].

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 [70] and as previously described [19]. SDS-PAGE samples were formulate 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 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% β-mercaptoethanol (v/v) for 5 min and then run on 4% stacking and 12.5 resolving gels containing 0,1% SDS, with a running buffer containing Tris (25 mM), glycine (192 mM), 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 the 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, the 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 polyclonal antibody, that cross reacts with the human protein, at 1:2500 dilution in TBS-T 1%. The filters 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 air dried, incubating with 200 ml 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 onto 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, we 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

3.1. Production and Solubilization of hMB-COMT

As referred previously, larger amounts of protein become the purification stage easier and increase the chances of obtain significant quantities of protein in a highly pure, stable and conformational active state. The human membrane associated COMT , like most of membrane proteins, is present in cells at low concentrations [60]. So, a suitable heterologous expression system is a critical requirement to obtain large amounts of our target protein.

As the hMB-COMT was successfully produced in a *Brevibacillus* expression system, using as a construct the plasmid pNCMO2-hMB-COMT, we also use this strategy to obtain hMB-COMT for the posterior purification. So, the first part of this work consisted in hMB-COMT production and solubilization. For production we use the same conditions previously described in literature [60].

Despite *Brevibacillus choshinensis* are able to secrete heterologous proteins to extracellular compartments; our target protein was mostly present in the membrane fraction (Figure 8). Generally, the next step would be the transference of hMB-COMT from membrane fraction to an appropriate hydrophobic environment applying a suitable detergent. This procedure is limited since detergents binds strongly to these matrix. Then, we decided to inject crude *Brevibacillus choshinensis* lysates resultant from solubilization of cellular lysis pellet with an appropriate buffer. Indeed, SDS-PAGE, western and dot blot analysis indicate the presence of hMB-COMT in the solubilized lysis pellet. Figure 8 depicted the results obtained.
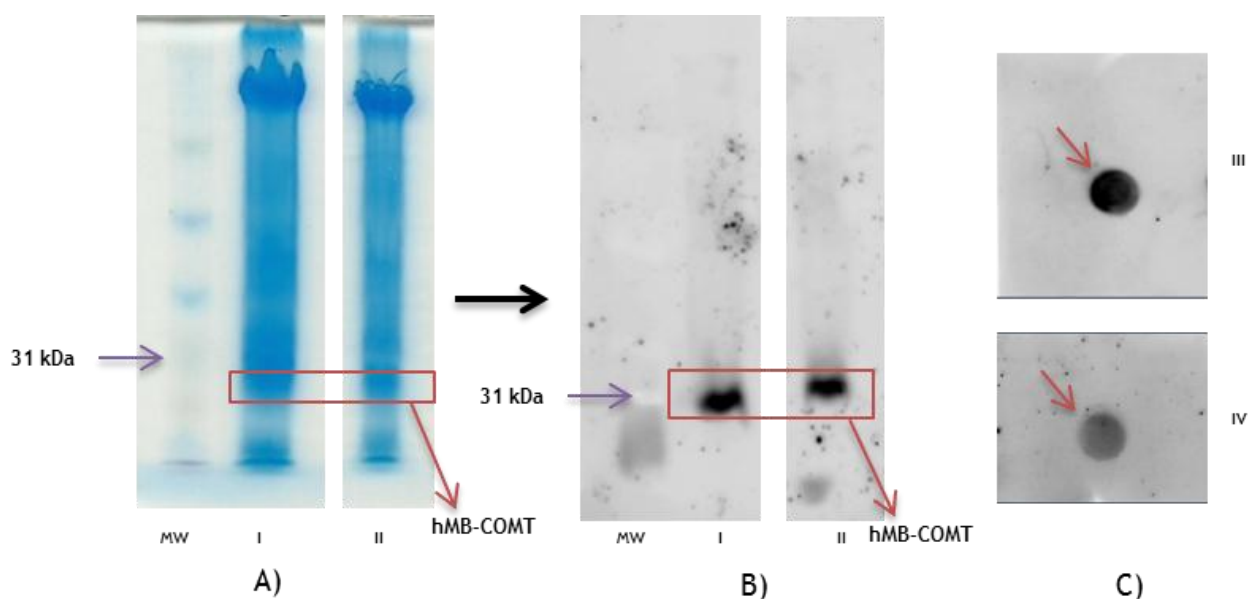
Figure 8 - SDS-PAGE (A), Western-blot (B) and Dot blot (C) analysis shows the presence of hMB-COMT in crude *Brevibacillus choshinensis* lysates injected on hydrophobic adsorbents. Lanes MW – molecular weight standards; Lanes I e II – solubilized lysis pellet injected on butyl adsorbent analyzed by SDS-page and western blot and Lanes III and IV - solubilized lysis pellet injected on butyl adsorbent analyzed by Dot blot. Human MB-COMT position is indicated by the red arrow.

3.2. Purification Trials onto HIC

HIC stationary phases have been use for partial or total purification of high-value therapeutic biomolecules. In fact, HIC is mentioned as a gentler process, leading to it widespread use in downstream purification of biopharmaceuticals [18,19,63]. One of the successful cases in application of hydrophobic adsorbents was S-COMT purification [19, 34, 63]. Owing to similarities between the two isoforms is expected that HIC may be a suitable method for hMB-COMT isolation. HIC was reported on isolation of membrane proteins such cytochrome F-450 [5], a membrane-associated esterase [71] a zinc transporter [10] and others. Hence, in this work we compared the performance of three commercial hydrophobic adsorbents, Butyl-, Epoxi- and Octyl-Sepharose, in terms of retention and recovery elution conditions of recombinant hMB-COMT isolation.

In general, for membrane proteins purification by HIC is required a choice of a suitable salt, detergent and the selection of the most relevant parameters for protein stability. The use of phosphates is highly recommended in HIC [5]. Application of a sample at moderate phosphate concentrations can offers some advantages. Indeed, contaminations are effectively removed by pass-through on column, while hydrophobic adsorption of loaded protein is reinforced [5][18]. Since sodium phosphate precipitates at concentrations above 150 mM at 4°C, purification trials must be driven at room temperature [5]. This may be a limitation if we are working with a thermolabile protein or susceptible to proteolysis (phenomenon minimize at

low temperatures). On the other hand, low temperatures may weaken hydrophobic interactions which is prejudicial to cold-sensitive proteins [5].

Therefore, in initial trials we pretended understand the phosphate effects on hMB-COMT retention onto the Butyl adsorbent. Primarily, we tested higher NaH_2PO_4 concentrations. These initial trials consisted in a sample loading at 1M [Figure 9(A)] or 500 mM [Figure 9(B)] NaH_2PO_4 concentrations followed by a decreasing ionic strength gradient to 0 mM of NaH_2PO_4 and a last step with 10 mM Tris-HCl buffer.

The screening of chromatographic profile (Figure 9(B)) by SDS-PAGE (Figure 10) demonstrated that at intermediate NaH_2PO_4 concentrations, hMB-COMT adsorption is promoted on butyl-adsorbent. The higher molecular weight contaminants are eliminated in peak II [figure 9(B)] with 500 mM of salt but the protein of interest is retained on matrix as judge by a signal absence onto Western blot (Figure 10). So, a low level of contaminants is likely to be eluted with the target protein.

Despite the hMB-COMT presence in *B. choshinensis* lysates extracts (Figure 10), the enzyme doesn`t elute in any of the peaks obtained. So, a mobile phase with basal ionic strength is not sufficient to promote elution. The major reasons for this behavior are probably the higher ligand density of Butyl support and stronger interactions established between hMB-COMT and matrix. .

In general, organic solvents, detergents and chaotropic agents are aggressive elution's agents apply when   strong interactions are establish between the target protein and hydrophobic supports [18]. Therefore, for the refinement of our elution strategy, we used detergents in desorption buffers while they bind strongly to matrix, promoting a selective elution of our biomolecule.
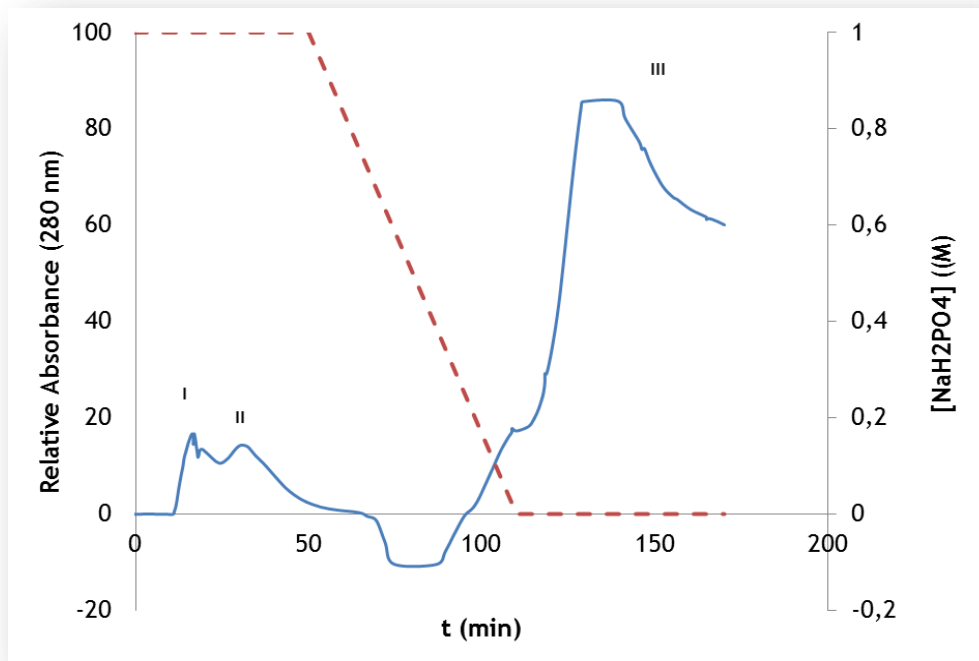
(A)
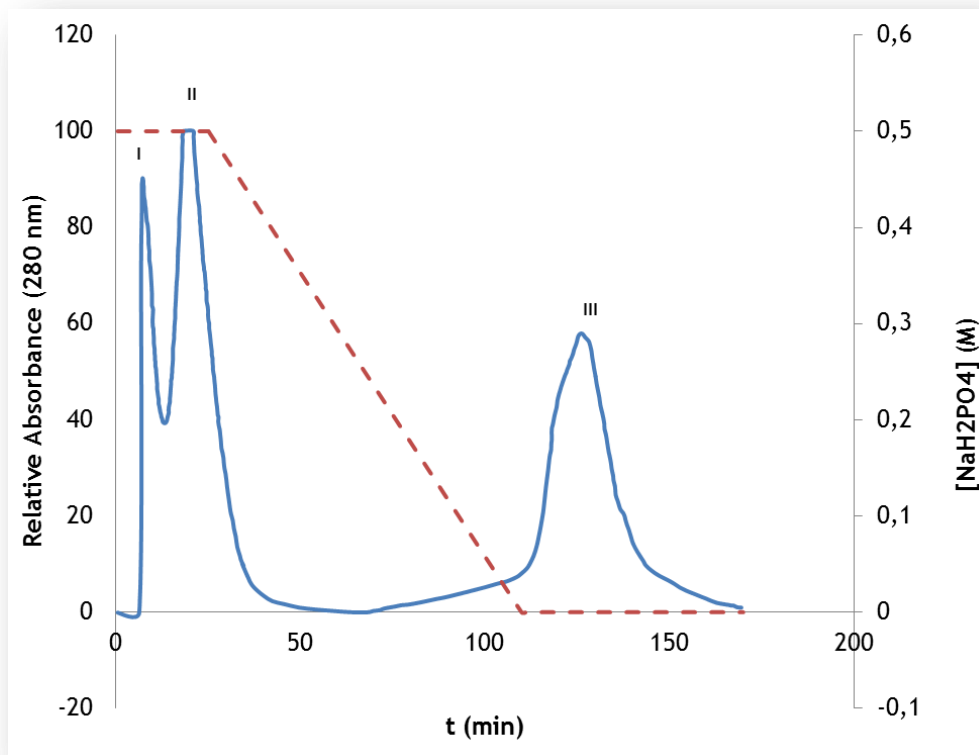
(B)

Figure 9 – Initial purification trials on Butyl-Sepharose. Absorption was performed at NaH_2PO_4 concentrations of 1 M (A) and 0.5 M (B) in 10 mM Tris-Cl buffer (pH 7.8), followed by a decreasing ionic strength gradient to 0 mM of NaH_2PO_4. Desorption was performed with 10 mM Tris–Cl buffer pH 7.8. Blue line represents the absorbance at 280 nm and the dashed red line represents NaH_2PO_4 concentrations.
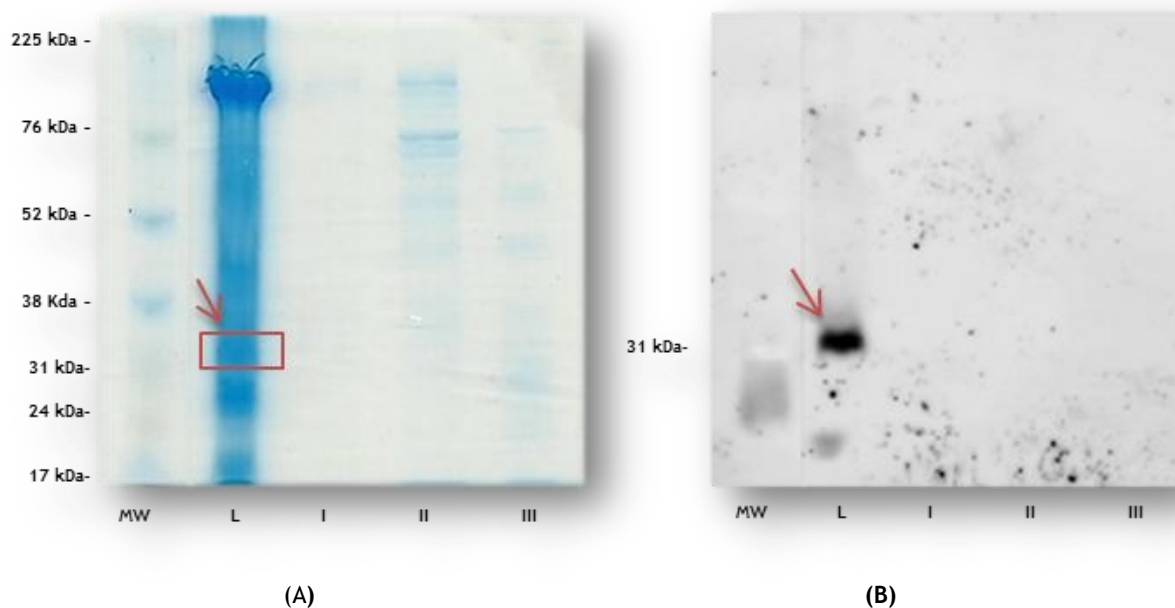
(A)   (B)

Figure 10 – SDS-PAGE (A) and Western blot analysis (B) of samples collected on chromatographic profile of figure 9(B). Lane MW – molecular weight standards; Lane L – solubilized lysis pellet injected on butyl adsorbent; Lane I and II – Peaks I and II obtained at 500 mM NaH2PO4 on butyl- and Lane II – Peak II obtained with Tris-HCl buffer on butyl-. Human MB-COMT position is indicated by the red arrow.

The choice of detergent for purification trials was based in previous studies focusing the influence of detergent selection in biological activity of recombinant human MB-COMT [60]. These studies demonstrate that hMB-COMT solubilization with digitonin increase its biological activity near 450 % relatively to positive control [60]. However, we prefer the application of a less toxic detergent such as Triton X-100 and also efficient in enzyme activity recovery. Triton X-100 is a mild and cheap detergent often used in membrane protein isolation. Also, reduced Triton X-100 with low UV absorbance at 280 nm (such as Sigma's product) may precipitate at cold environmental conditions [5].

Subsequently, we analyzed Triton X-100's effects in hMB-COMT's isolation. Standard procedures in HIC include sample application at high salt concentrations but, due to high hMB-COMT hydrophobicity, we hypothesized its adsorption to resin with a weak ionic strength. Therefore, we firstly applied the sample only with Tris-HCL, followed by an increasing Triton X-100 gradient until 1% detergent in mobile phase and a final step with 1% Triton in Tris-HCl buffer.

The dot blot analysis demonstrated that a Tris-HCl buffer's as adsorption mobile phase doesn't allow hMB-COMT retention onto the matrix, being mainly eluted on the first step as we can observed in figures 11 and 12. So, salt application is required to hMB-COMT's total retention from a butyl-Sepharose column.
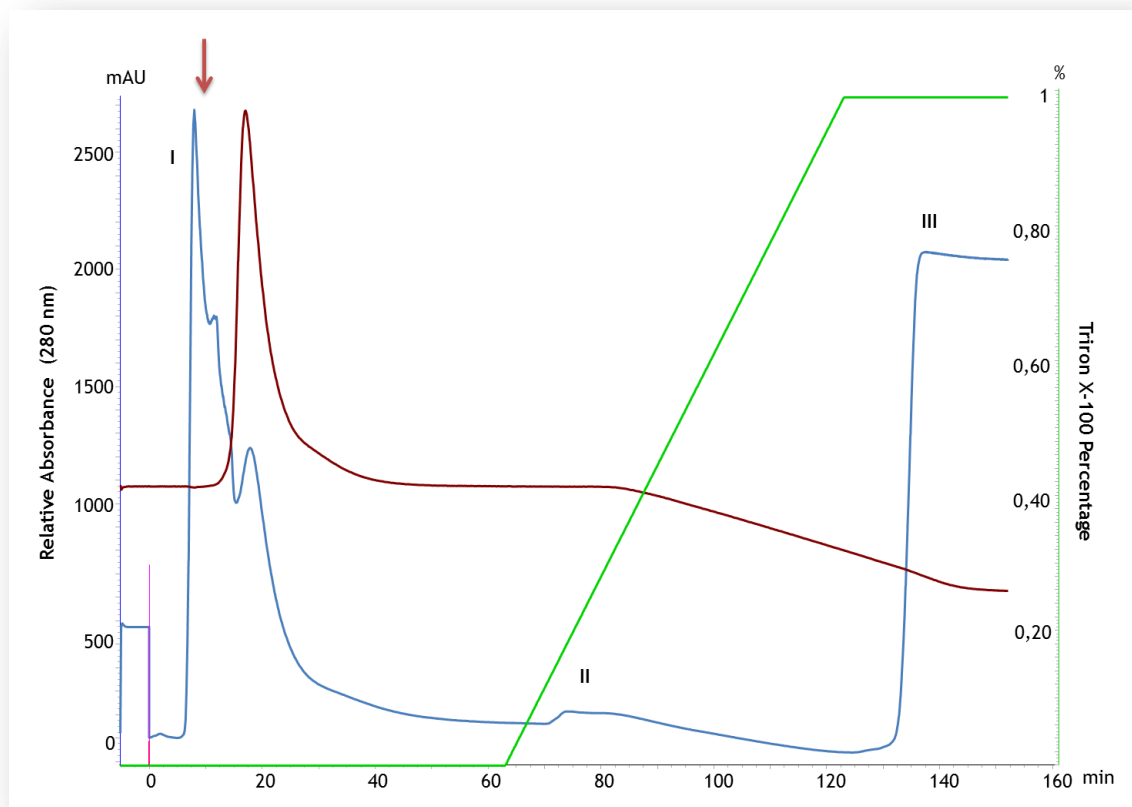
Figure 11 - Initial purification trials on Butyl-Sepharose by HIC. Adsorption was performed with 10 mM Tris-Cl buffer, pH 7.8; followed by an increasing Triton X-100 gradient until a 1% Triton X-100 percentage. Desorption was performed at 1% Triton in 10 mM Tris-Cl buffer, pH 7.8. Blue line represents the absorbance at 280 nm, the green red line the Triton X-100 percentage in mobile phase and the brown line the conductivity.
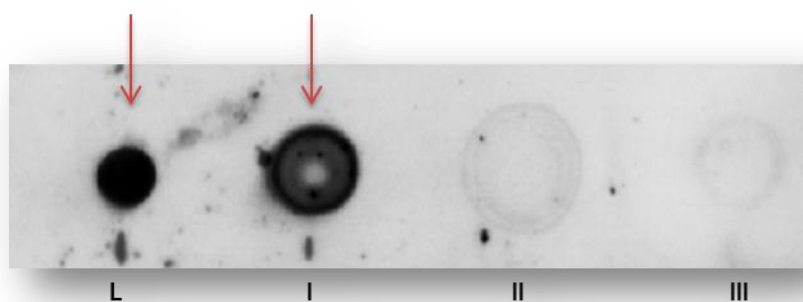
Figure 12 – Dot blot analysis of samples collected in initial chromatographic trials with a Triton X-100 profile on butyl-sepharose (Figure 11). Lane MW – molecular weight standards; Dot L – solubilized lysis pellet injected on butyl adsorbent; Dot I – Peaks I obtained with 10 mM Tris-HCl, Dot II – Peak II obtained at 0,2 % Triton X-100 in Tris-HCl buffer. Human MB-COMT position is indicated by the red arrow.

3.3. Isolation of hMB-COMT on butyl-sepharose by HIC

First trials results described above showed that ionic strength's decrease doesn't contribute to enzyme's elution being necessary the application of a specific detergent. So, for the optimization of chromatographic process, the correct salt and detergent concentration is necessary to allow hMB-COMT's total retention and elution, respectively.

The hMB-COMT isolation strategy comprises cells lysates load at low NaH_2PO_4 concentrations to promote enzyme retention, followed by a Tris-HCl buffer step to remove moderate hydrophobic and weakly retained protein contaminants. An increasing detergent's gradient until 1% was the next step in order to screening the Triton X-100 concentrations favorable to promote elution of the target protein. In the last chromatographic step, we apply 1% Triton concentration to elute totally the high hydrophobic components that may still in the column. Chromatographic assays performed for optimization of hMB-COMT purification are summarized in table 3.

Table 3 – Summary of salt and Triton X-100, used in detergent gradient, concentrations and hMBCOMT elution behavior onto a butyl-Sepharose support.

	[NaH$_2$PO$_4$] (mM)	hMBCOMT retention	hMB-COMT elution behaviour		[Triton X-100] elution (%)
			Tris Buffer	Triton X-100	
Chromatographic methodologies	100	+	++	Residual	0.8 1.0
	250	+	++	+	0 – 1 1.0
	350	++	++	++	0 – 1 1.0
	375	+++	Residual	+++	0.25 0.7 1
	500	+++	-	+++	0-1 1.0

(−) to (+) denotes respectively no retention/elution to total retention of hMBCOMT (+++) on butyl-Sepharose

After the discovery of the correct salt concentration that promotes total hMB-COMT adsorption and screening of detergent percentages that allowing the elution, the following procedure employed was a stepwise strategy. As stated in table 3, a 375 mM NaH_2PO_4 and application of 0.25, 0.7 and 1% Triton X-100 we achieved the total hMB-COMT retention and elution, respectively. Application of 500 mM NaH_2PO_4 was discarded due to the possibility of

contaminants elution together with protein, decreasing the purity level and chromatographic selectivity.
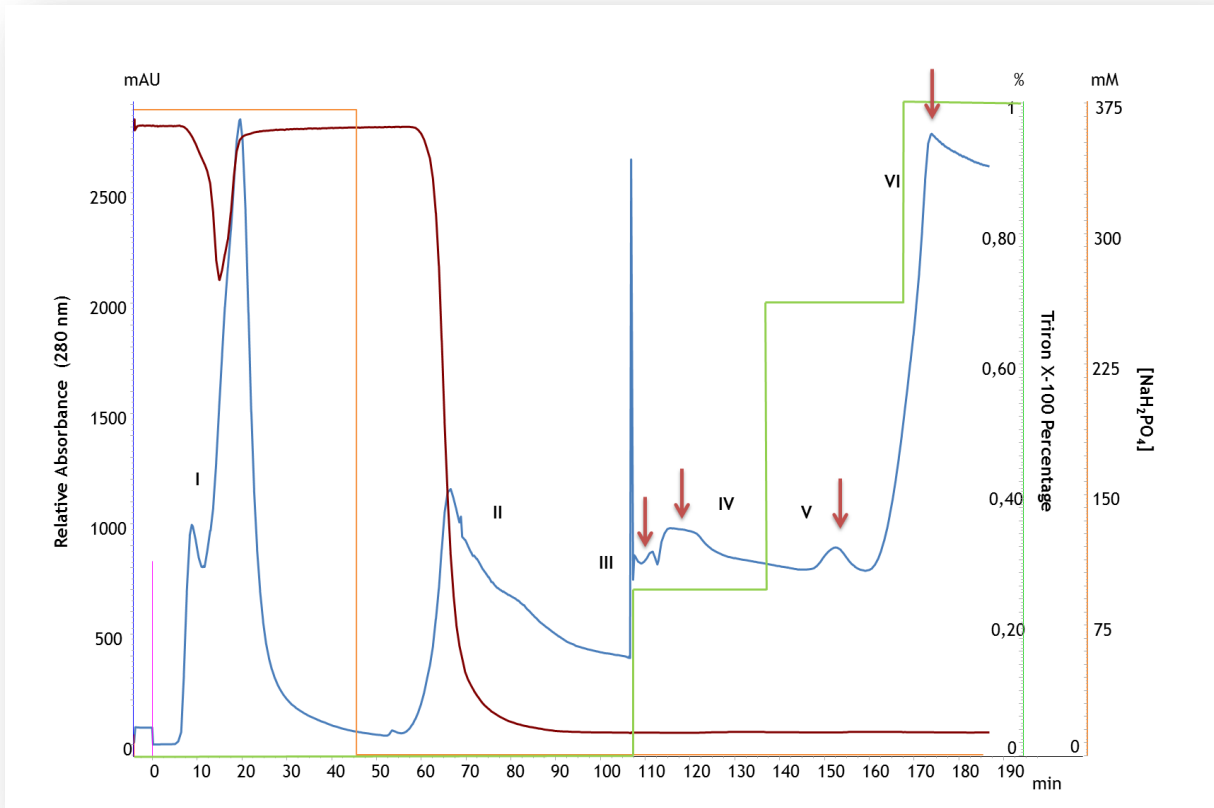

Figure 13 – Chromatographic profile for hMB-COMT isolation on Butyl-Sepharose (HIC). Adsorption was performed at 375 mM NaH$_2$PO$_4$ in 10 mM Tris-Cl buffer, pH 7.8; followed by Tris-HCl buffer step. Desorption was performed at 0.25, 0.7 and 1% Triton in 10 mM Tris-Cl buffer, pH 7.8. Blue line represents the absorbance at 280 nm, orange line the NaH$_2$PO$_4$ concentrations in mobile phase, green red line the Triton X-100 percentage in mobile phase and the brown line the conductivity. hMB-COMT active fractions are represented by a red arrow.

The hMB-COMT chromatographic isolation was achieved with salt and detergent concentrations mentioned above. The hMB-COMT, as the soluble form, was found to elute at four different fractions and distinct detergent concentrations (figure 13). The fraction with stronger interaction can only be eluted with 1% Triton. This multiple peak tendency can be related to different adsorption faces, dimer and trimer formation and some sample contaminants. This fact emphasizes the possibility of different adsorption patterns related to the interaction protein-matrix. Protein position on the column influences the interaction to butyl-, being that if a more hydrophobic zone is oriented to the stationary phase, like the enzyme's hydrophobic tail responsible of cell membrane connection, a stronger interaction is created and so an aggressive elution is require.
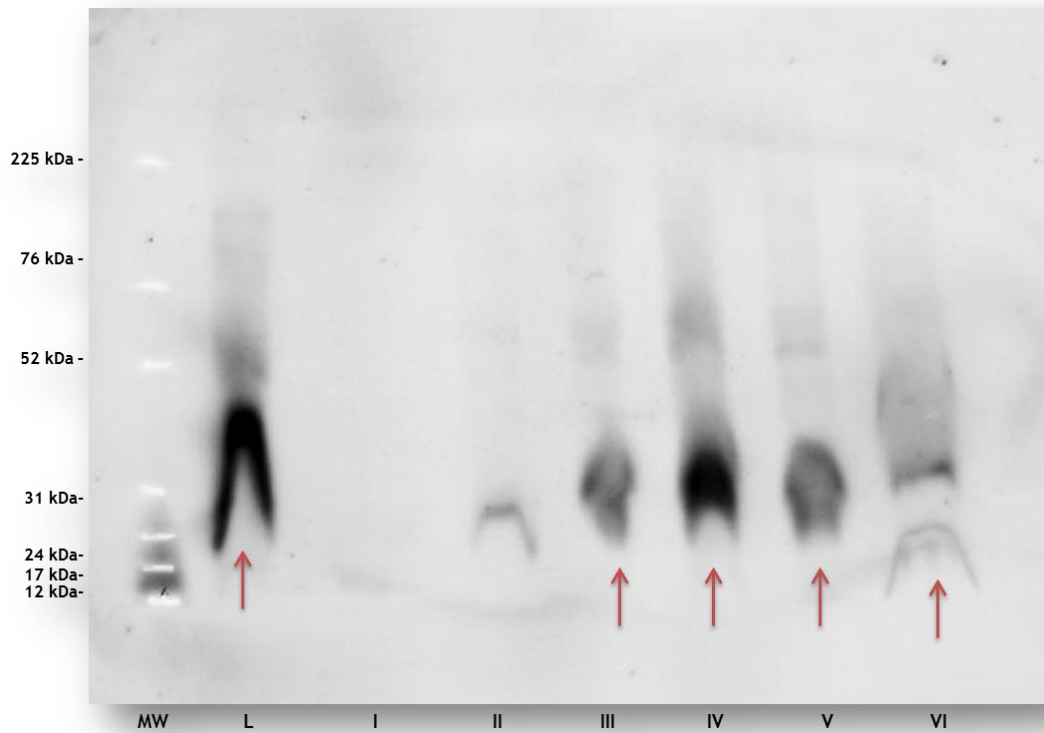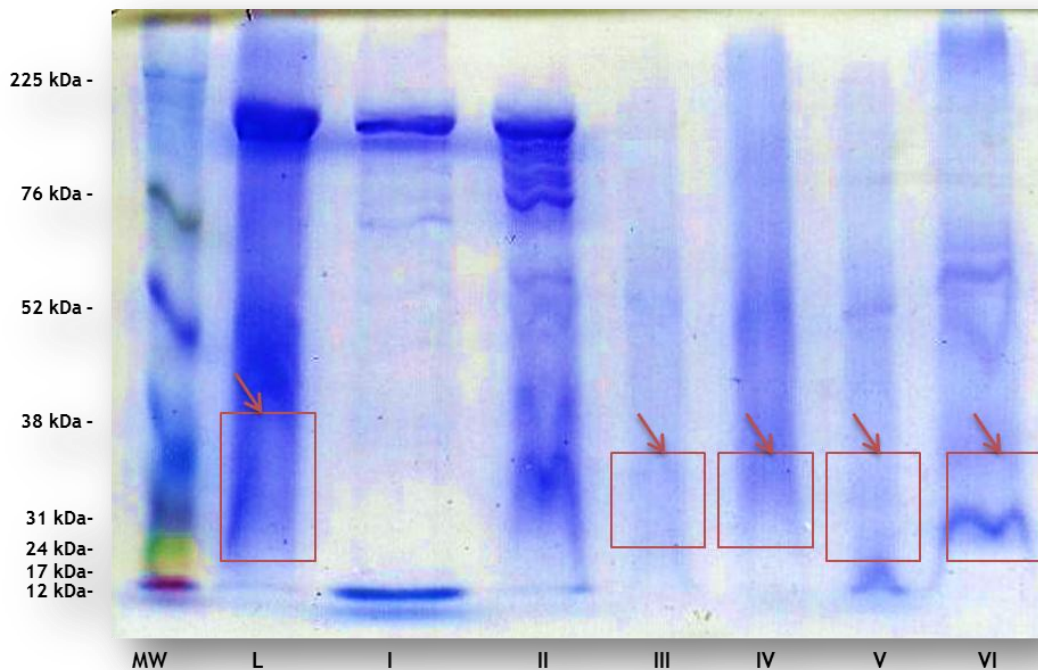
Figure 14 – Western Blot (A) and SDS-Page analysis (B) of samples collected on hMB-COMT isolation chromatographic assay [figure 13]. Lane MW – molecular weight standards; Lane L – solubilized lysis pellet injected on butyl adsorbent; Lane I – Peaks I obtained at 375 mM NaH$_2$PO$_4$; Lane II – Peak II obtained with Tris-HCl buffer; Lanes III and IV – Peaks III and IV obtained at 0.2% Triton X-100 and Lanes V and VI – Peaks V and VI obtained at 0.7 and 1% Triton X-100, respectively. Human MB-COMT position is indicated by the red arrow.

From the analysis of the SDS-PAGE and Western Blot (figure 14-A/B), we can observed above the 52 kDa marker line, the presence of some strong bands stained as immunologically active to the protein. Considering the double value (60 kDa) in relationship to the molecular weight of hMB-COMT (30 kDa) we can hypothesize the dimer formation, a common state due to membrane proteins' hydrophobicity. In order to obtain the target protein in a monomeric form and well resolved form these "isoagregates", we can apply some different techniques like size-exclusion chromatography or some soft denaturant conditions. Despite this aggregation phenomenon, hMB-COMT was successfully purified, on the experiment conditions stated above, in a multiple peak pattern with no significant contaminants level.

3.4. Purification trials on Epoxy- Sepharose

Epoxy is a mild hydrophobic adsorbent, the less hydrophobic matrix used in this study. As referred in literature, the use of mild hydrophobic ligands appears be a promising substitute to strongly bound protein elution since permit an adequate binding strength and softer elution conditions [18]. Therefore, epoxy- can be a suitable hydrophobic adsorbent to apply for an hMBCOMT isolation procedure.

Despite the data found about this adsorbent, preliminary results don't demonstrate the statements published for other target proteins (Table 4). In table 4, high salt concentrations

Table 4 - Summary of salt and Triton, used in detergent gradient, concentrations applied for understand the hMBCOMT elution behavior on an Epoxy-Sepharose support.

	$[NaH_2PO_4]$ (mM)	hMBCOMT retention	hMBCOMT elution behaviour		$[Triton\ X\text{-}100]_{elution}$ (%)
			Tris Buffer	Triton X-100	
	375	Residual	-	Residual	0.44
	500	Residual	-	Residual	0.07
Chromatographic methodologies	750	+	+	——	——
	800	+	Residual	++	0.04 0.33 0.52
	850	++	+	++	0.07 0.33 0.48

(−) to (+) denotes respectively no retention/elution to total retention of hMBCOMT (+++) on butyl-sepharose

are required to hMB-COMT retention in contrast to the fact stated in literature that medium salt concentrations are able to retain the protein of interest. [18]. In addition, detergents concentrations required to the elution process are softer than the necessary in butyl-. An advantage is that these trials lead to a faster chromatographic cycle due to a straightforward elution procedure while lower detergent concentrations are required.

As seen in figure 15, hMB-COMT is almost totally retained at 850 mM salt concentration. In spite this, there's elution in all the peaks, even so some peaks have only residual amounts. This fact demonstrates that, until now, a purification strategy based on an epoxy support for COMT purification can lead to a lower selective retention.

Also, sodium phosphate precipitates at concentrations above 150 mM and 4°C, so the higher concentrations require in epoxy (table 4) could be a major problem in the subsequent hMB-COMT concentration step. Indeed, lowers temperatures in the concentration step is determinant factor to obtain and minimize unusual enzyme forms and correct native protein folding. During the development of this work, high sample precipitation rates were achieved in the concentration step due to low temperatures in the centrifuge step. So, we were forced to shift to medium-high temperatures as the solution to this issue with a consequent high risk of hMB-COMT instability.

Figure 15 – Example of an hMB-COMT chromatographic profile on Epoxy-Sepharose by HIC. Adsorption was performed at 850 mM NaH$_2$PO$_4$ in 10 mM Tris-Cl buffer, pH 7.8; followed by Tris-HCl buffer step. Desorption was performed by an increased Triton X-100 gradient followed by 1% Triton in 10mM Tris-Cl buffer step. Blue line represents the absorbance at 280 nm, orange line the NaH$_2$PO$_4$ concentrations in mobile phase, green red line the Triton X-100 percentage in mobile phase and the brown line the conductivity. More hMB-COMT active fractions are represented by a red arrow. hMB-COMT is eluted in all the other peaks but at residual levels.

3.5. Purification trials on Octyl- Sepharose

Octyl-sehparose is a highly hydrophobic resin resulting of an higher length in the alkyl chain [18], [19]. Previous studies on octyl-sepharose absorbent on S-COMT isolation demonstrated that recovery of the target protein is allowed with mild elution conditions [19].

Indeed for MB-COMT preliminary purification trials showed that protein retention is achieved at low phosphate concentrations. Also, it was verified that in spite of mild retention conditions, in general it is necessary higher detergent concentrations to promote elution. First experiments are summarized in table 5 considering salt and detergent concentrations required and protein behavior.

Table 5 - Summary of salt and Triton X-100, used in detergent gradient, concentrations and hMBCOMT elution behavior on Octyl-Sepharose support.

	$[NaH_2PO_4]$ (mM)	hMBCOMT retention	hMBCOMT elution behaviour		$[Triton X-100]_{elution}$ (%)
			Tris Buffer	Triton X-100	
Chromatographic methodologies	250	++	++	++	1
	350	++	++	++	0 – 1 1.0
	375	+++	Residual	+++	1
	500	+++	-	+++	0-1 1.0

(−) to (+) denotes respectively no retention/elution to total retention of hMBCOMT (+++) on octyl-sepharose

An example of a typical hMB-COMT chromatographic profile on octyl resin is showed in figure 16. By the chromatogram is observed that at 250 mM of NaH_2PO_4 almost all hMB-COMT is retained. SDS-PAGE analyses (data not show) demonstrated that hMB-COMT is eluted without a significant level of contaminants. This indicates that few more trials are required to achieve optimal conditions to enzyme isolation. Also, is verified that with a specific salt concentration, lower detergent concentration are required to protein elution since that large amounts of retained hMB-COMT are eluted only with 10 mM of Tris buffer (figure 17).

So, is expected that octyl-sepharose, despite of their high hydrophobicity and lower ligand density, allows high protein recovery and selectivity using mild elution conditions.
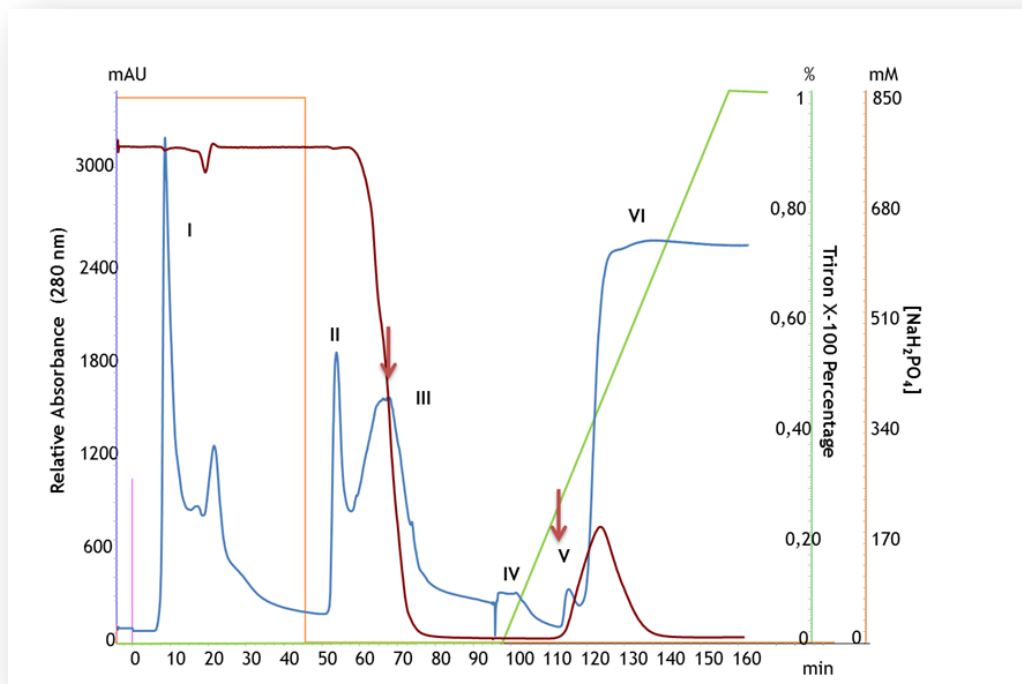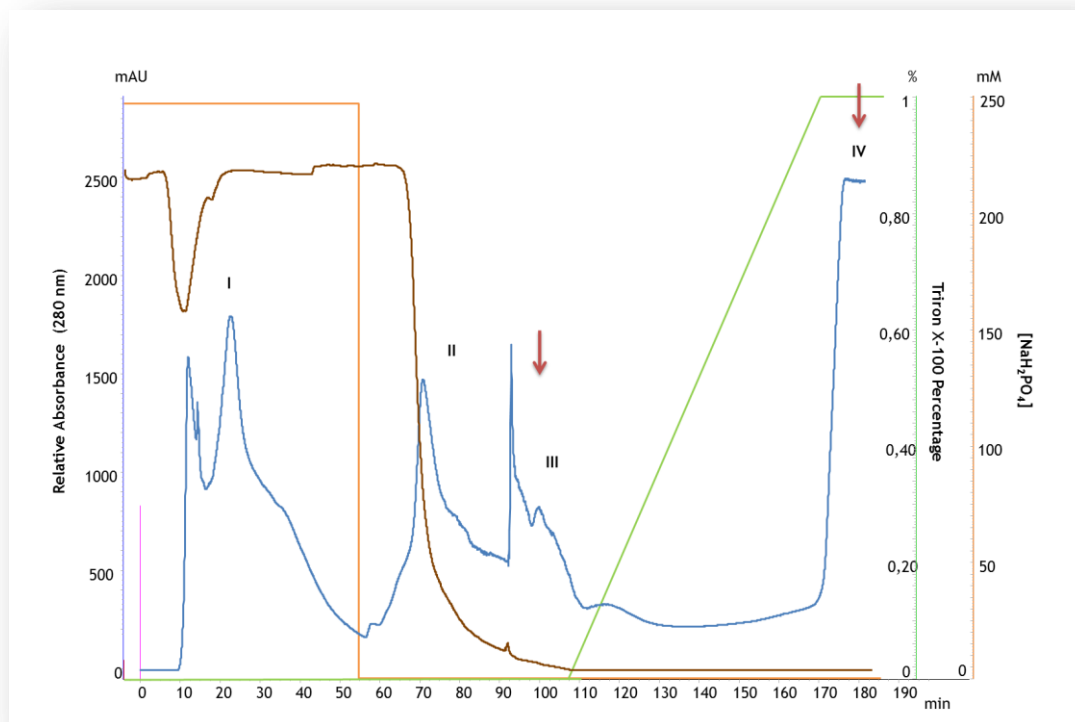
Figure 16 - Example of an hMB-COMT chromatographic profile on Octyl-Sepharose by HIC. Absorption was performed at 250 mM NaH_2PO_4 in 10 mM Tris-Cl buffer, pH 7.8; followed by Tris-HCl buffer step. Desorption was performed by an increased Triton X-100 gradient followed by 1% Triton in 10 mM Tris-Cl buffer step. Blue line represents the absorbance at 280 nm, orange line the NaH_2PO_4 concentrations in mobile phase, green red line the Triton X-100 percentage in mobile phase and the brown line the conductivity. More hMB-COMT active fractions are represented by a red arrow. hMB-COMT is eluted in peaks I and II at residual amounts.
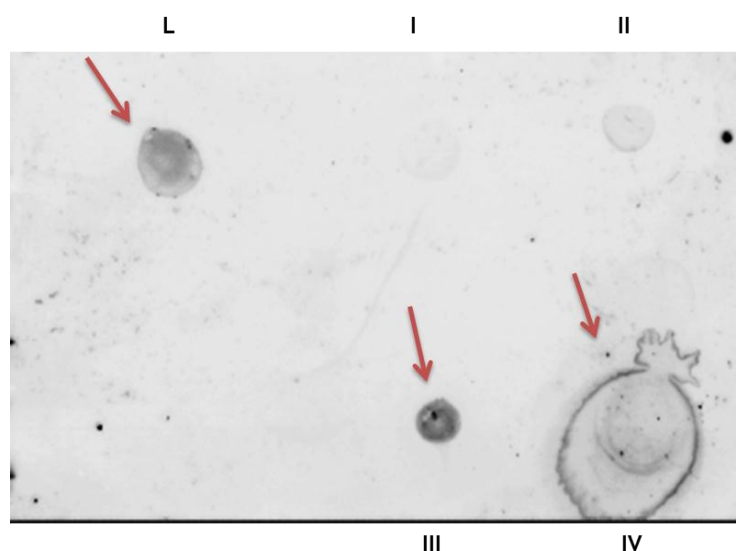
Figure 17 - Dot blot analysis of samples collected in initial chromatographic trials on octyl resin (Figure 16). Dot L – solubilized lysis pellet injected on octyl adsorbent; Dot I – Peaks I obtained at 250 NaH_2PO_4 in 10 mM Tris-HCl, Dot II – Peak II obtained with Tris-HCl buffer, Dot III – Peaks III obtained with Tris buffer and at low detergent percentages and Dot IV – Peak IV – Peak IV obtained at 1% Triton X-100. Human MB-COMT position is indicated by the red arrow.

3.6. Comparative study of hMB-COMT interaction on butyl-, octyl- and epoxy-sepharose

In HIC, the protein retention is largely affected by hydrophobicity of resins. Using the same retention and elution strategy we are able to study the hydrophobicity resin intrinsic characteristics effects on hMBCOMT isolation. An increase in the chain length, such as verify on octyl and butyl structures in figure 18, increases the hydrophobic interaction strength.
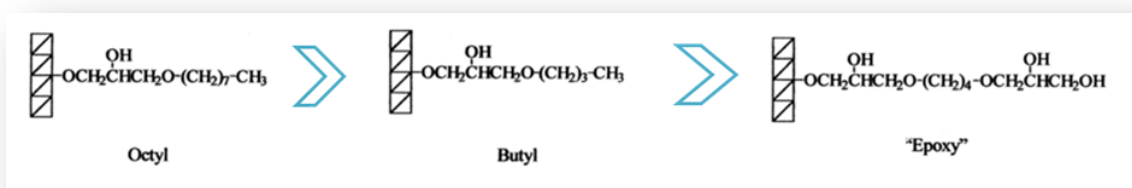
Figure 18 - Hydrophobicity scale and resin structure [18].

Therefore, we compared the performance of butyl-, epoxy- and octyl-sepharose in hMB-COMT isolation with same retention and elution conditions. Chromatograph analysis (figure 18) shows that at same phosphate concentrations as the resin hydrophobicity increase it can be observed higher hMB-COMT retention.

Therefore, at 375 mM NaH_2PO_4 it was not possible the protein retention in low hydrophobic epoxy- but in higher hydrophobic resins such as octyl- and butyl- the protein of interest was totally retained.

Matrix hydrophobicity influences, also, the elution strategy, being that for higher hydrophobic matrices stronger conditions must be applied. This fact is verified comparing elution's strategies between octyl- and butyl-. In octyl, elution is only possible with increasing detergent concentrations. In contrast, for butyl- protein begins to elute at medium Triton X-100 concentrations.
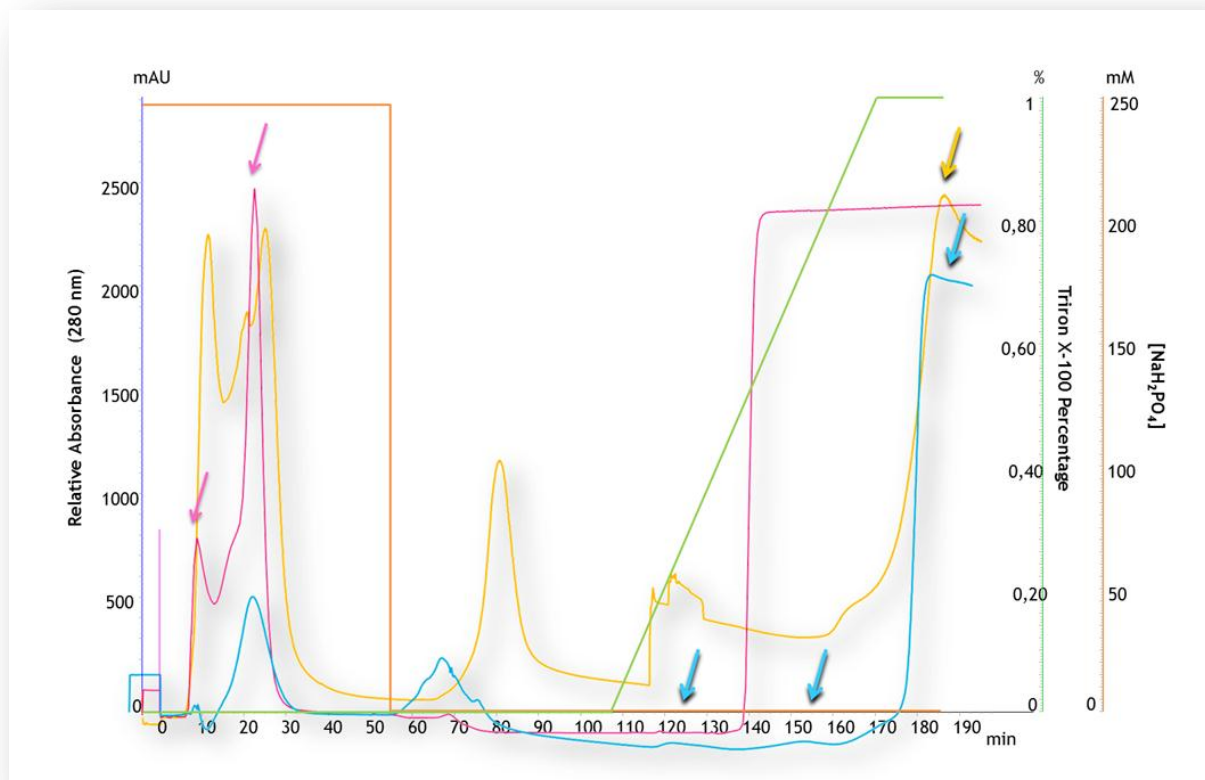
Figure 19 – Comparison of hMB-COMT chromatographic profiles on butyl-, epoxy- and octyl-sepharose and their relative absorbance (280 nm) are represented, respectively, by blue, pink and yellow lines. Adsorption was performed at 375 mM NaH_2PO_4 in 10 mM Tris-Cl buffer, pH 7.8; followed by Tris-HCl buffer step. Desorption was performed by an increased Triton X-100 gradient followed by 1% Triton in 10 mM Tris–Cl buffer step. Orange line represents the NaH_2PO_4 concentrations in mobile phase and green line the Triton X-100 percentage in mobile phase. hMB-COMT active fractions are represented, respectively by a blue, a yellow and a red arrow on Butyl-, Epoxy- and Octyl-Sepharose resins.

Chapter IV

Conclusions

Over several years, chromatographic separation procedures were used in COMT purification. Nevertheless, only few studies in literature focused in the purification of the membrane form and, therefore, a comprehensive and exhaustive study must be done. Until now, only a reference to a partial purification of rat MB-COMT from microssomal fraction of rat liver homogenates by anion exhange chromatography was reported in literature so this process and their problems is not correctly investigated.

For the first time, it was possible the hMB-COMT isolation on a hydrophobic adsorbent butyl-sepharose by hydrophobic interaction chromatography.. In general, the isolation of the enzyme is possible only with suitable adjusts on two parameters: ionic strength and the detergent concentration on mobile phase.

First chromatographic trials on butyl-Sepharose demonstrated that salt and detergent applications on mobile phase are crucial to, respectively, hMB-COMT adsorption and elution.

Preliminary trials on epoxy indicate that high phosphate concentrations are required to protein adsorption. Also, it was verified that after this chromatographic step at high salt concentrations there are a tendency for NaH_2PO_4 precipitate during the concentration process. So, if protein isolation on epoxy-sepharose was highly desired, the use of another salt type is advisable.

On the other hand, are expected that octyl-sepharose, despite of its high hydrophobicity, allows high protein recovery and excellent selectivity using only mild salt conditions.

Comparing both isoforms isolation (S-COMT and MB-COMT), with the same hydrophobic resins, we verified that low salt concentrations are necessary to the correct retention of the membrane form due to hydrophobicity structural differences. Furthermore, application of an specific detergent is necessary to MB-COMT elution whereas for S-COMT's elution it's only necessary a decrease in ionic strength. This specific detergent concentration is different for each resin, being that more hydrophobic ones need higher concentrations for elution.

In summary, HIC may be a suitable method for the hMB-COMT isolation due to strong hydrophobic characteristics of membrane anchor region.

Chapter V

Future perspectives

Chromatography has been used for centuries as a means of separation and, over time, has developed into a sophisticated analytical technique. On the other hand, optimization of chromatographic procedure takes a long time and it's multifactorial. Moreover, the process conditions that allow the protein isolations are largely an empirical method.

Several parameters such as temperature, pH, ionic strength, detergent characteristics affect hydrophobic interactions on HIC, which may influence protein stability and structure. Factorial design was immerge as advantageous tool that permits understanding the effects of independent variables upon a single dependent variable. In order to establish the optimized conditions for maintenance of hMB-COMT physiologically relevant characteristics factorial design studies should be applied.

Also, ultrafiltration may be interesting to apply in protein purification. At the moment, several membrane proteins purification procedures apply ultrafiltration as the main capture step. Ultrafiltration, besides allowing samples concentration after a chromatographic step, may remove some residual contaminants coeluting with the target protein.

Chapter VI

References:

[1] E. Carpenter, K. Beis, and A. Cameron, "Overcoming the challenges of membrane protein crystallography," *Current opinion in structural*, vol. 18, pp. 581-586, 2008.

[2] E. Pebay-Peyroula, "Determining membrane protein structures: still a challenge!," *Trends in biochemical*, vol. 32, no. 6, 2007.

[3] D. C. Rees, *Membrane Proteins: Advances in protein chemistry*, First edit., vol. 63. San Diego, California: Academic Press, 2003, p. 345.

[4] I. Mus-Veteau, "Heterologous expression of membrane proteins for structural analysis.," *Methods in molecular biology (Clifton, N.J.)*, vol. 601, pp. 1-16, Jan. 2010.

[5] C. Hunte, G. von Jagow, and H. Schagger, *MEMBRANE PROTEIN PURIFICATION AND CRYSTALLIZATION*, 2nd Edition. Academic Press 2003.

[6] H. Palsdottir and C. Hunte, "Lipids in membrane protein structures," *Review Literature And Arts Of The Americas*, vol. 1666, pp. 2-18, 2004.

[7] Y. Sonoda, A. Cameron, S. Newstead, and H. Omote, "Tricks of the trade used to acelerate high-resolution structure determination of membrane proteins," *FEBS Letters*, vol. 584, 2010.

[8] D. Niegowski, M. Hedrén, P. Nordlund, and S. Eshaghi, "A simple strategy towards membrane protein purification and crystallization.," *International journal of biological macromolecules*, vol. 39, no. 1–3, pp. 83-7, Aug. 2006.

[9] A. M. Seddon, P. Curnow, and P. J. Booth, "Membrane proteins , lipids and detergents : not just a soap opera," *Marine Biotechnology*, vol. 1666, pp. 105-117, 2004.

[10] R. Prasad, "Purification, reconstitution, and functional characterization of zinc transporter from rat renal brush border membranes.," *Methods In Molecular Biology Clifton Nj*, vol. 228, pp. 195-221, 2003.

[11] M. Zoonens and B. Miroux, "Expression of membrane proteins at the Escherichia coli membrane for structural studies.," *Methods in molecular biology (Clifton, N.J.)*, vol. 601, pp. 49-66, Jan. 2010.

[12] A. G. Lee, "How lipids affect the activities of integral membrane proteins," *Biochemistry*, vol. 1666, pp. 62-87, 2004.

[13] M. Bannwarth and G. E. Schulz, "The expression of outer membrane proteins for crystallization," *Science*, vol. 1610, pp. 37-45, 2003.

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

[15] K. Duquesne and J. N. Sturgis, "Membrane protein solubilization.," *Methods in molecular biology (Clifton, N.J.)*, vol. 601, pp. 205-17, Jan. 2010.

[16] R. Staudinger and J. C. Bandrés, "Solubilization of chemokine receptors from cell membranes.," *Methods in molecular biology (Clifton, N.J.)*, vol. 228, pp. 103-9, Jan. 2003.

[17] C. G. Tate, "Practical considerations of membrane protein instability during purification and crystallisation.," *Methods in molecular biology (Clifton, N.J.)*, vol. 601, pp. 187-203, Jan. 2010.

[18] J. A. Queiroz, C. T. Tomaz, and J. M. S. Cabral, "Hydrophobic interaction chromatography of proteins," *Journal of Biotechnology*, vol. 87, pp. 143-159, 2001.

[19] 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 : BMC*, vol. 438, pp. 430-438, 2007.

[20] K. Duquesne and J. N. Sturgis, "Membrane protein solubilization.," *Methods In Molecular Biology Clifton Nj*, vol. 601, pp. 205-217, 2010.

[21] F. C. Chong, W. S. Tan, D. R. a. Biak, T. C. Ling, and B. T. Tey, "A preparative hydrophobic interaction chromatography for purification of recombinant nucleocapsid protein of Nipah virus from clarified Escherichia coli homogenate," *Separation and Purification Technology*, vol. 71, no. 1, pp. 97-101, Jan. 2010.

[22] B. S. Selinsky, *Membrane Protein Protocols*, vol. 228, no. 1. New Jersey: Humana Press, 2003, p. 98.

[23] R. Hatti-Kaul, *Isolation and purification of proteins*. NEW YORK • BASEL: , 2003, pp. 1-652.

[24] M. E. Lienqueo, A. Mahn, J. C. Salgado, and J. a. Asenjo, "Current insights on protein behaviour in hydrophobic interaction chromatography," *Journal of Chromatography B*, vol. 849, no. 1–2, pp. 53-68, Apr. 2007.

[25] C. Tanford, "Hydrophobic free energy, micelle formation and the association of proteins with amphiphiles.," *Journal of molecular biology*, vol. 67, no. 1, pp. 59-74, Jun. 1972.

[26] A. Mahn, M. E. Lienqueo, and J. a Asenjo, "Optimal operation conditions for protein separation in hydrophobic interaction chromatography.," *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, vol. 849, no. 1–2, pp. 236-42, Apr. 2007.

[27] 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, no. 3, pp. 352-379, 2007.

[28] 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, no. 4, pp. 593-628, Dec. 1999.

[29] M. J. Bonifácio and P. Soares-da-Silva, "Purification of membrane-bound catechol-O-methyltransferase.," *Methods in molecular biology (Clifton, N.J.)*, vol. 228, pp. 231-8, Jan. 2003.

[30] J. A. Roth, "Membrane-Bound Catechol-O-Methyltransferase: A Reevaluation of Its Role in the O-Methylation of the Catecholamine Neurotransmitters," vol. 120, 1992.

[31] M. Käenmäki et al., "Importance of membrane-bound catechol-O-methyltransferase in L-DOPA metabolism: a pharmacokinetic study in two types of Comt gene modified mice.," *British journal of pharmacology*, vol. 158, no. 8, pp. 1884-94, Dec. 2009.

[32] B. Bertocci, V. Miggiano, M. Da Prada, Z. Dembic, H. W. Lahm, and P. Malherbe, "Human catechol-O-methyltransferase: cloning and expression of the membrane-associated form.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 4, pp. 1416-20, Feb. 1991.

[33] W. J. Lee and B. T. Zhu, "Inhibition of DNA methylation by caffeic acid and chlorogenic acid, two common catechol-containing coffee polyphenols.," *Carcinogenesis*, vol. 27, no. 2, pp. 269-77, Feb. 2006.

[34] V. Nunes and J. Queiroz, "Assessment of COMT isolation by HIC using a dual salt system and low temperature," *Biomedical*, vol. 2009, no. December 2009, pp. 858-862, 2010.

[35] V. Bonifati, G. Meco, A. D. Inhibitors, and C. O. Methyltransferase, "New , Selective Catechol- O -Methyltransferase Inhibitors as Therapeutic Agents in Parkinson ' s Disease PHYSIOLOGICAL ROLES OF FOR POSITRON EMISSION," *Science*, vol. 81, no. 1, pp. 1-36, 1999.

[36] V. A. McKusick, C. L. Kniffin, and J. L. Black, "+116790 CATECHOL-O-METHYLTRANSFERASE; COMT," 1987. [Online]. Available: http://omim.org/entry/116790. [Accessed: 17-Jun-2012].

[37] M. A. Vieira-Coelho and P. Soares-da-Silva, "Effects of tolcapone upon soluble and membrane-bound brain and liver," *Brain Research*, pp. 69-78, 1999.

[38] H.-woo Bai, J.-youn Shim, J. Yu, and B. T. Zhu, "Biochemical and Molecular Modeling Studies of the O-Methylation of Various Endogenous and Exogenous Catechol Substrates Catalyzed by Recombinant Human Soluble and Membrane-Bound," no. 20, pp. 1409-1425, 2007.

[39] A. Illi et al., "Catechol-O-methyltransferase val108 / 158met genotype , major depressive disorder and response to selective serotonin reuptake inhibitors in major depressive disorder," *Psychiatry Research*, vol. 176, no. 1, pp. 85-87, 2010.

[40] A. G. Nackley and L. Diatchenko, "Assessing potential functionality of catechol-O-methyltransferase (COMT) polymorphisms associated with pain sensitivity and temporomandibular joint disorders.," *Methods in molecular biology (Clifton, N.J.)*, vol. 617, pp. 375-93, Jan. 2010.

[41] V. Witte, "Effects of COMT polymorphisms on brain function and behavior in health and disease," *Brain Research Bulletin*, 2011.

[42] I. Massat et al., "Association between COMT (Val158Met) functional polymorphism and early onset in patients with major depressive disorder in a European multicenter genetic association study.," *Molecular psychiatry*, vol. 10, no. 6, pp. 598-605, Jun. 2005.

[43] S. Nohesara, M. Ghadirivasfi, S. Mostafavi, and M.-reza Eskandari, "DNA hypomethylation of MB-COMT promoter in the DNA derived from saliva in schizophrenia and bipolar disorder," *Journal of Psychiatric Research*, vol. 45, no. 11, pp. 1432-1438, 2011.

[44] N. Hevir, J. Šinkovec, and T. L. Rižner, "Disturbed expression of phase I and phase II estrogen-metabolizing enzymes in endometrial cancer: Lower levels of CYP1B1 and increased expression of S-COMT," *Molecular and Cellular Endocrinology*, vol. 331, no. 1, pp. 158-167, Jan. 2011.

[45] J. Lépine et al., "Circulating estrogens in endometrial cancer cases and their relationship with tissular expression of key estrogen biosynthesis and metabolic pathways.," *The Journal of clinical endocrinology and metabolism*, vol. 95, no. 6, pp. 2689-98, Jun. 2010.

[46] P. Sciences and R. July, "Role of Catecholamine Metabolism in Blood Pressure Regulation Using Chemiluminescence Reaction Detection," vol. 128, no. 11, pp. 1589-1594, 2008.

[47] B. T. Zhu, "On the mechanism of homocysteine pathophysiology and pathogenesis : a unifying hypothesis," pp. 1283-1291, 2002.

[48] M. Karayiorgou et al., "Genotype determining low catechol-O-methyltransferase activity as a risk factor for obsessive-compulsive disorder.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 9, pp. 4572-5, Apr. 1997.

[49] M. Gabrovsek et al., "Combined family trio and case-control analysis of the COMT Val158Met polymorphism in European patients with anorexia nervosa.," *American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics*, vol. 124B, no. 1, pp. 68-72, Jan. 2004.

[50] E. Michaelovsky et al., "Haplotype analysis of the COMT-ARVCF gene region in Israeli anorexia nervosa family trios.," *American journal of medical genetics.*

Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics, vol. 139B, no. 1, pp. 45-50, Nov. 2005.

[51] S. Shifman et al., "A highly significant association between a COMT haplotype and schizophrenia.," *American journal of human genetics*, vol. 71, no. 6, pp. 1296-302, Dec. 2002.

[52] N. J. Bray et al., "A haplotype implicated in schizophrenia susceptibility is associated with reduced COMT expression in human brain.," *American journal of human genetics*, vol. 73, no. 1, pp. 152-61, Jul. 2003.

[53] J. Tenhunen, P. Heikkilä, A. Alanko, E. Heinonen, J. Akkila, and I. Ulmanen, "Soluble and membrane-bound catechol-O-methyltransferase in normal and malignant mammary gland," *Cancer Letters*, vol. 144, no. 1, pp. 75-84, Sep. 1999.

[54] 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, no. 7–8, pp. 1098-105.

[55] 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, no. 22, pp. 23710-8, May 2004.

[56] C. Sissi, E. Marangon, a Chemello, C. G. Noble, a Maxwell, and M. Palumbo, "The effects of metal ions on the structure and stability of the DNA gyrase B protein.," *Journal of molecular biology*, vol. 353, no. 5, pp. 1152-60, Nov. 2005.

[57] A. Chefson, J. Zhao, and K. Auclair, "Sugar-mediated lyoprotection of purified human CYP3A4 and CYP2D6.," *Journal of biotechnology*, vol. 130, no. 4, pp. 436-40, Jul. 2007.

[58] 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, no. 17–18, pp. 2249-53, May 2002.

[59] M and A, "Importance of membrane-bound catechol-O-methyltransferase in L-DOPA metabolism: a pharmacokinetic study in two types of Comt gene modified mice," *British journal of*, pp. 1884-1894, 2009.

[60] 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," *Journal of Biotechnology*, vol. 156, no. 2, pp. 141-146, 2011.

[61] I. Introduction, "Brevibacillus Expression System_TAKARA," *Transformation*, pp. 1-19.

[62] 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,*

Analytical technologies in the biomedical and life sciences, vol. 879, no. 19, pp. 1704-6, Jun. 2011.

[63] 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, no. 2, pp. 287-96, Jan. 2008.

[64] P. A. Gulliver and C. W. Wharton, "Novel ligands for the purification of catechol-O-methyltransferase by affinity chromatography.," *Biochemical pharmacology*, vol. 25, no. 18, pp. 2033-8, Sep. 1976.

[65] 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, no. 2, pp. 995-1002, Jan. 1991.

[66] P. Darmenton, L. Cronenberger, and H. Pachéco, "[Purification and properties of rat kidney catechol-O-methyltransferase].," *Biochimie*, vol. 58, no. 9, pp. 1031-45, Nov. 1976.

[67] B. Bertocci et al., "Immunoaffinity purification and partial amino acid sequence analysis of catechol-O-methyltransferase from pig liver.," *Biochimica et biophysica acta*, vol. 1080, no. 2, pp. 103-9, Oct. 1991.

[68] P. A. Gulliver and K. F. Tipton, "The purification and properties of pig-liver catechol-O-methyl transferase.," *European journal of biochemistry / FEBS*, vol. 88, no. 2, pp. 439-44, Aug. 1978.

[69] L. a Passarinha, M. J. Bonifácio, and J. a Queiroz, "The effect of temperature on the analysis of metanephrine for catechol-O-methyltransferase activity assay by HPLC with electrochemical detection.," *Biomedical chromatography : BMC*, vol. 20, no. 9, pp. 937-44, Sep. 2006.

[70] U. K. LAEMMLI, "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4," *Nature*, vol. 227, no. 5259, pp. 680-685, Aug. 1970.

[71] L. Pastrana, M. A. Longo, and J. A. Hermoso, "An esterase from Thermus thermophilus HB27 with hyper-thermoalkalophilic properties: Purification, characterisation and structural modelling," *Journal of Molecular Catalysis B*, 2011.

[72] E. Nissinen, *Basic aspects of Catechol-O-methyltransferase and the clinical applications of its inhibitors*, vol. 95. Academic Press, 2010.

Chapter VII

Appendices

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

225 K	Blue
76 K	Yellow
52 K	Purple
38 K	Blue
31 K	Orange
24 K	Green
17 K	Blue
12 K	Red