Universidade da Beira Interior
Departamento de Química
Centro de investigação em Ciências da Saúde

"Screening of Bioaffinity Ligands for Human Soluble COMT Purification"

Sara Rute Afonso Costa

Dissertation for purpose of Master Degree in Biochemistry

Supervisors: Luís António Paulino Passarinha, PhD
João António de Sampaio Rodrigues Queiroz, PhD

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“By three methods we may learn wisdom: First, by reflection, which is noblest; Second, by imitation, which is easiest; and third by experience, which is the bitterest.”

Confucius

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Abbreviations list

AADC - Aromatic amino acid decarboxylase

AAIL's - Amino acids immobilized ligands

AdoMet - S-adenosyl-L-methionine

BSA - Bovine serum albumine

COMT – Cathecol-*O*-methyltransferase

DTT - Dithiothreitol

FPLC - Fast performance liquid chromatography

HPLC - High pressure liquid chromatography

hSCOMT - human soluble Cathecol-*O*-methyltransferase

IPTG - isopropylthiogalactosidase

MB-COMT – Membrane-bound Cathecol-*O*-methyltransferase

OD - Optical density

PD - Parkinson's disease

pI - Isoeletric point

PVDF - Polyvinylidene difluoride

RT - Room temperature

S-COMT - Soluble Cathecol-*O*-methyltransferase

SDS - Sodium dodecyl sulfate

SDS-PAGE - Sodium dodecyl sulfate -polyacrylamide gel electrophoresis

TBS-T - Tris buffer saline-Tween 20

Tris - Tris (hydroxymethyl)aminomethane

Resumo

Nos finais de 1950, Alxelrod e co-autores descreveram a enzima responsável pela *O*-metilação de catecolaminas e outros catecóis, e no mesmo ano a Catecol-*O*-metiltransferase (COMT) foi parcialmente purificada e caracterizada. Desde então, foram efectuados vários estudos para caracterizar os três polimorfismos mais frequentes da forma humana da COMT, responsáveis por lhe conferir susceptibilidade térmica e oxidativa. Estes polimorfismos podem ter implicações clínicas, e são o factor genético preponderante em várias doenças neurodegenerativas que envolvam os sistemas catecolaminergicos. Nos últimos 40 anos, foram descritas várias estratégias de purificação, utilizando vários extractos biológicos, e consequentemente vários métodos bioquímicos de separação. Apesar de todos os desenvolvimentos tornam-se necessários métodos de purificação mais simples, rápidos e eficazes que confiram um grau de pureza mais elevado sem comprometer a actividade enzimática da Catecol-*O*-metiltransferase humana na forma solúvel (hSCOMT).

O objectivo global deste trabalho foi desenvolver/melhorar os processos de purificação da hSCOMT, aumentando o grau de purificação e de recuperação da actividade enzimática para aplicação em domínios farmacológicos, cinéticos e estruturais. Deste modo o trabalho desenvolvido foi assente em três objectivos; os dois primeiros utilizando Cromatografia de Interacção Hidrofóbica (HIC), sendo que no suporte Octil-sepharose o objectivo foi analisar o efeito de agentes destabilizadores, a L-arginina; e no suporte Epoxi-sefarose foi analisar o efeito do sistema de dois sais nomeadamente, citrato de sódio ($Na_3C_6H_5O_7$) e sulfato de amónio (NH_2SO_4). Por último, utilizar cromatografia de pseudo-bioafinidade em suportes com aminoácidos imobilizados, com o objectivo de analisar a selectividade destes suportes utilizando estratégias de eluição não específicas, sal e alteração de pH.

Estudos realizados pelo nosso grupo de investigação, demonstraram que a estratégia do sistema de dois sais apresenta uma selectividade adequada e consequentemente um factor de purificação incrementado, reduzindo-se assim os efeitos de desnaturação da proteína hSCOMT na presença de elevadas concentrações de sal. Deste modo, tornou-se conveniente analisar a aplicação de uma estratégia de dois sais no suporte Epoxi-sefarose com o objectivo de melhorar o processo de purificação da enzima hSCOMT. Especialmente os resultados demonstraram que, neste suporte, as concentrações de sal necessárias para promover a retenção da hSCOMT são superiores a

0,7 M NH_2SO_4/0,15 M $Na_3C_6H_5O_7$, tornando assim esta estratégia inadequada em comparação com estratégias já descritas. Em geral, devido à forte ligação hidrofóbica entre proteínas e suportes cromatográficos, a eluição poderá ser comprometida, não permitindo eluições com a simples diminuição da concentração de sal. Este facto é relevante na interacção entre hSCOMT-Octil, em que a utilização dos sistemas de dois sais necessita da diminuição da temperatura de modo a promover a eluição completa da proteína alvo.

Em geral, os amino ácidos, têm sido amplamente utilizados, para melhorar estratégias de purificação ou para estabilizar proteínas durante o processo cromatográfico. Especificamente, a L-arginina é altamente eficiente melhorando o desempenho de colunas cromatográficas. Neste caso, torna-se conveniente a optimização dos métodos de fraccionamento no suporte Octyl, testando condições de eluição destabilizadoras pela incorporação da L-arginina na fase móvel. No trabalho desenvolvido, estudos preliminares mostram que este aminoácido poderá interferir na estrutura nativa da COMT, promovendo uma diminuição da actividade específica. Adicionalmente, o grau de purificação alcançado denota que a estratégia apresentada não cumpre os requisitos necessários para a purificação da proteína alvo.

Os suportes de aminoácidos têm sido utilizados na separação de várias biomoléculas, no entanto, estes suportes nunca foram utilizados na purificação da proteína hSCOMT. Deste modo, o último objectivo é analisar a performance de ligandos típicos de pseudo-bioafinidade (L-arginina, L-metionina, L-histidina, L-aspartato, L- glutamina and L-leucina) como etapa central no isolamento da COMT, de modo a compreender a selectividade alcançada. Especialmente, foram utilizados perfis de eluição não específicos baseados na manipulação do pH e na concentração de sal. As condições utilizadas de forma a promover a retenção da hSCOMT mostraram que: 1) são necessárias altas concentrações para promover a retenção da proteína, que favorecem o decréscimo da actividade da proteína; 2) na manipulação de pH, a necessidade de pH's ácidos para promover a retenção da hSCOMT, levam a uma alteração do peso molecular e consequente perda de actividade enzimática. Em síntese conclui-se, comparando as duas estratégias utilizadas, que o factor crítico para este facto é independente dos suportes utilizados, e altamente influenciável pelas condições estabelecidas para promover a ligação da hSCOMT.

Como conclusão, a comparação entre as três abordagens descritas na dissertação demonstram as limitações existentes no delineamento das estratégias de purificação

desta proteína a partir de misturas biológicas complexas. No entanto, comparando entre as três partes exploradas, e apesar de serem necessários estudos estruturais de modo a caracterizar e compreender o mecanismo de ligação, os suportes de aminoácidos tornam-se mais vantajosos devido a simplicidade e eficiência nos processos cromatográficos.

Palavras-Chave

Catecol-*O*-metiltransferase humana, Cromatografia de Interacção Hidrofobica (HIC), Cromatografia de Pseudo-Bioafinidade, Purificação.

Abstract

Catechol-*O*-methyltransferase (COMT), play an important role in the metabolism of catecholamines, catecholestrogens and catechol drugs and consequently has a closer relationship with several mental disorders. As a result, while the development of pharmaceutical Human Soluble COMT (hSCOMT) trials for a rational drug design depends on the availability of high purified samples, more suitable purification strategies must be developed and emerged in order to fulfil the requirements of pharmaceutical industry.

In this context, the global aim of this work was the improvement of recovery yields and activity protein results in a hSCOMT purification process. Therefore, the work was being oriented according to three intermediate goals. Two first's concerning Hidrophobic Interaction Chromatography; (1) Octyl-sepharose support in order to analyse new fractionation methods by testing destabilizing elution conditions with the incorporation of L-arginine in the mobile phase; (2) the application of a dual salt system in Epoxy-sepharose resin; (3) the pseudo-bioaffinity chromatography using six commercial resins with immobilized amino acids, examining the ligands performance, in order to understand the selectivity's achieved based on non-specific desorption profiles supported on pH and ionic strength manipulation.

In general for Epoxy-sepharose support, the concentrations of dual salt system required to allow hSCOMT retention was above 0,7 M NH_2SO_4/0,15 M $Na_3C_6H_5O_7$. Indeed, this strategy is needless, considering previous purification strategies described that allow satisfatory protein selectivity and purification factor with less salt concentration; and consequently with a reduction in hSCOMT denaturation effects.

The L-arginine is highly effective in improving the performance of various chromatographic columns. However, is this study preliminary stability assays showed that this amino acid decreases hSCOMT specific activity; showing that this strategy do not comprise hSCOMT purification strategy requests.

This is the first report using amino acids as immobilized ligands (AAIL's) as pseudo-bioaffinity supports in hSCOMT isolation. Based on non-specific desorption profiles the results show that; (1) the high concentrations necessary to promote hSCOMT retention, decreasing product activity recovery; (2) for pH approach, the requirement of acidic pH's to allow hSCOMT retention leads to a molecular weight alteration and consequently loss of enzymatic activity. The comparison between these

two strategies, reveal that the hSCOMT molecular weight discrepancy are not AAIL dependent, but due to binding conditions.

In conclusion, the comparison of these three approaches in this dissertation demonstrated the complexity of hSCOMT purification processes. In spite of, structural studies are need to understand the hSCOMT-AAIL binding mechanism, these supports have furthermost advantages over the earlier methods published due of its simplicity and efficiency for hSCOMT purification.

Keywords

Human Cathecol-*O*-methyltransferase, Hydrophobic Interaction Chromatography (HIC), Pseudo-bioaffinity chromatography, Purification.

Chapter I. Catechol-*O*-methyltransferase: An overview

In the late of 1950s, Axelrod and coworkers described the enzyme catalyzed-*O*-methylation of catecholamines and other catechols (Axelrod *et al.*, 1958), in the same year Cathecol-*O*-methyltransferase (COMT) was partly purified and characterized (Axelrod and Tomchick, 1958). The subsequent basic research occured since 1958 as far as 1975, and the protein purification has revealed some heterogeneity in COMT activity, stability and molecular weights (Guldberg and Marsden, 1975). Also multiple forms of COMT have been demonstrated in a variety of tissues, supporting the hypothesis of isozymes (Assicot and Bohuon, 1971; Huh and Friedhoff, 1979). The enzyme has been found in organisms at various phylogenic levels, from bacteria and yeast to man (Guldberg and Marsden, 1975; Männisto, 1994), Afterwards two distinct forms of COMT have been identified, based on subcellular fractionation studies, a soluble (S-COMT) and a membrane-bound form (M-COMT) (Roth, 1992).

Although, the interest in COMT was rekindled in the late 1980s, when the potent and selective second-generation COMT inhibitors were developed (Mannisto and Kaakkola, 1989, 1990) as adjuncts in L-DOPA therapy for Parkinson's diseases (PD). Since that, much work on the biochemical characterization of COMT was done with purified enzyme from rat tissues and human placenta. However, with the disclosure of mammalian COMT cDNAs (Bertocci *et al.*, 1991; Lundstrom *et al.*, 1991) and genes it was possible to express both rat and human S- and MB-COMT in heterologous systems, eukaryotic (Tilgmann *et al.*, 1992; Ulmanen *et al.*, 1997) and prokaryotic (Bonifacio *et al.*, 2001; Lundstrom *et al.*, 1992; Malherbe *et al.*, 1992; Passarinha *et al.*, 2007), allowing a more detailed characterization of the functional properties of the enzyme, its subcellular localization, and its three-dimensional structure (Vidgren *et al.*, 1994).

In spite of the crystal structures of rat COMT have provided a useful basis for development of COMT inhibitors used in the primary treatment of PD (Bonifacio *et al.*, 2002; Kaakkola *et al.*, 1994; Learmonth *et al.*, 2005; Learmonth *et al.*, 2004; Lerner *et al.*, 2003; Lerner *et al.*, 2001; Masjost *et al.*, 2000; Palma *et al.*, 2003; Palma *et al.*, 2006), and Entacapone and Tolcapone (first COMT inhibitor commercialized in 2003) are currently being used clinically in treatment of Parkinson disease, structures of the human protein are desirable. However, was only in 2009 the first succeeded crystallization of soluble form of Human COMT (hSCOMT) (Rutherford *et al.*, 2008b).

In the last years, several studies have been performed in order to characterize the polymorphism of human COMTs. Three common polymorphisms were described (A22S, A52T, and V108M), two of which (A22S and V108M) render the protein

susceptible to deactivation by temperature or oxidation (Rutherford and Daggett, 2009). Tipically, polymorphisms could have clinical implications, and are a candidate gene in many neurologic disorders involving catecholaminergic systems.

1.1 Enzymatic reaction and physiological role

COMT enzyme catalyzes the transfer of a methyl group from the coenzyme S-adenosyl-L-methionine (AdoMet) to one of the hydroxyls (preferentially 3-hydroxyl) in a variety of endogenous and exogenous catechol or substituted catechols substrates in the presence of an Mg^{+2} ion (Figure 1). The chemical step of the reaction was revealed to be an S_N2-like process (Woodard *et al.*, 1980), and likely proceeds by sequential order kinetics mechanism with AdoMet-binding first, followed by an Mg^{2+} ion, and then by the catechol substrate. S-adenosyl-L-homocysteine the last ligand, wich is released in the catalytic cycle (Lotta *et al.*, 1995).

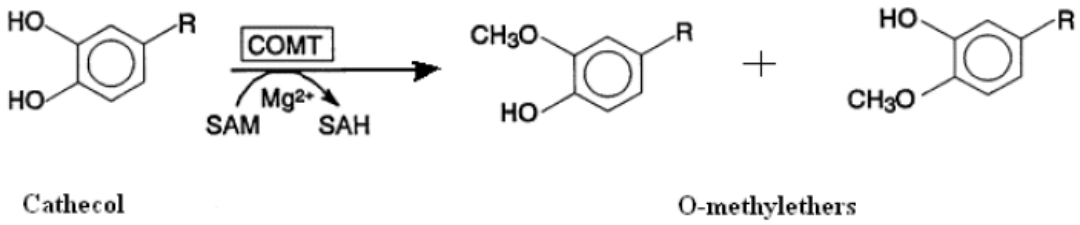


Figure 1. Reaction mechanism of COMT. AdoMet and S-adenosyl-L-homocysteine. (Adapted from (Lundstrom *et al.*, 1995)).

This enzymatic *O*-methylation plays an important physiological role in the inactivation of biologically active and toxic catechols. These substrates include: a wide variety of catechols, namely catecholamines with hormonal and neurotransmission activities (dopamine, norepinephrine, epinephrine, catecholestrogens) and their hydroxylated metabolites, ascorbic acid (Guldberg and Marsden, 1975; Kopin, 1985; Mannisto *et al.*, 1992), dietary phytochemicals, indolic intermediates of melanin metabolism (Smit *et al.*, 1990), xenobiotic catechols like carcinogenic catechol-containing flavonoids (Zhu *et al.*, 1994) and a multitude of drugs with a catechol structure by methylation and consequently inactivation (Guldberg and Marsden, 1975).

1.2 Gene and Protein characterization

1.2.1 The COMT gene: localization and structure

In humans, COMT gene was localized in *locus* 22q11.2 (Grossman *et al.*, 1992a; Winqvist *et al.*, 1992). The regulation of the COMT gene expression seems to occur at several levels, (i) transcription initiation, (ii) translation initiation and (iii) mRNA splicing. The gene organization together with the complex regulation enables the expression of two different COMT proteins, S- and MB-COMT (Lundstrom *et al.*, 1995).

The overall structure of the COMT gene, showed in Figure 2, is composed of six exons. At 5' region, the first two exons are noncoding and the translation initiation codons for the membrane bound (MB-ATG for MB-COMT polypeptide) and soluble (S-ATG for S-COMT form) isoforms are located on the third exon.

The size of the 3' untranslated region is 274 bp and the larger COMT mRNA also has several 5' ends (Lundstrom *et al.*, 1995). The shorter transcript initiates at multiple sites in the region between the two translation initiation ATG codons, and these two alternative splicing products have different capacity for the translation of MB- and S-COMT polypeptides (Tenhunen and Ulmanen, 1993).

The expression of the COMT gene is controlled by two distinct promoters located in exon 3 (Lundstrom *et al.*, 1991). The upper promoter (P2) is constitutively expressed. In contrast, the lower promoter (P1) is regulated in a tissue-specific manner and so, the amount of the shorter transcript varies from one tissue to another (Lundstrom *et al.*, 1995).

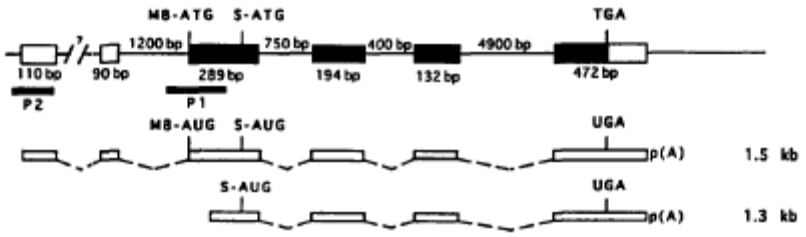


Figure 2. The structure of human COMT gene. Thin line represents introns and the boxes the exons. The black boxes indicate the protein coding regions. Two identified promoters, P1 and P2, are shown by black bars. COMT mRNA species expressed from the genes (1.3 and 1.5 kb) are presented as white bars. The positions of translation initiation codons for MB-COMT polypeptide (MB-ATG, MB-AUG), S-COMT polypeptide (S-ATG, S-AUG) and for translation stop codons (TGA, UGA), and the sizes of exons and introns are also shown (Lundstrom *et al.*, 1995).

1.2.2 The COMT protein: localization and characterization

The COMT protein is a monomeric (Tilgmann and Kalkkinen, 1991) and nonglycosylated enzyme (Tilgmann and Ulmanen, 1996). This protein exists as two isozymes: a ubiquitous 221-residue soluble form and a 271-residue membrane-bound form (Bertocci *et al.*, 1991; Lotta *et al.*, 1995), and their molecular weight are 24.4 and 29 kDa, respectively (Bertocci *et al.*, 1991; Lundstrom *et al.*, 1995). In addition, chromatofocusing revealed that isoelectric points of S-COMT is 5.5 (White and Wu, 1975).

The membrane isoform of COMT is an integral membrane protein with the catalytic portion of the enzyme oriented toward the cytoplasmic side of the membrane (Ulmanen and Lundstrom, 1991). Their 50 additional amino acids can owning a stretch of 17 hydrophobic amino-acid residues (Bertocci *et al.*, 1991; Lundstrom *et al.*, 1991). The arrangement of positively charged amino acids spanning the putative anchor sequence, suggests that MB-COMT may be oriented towards the cytoplasmic side of the membrane, reminding the membrane proteins of the class Ib type (Singer, 1990; von Heijne and Gavel, 1988). Nevertheless, the protein appears mostly in a soluble form (S-COMT), and only a minor fraction is in the particular form (MB-COMT) (Guldberg and Marsden, 1975; Roth, 1992).

1.2.2.1 Subcellular localization

In general, the indication of COMT subcellular distribution was obtained through differential centrifugation. The major S-COMT activity is in the non-sedimenting and cytoplasmic fractions (Guldberg and Marsden, 1975; Roth, 1992; Tilgmann *et al.*, 1992), while MB-COMT was previously assigned to the outer mitochondrial and plasma membrane (Lundstrom *et al.*, 1992; Tilgmann *et al.*, 1992). Indeed, currently MB-COMT subcellular localization was described in the rough endoplasmic reticulum (Tilgmann *et al.*, 1992; Ulmanen *et al.*, 1997).

1.2.2.2 Tissues distribution

In mammals, COMT is widely distributed throughout the organs. The S-COMT form represents approximately 70-80% of the total COMT proteins whereas in brain only 30% of COMT proteins is in S-form (Table 1) (Lundstrom *et al.*, 1995; Tenhunen *et al.*, 1994). The highest activity is found in the liver, followed by the kidneys and gastrointestinal tract (both stomach and intestine) (Lundstrom *et al.*, 1995; Tenhunen and Ulmanen, 1993).

As observed in table I, the difference in the expressed protein levels can be explained by different translation efficiency of COMT polypeptides from the larger COMT mRNA (Mannisto *et al.*, 1992; Nissinen *et al.*, 1988). In most tissues the level of the shorter COMT mRNA, capable of expressing only the S-COMT polypeptide, exceeds the longer transcript. Tipically, the long transcript has been found in all tissues analyzed, with higher levels in human liver, brain, kidneys, adrenals, and lungs. On the other hand, the short transcript, is particularly abundant in liver, kidneys, and mammary glands and it is found in very small amounts in the human brain (Hong *et al.*, 1998; Tenhunen *et al.*, 1994).

Table I. Quantization of S- and MB-COMT polypeptides in human tissues expressed as % of total COMT (Lundstrom *et al.*, 1995; Tenhunen and Ulmanen, 1993).

	S-COMT	**MB-COMT**
Liver	85	15
Kidney	77	23
Adrenal medulla	74	26
Duodenum	89	11
Brain	30	70

1.2.2.3 Differences of S- and MB-COMT

Total In spite of different molecular weight, and subcellular localization, S-COMT and MB-COMT have a similar kinetic mechanism (Mannisto *et al.*, 1992), similar affinities for AdoMet (Jeffery and Roth, 1987; Lotta *et al.*, 1995), magnesium, inhibition by calcium, and optimal pH for the activity (Mannisto and Kaakkola, 1999). However, the most distinct difference between S- and MB-COMT is the different substrate specificities, and the regioselectivity of methylation (Gordonsmith *et al.*, 1982; Lau and Bruice, 1998; Lotta *et al.*, 1995; Malherbe *et al.*, 1992; Mannisto and Kaakkola, 1999). As S-COMT and MB-COMT seem to exhibit different functions, and is the membrane isoform that is more relevant in the inactivation of xenobiotic catechols and the latter playing an important role in the termination of catecholaminergic neurotransmission (Lotta *et al.*, 1995; Roth, 1992).

1.2.2.3.1 Substrate specificities and regioselectivity

MB-COMT has a higher affinity for dopamine and others catechols (Gordonsmith *et al.*, 1982; Malherbe *et al.*, 1992). The adjacent membrane with a charged structure or an additional structural part in the amino end of MB-COMT contributes significantly to the higher binding affinity of the substrates (Lotta *et al.*, 1995). Indeed, for an extended charged side chain of the substrate, the membrane anchor region of MB-COMT (a possible helix) or the membrane itself causes a more favorable binding interactions (Lotta *et al.*, 1995). Also, physiological substrate concentrations and possible differences in substrate selectivity have to be considered when the relative importance of either enzyme subtype is assessed (Mannisto and Kaakkola, 1999).

It is well known that, both enzyme isoforms promote 3-*0*-methylation, and MB-COMT is even more regioselective than S-COMT. The *meta*/*para* ratio is higher, 22 to 88 (depending on the substrate) in MB-COMT than in S-COMT, 4 to 15 (depending on the substrate) (Gordonsmith *et al.*, 1982; Lotta *et al.*, 1995). The reason may be that as the *p*-hydroxyl group (i.e., 4-*O*-hydroxyl) approaches the AdoMet; this forces the side chain to become orientated in an unfavorable position with the hydrophobic protein residues of the catalytic site (Gordonsmith *et al.*, 1982; Lau and Bruice, 1998).

1.2.2.4 COMT structure: Crystallographic studies

The first COMT crystallization was obtained in 1994 (Vidgren *et al.*, 1994), of rat soluble Cathecol-*O*-methyltransferase. In XXI century, crystallization of rSCOMT structure, complexed with various inhibitors, had provided considerable insight into the recognition of substrates (Bonifacio *et al.*, 2002; Lerner *et al.*, 2001; Palma *et al.*, 2006). Rat and human COMT share 81% sequence identity and both belong to the highly structurally conserved AdoMet-dependent methyltransferase fold family (class I) (Cheng and Roberts, 2001; Martin and McMillan, 2002). In spite of, rat and human COMT similarities the structure of the human COMT are desirable because the proteins of two species differ in their kinetic properties (Km values generally are higher in the rat enzyme than in human COMT, and the relative specificities for specific catechols can differ). However, only in 2008 hSCOMT was crystallized. (Rutherford *et al.*, 2008b).

Specifically, the structure of hSCOMT is composed of a seven-stranded β-sheet core (3↑2↑1↑4↑5↑7↓6↑) and wiched between two sets of α-helices (Figure 3). The active site of COMT consists of the AdoMet binding domain and the actual catalytic site (Veerapandian, 1997). The results of crystallographic studies indicated that the catalytic site of S-COMT was formed by the Mg^{2+} ion and relevant amino acids for substrate-binding and methylation catalysis. The binding motif of the AdoMet site is similar to the Rossman fold, which is a common feature of many nucleotide binding proteins (Mannisto and Kaakkola, 1999).

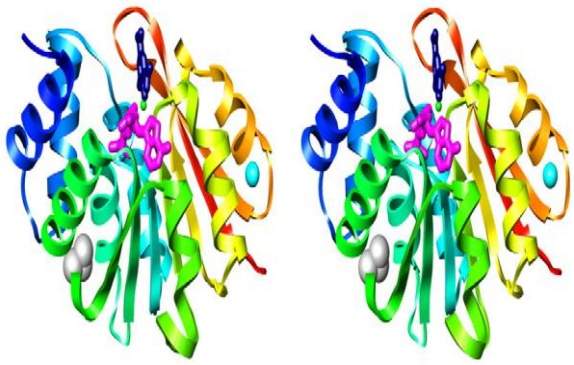


Figure 3. Crystal structure of hSCOMT. Ribbon diagrams of human 108V S-COMT colored from blue (N-terminus) to red (C-terminus) are shown in stereo. AdoMet (magenta) and 3,5-dinitrocatechol (dark blue) are shown in stick representation. K+ (cyan), Mg2+ (green), and the side chain of residue V108 (gray) are shown in space-filling representation (Rutherford *et al.*, 2008b).

In summary, the Lys144 and Glu199 residues participated in the methylation reaction (Veerapandian, 1997; Woodard *et al.*, 1980), and Trp38 and Pro174 residues were positioned at the surface of the enzyme and sandwiched the planar catechol ring system (Bonifacio *et al.*, 2002; Lerner *et al.*, 2001; Vidgren *et al.*, 1994), in order to maintain the proper positioning for catalytic reaction. The Leu198, Met201, and Trp38 residues made a hydrophobic wall around the ligand binding site, while Met201 residue has variable conformations depending on the bound ligand and adjusts the size of the ligand-binding site (Bonifacio *et al.*, 2002). The Pro174 and Leu198 residues are known to contribute significantly to the stabilization of the complex (Learmonth *et al.*, 2004). Furthermore, lipophilic Leu198 residue influences the regioselectivity of ortho- and meta-nitrated inhibitors (Palma *et al.*, 2006). Indeed, positively charged Lys144 and S-Met group of AdoMet influence the electrostatic effects of catechol ring substitution and the selectivity of several inhibitors.

1.3 Expression of recombinant COMT proteins

Recombinant production of proteins is a major step forward in the development of biotechnology products that became possible with the advent of recombinant DNA technologies.

In the past decades, with the disclosure of mammalian COMT gene and cDNA synthesis (Bertocci *et al.*, 1991; Lundstrom *et al.*, 1991) it was possible to express recombinant COMT proteins in heterologous system such as eukaryotic (insect (Tilgmann *et al.*, 1992) and mammalian cells (Malherbe *et al.*, 1992; Tilgmann *et al.*, 1992; Ulmanen *et al.*, 1997)) and prokaryotic (Bonifacio *et al.*, 2001; Lundstrom *et al.*, 1992; Malherbe *et al.*, 1992; Passarinha *et al.*, 2007; Xu *et al.*, 1999) systems. The last one used different strains of *Escherichia coli (E. coli)*. In spite of all the afore-mentioned systems having produced functional forms of the protein, the improvement of recombinant COMT, in large scale expression *E. coli* is a promising host. Since it is completely lacks endogenous COMT enzyme (Tilgmann and Ulmanen, 1996) and in order to improve the volumetric and mass productivity for several biopharmaceutical and neurological domains (Bonifacio *et al.*, 2001). Indeed, this expression system allows the highest levels of expressed soluble, non-glycosilated and moderate size proteins (Ibdah *et al.*, 2003; Vilbois *et al.*, 1994), such as COMT.

1.4 Purification procedures of COMT proteins

The development of techniques and methods for the separation and purification of proteins have been essential for several advancements in biotechnology research. The purity of a protein is a pre-requisite that has to be established for structure and function studies or its potential application.

During the last 40 years, several purification procedures were been described to COMT from different biological extracts. The enzyme has been enriched by various biochemical separation methods and strategies, such as differential centrifugation, ammonium sulfate (NH_2SO_4) fractionation (Ball *et al.*, 1971), size exclusion (Lundstrom *et al.*, 1992) and anion (Ball *et al.*, 1971; Lundstrom *et al.*, 1992) or cation exchange chromatography (Tilgmann and Ulmanen, 1996; White and Wu, 1975). Recently, affinity chromatographic methods have been described for the purification of recombinant rat and human COMT as a fusion protein (Bonifacio *et al.*, 2001; Cotton *et al.*, 2004). In spite of this chromatographic procedure leads to low percentage recovery, it allows the purification of the recombinant enzyme in sufficient amounts for structure–function studies (Bonifacio *et al.*, 2002; Palma *et al.*, 2003; Rodrigues *et al.*, 2005). Also, preparative reversed-phase chromatography is described for the purification of human COMT (Lundstrom *et al.*, 1992), but the denaturing conditions made it impossible to use the enzyme for biochemical and functional analysis. Only a few authors described hydrophobic interaction chromatography (HIC) as part of the downstream purification process for this recombinant protein (Nunes *et al.*, 2009; Passarinha *et al.*, 2006; Passarinha *et al.*, 2008). In fact, this strategy could offer great advantages in terms of increased yields and reduced the number of chromatographic steps.

Accordingly, most of the formerly published purification protocols for COMT have presented multi-step procedures requiring application of multiple chromatography types and chemical and physical manipulations in order to achieve the desired level of purity. Therefore, the demands of more simple, reliable and rapid purification methods are needed to recover highly pure, homogeneous and active COMT protein.

1.4.1 COMT stability

The global aim of a protein purification process is not only the removal of contaminants, but also the concentration of the desired protein and their transfer to an environment where it is stable and in a formulation for the intended application. In general, proteins for pharmaceutical applications must be stable over 2 years or longer, against several stress factors encountered during storage, shipping and handling. Various additives (designed as excipients) are used to enhance stability and reduce aggregation of the proteins against these effects (Arakawa *et al.*, 2007a).

Several purification data suggested that COMT is fairly labile and loses rapidly its activity during the isolation process and storage (Tilgmann and Kalkkinen, 1990), probably due to the oxidation of the free cysteine-SH groups. Indeed, S-COMT highly purified fractions show a more rapid decrease in activity (Cotton *et al.*, 2004). Experimental observations reveal that EDTA and $MgCl_2$ in equimolar concentrations (Ball *et al.*, 1971) conjugated with the reducing agent DTT (Cotton *et al.*, 2004) into buffers, have a stabilizing effect on all enzyme preparations. Similarly, the use of reducing agents as β–mercaptoethanol, DTT or L-cysteine by other investigators had stabilized the enzyme and allowed the purification and partial characterization of human COMT (Assicot and Bohuon, 1970). In this context, it can be concluded that since reducing agents can restore COMT activity, the rapid inactivation observed in purified S-COMT samples can be a result of the oxidation of sulphydryl group(s) (Assicot and Bohuon, 1970).

1.5 Genetic polymorphism of COMT and diseases association

The human COMT gene contains three common coding polymorphisms: A22S, A52T, and V108M (Cargill *et al.*, 1999; Lee *et al.*, 2005; Saito *et al.*, 2001; Shield *et al.*, 2004), leaving the protein susceptible to deactivation by temperature or oxidation. Although, the structural and epidemiological effects of the V108M mutation are the best characterized. The side chain of residue 108 is buried within a hydrophobic pocket in a loop between R5 and β3 from the protein's active site. The mutation distorted the overall structure of COMT, increasing the solvent exposure of both the AdoMet- and catechol-binding sites, as showed in Figure 4 (Rutherford *et al.*, 2006). While the wild-type and V108M proteins display similar kinetic properties *in vitro* (Chen *et al.*, 2004; Goodman *et al.*, 2002; Lotta *et al.*, 1995), the V108M polymorphism destabilizes the

protein, increasing its susceptibility to thermal (Rutherford *et al.*, 2008a; Shield *et al.*, 2004) and chemical denaturation (Rutherford *et al.*, 2008a) as well as oxidative stress (Cotton *et al.*, 2004; Li *et al.*, 2005; Li *et al.*, 2004). This destabilization results in the protein levels decrease (Chen *et al.*, 2004; Doyle *et al.*, 2004; Doyle and Yager, 2008), and therefore activity (Boudikova *et al.*, 1990; Grossman *et al.*, 1992b; Scanlon *et al.*, 1979; Spielman and Weinshilboum, 1981; Weinshilboum and Dunnette, 1981) *in vivo* relative to the wild-type protein.

In the A22S mutation, residue 22 is positioned in a surface loop between R1 and R2, 13 Å from the AdoMet-binding site and ~20 Å from the catechol-binding site. This polymorphism significantly increases COMT's affinity for AdoMet but decreases COMT activity by 30% relative to the wild-type protein (Li *et al.*, 2005; Rutherford and Daggett, 2009).

In addition, while the A52T mutation slightly decreases COMT's affinity for AdoMet, it has no significant effect on COMT activity (Li *et al.*, 2005). Typically, residue 52 is positioned on the protein surface in helix R3 which contains critical residues for the binding of AdoMet and catechol substrates. The 52T protein have a T50 (temperature resulting in 50% inactivation) value more low to wild-type (Shield *et al.*, 2004).

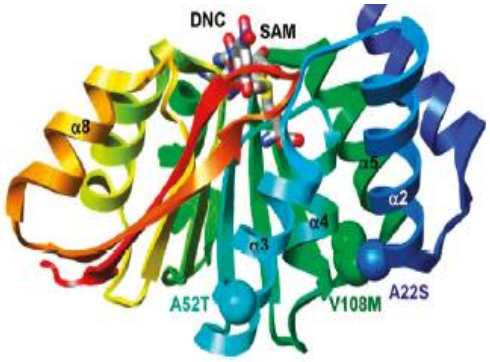


Figure 4. A22S, A52T, and V108M COMT polymorphisms. Ribbon diagram of wild-type COMT colored from blue (N-terminus) to red (C-terminus). AdoMet and 3,5-dinitrocatechol are shown in stick representation and colored by atom type. Polymorphic residues 22, 52, and 108 are shown in space-filling representation and colored blue, cyan, and green, respectively (Rutherford and Daggett, 2009).

In spite of both the 22S and 108M alleles have been associated with neuropsychiatric dysfunction (Frisch *et al.*, 2001; Glatt *et al.*, 2003; Karayiorgou *et al.*, 1997; Karege *et al.*, 1987; Kirov *et al.*, 1998; Lee *et al.*, 2005; Li *et al.*, 1996; Papolos *et*

al., 1998; Rujescu *et al.*, 2003; Woo *et al.*, 2002), only the allele 108M is known to be related to an increased risk of cancer (Lavigne *et al.*, 1997; Yim *et al.*, 2001). Interestingly, recent studies have linked the 108M allele with increased sensitivity to pain (Woo *et al.*, 2002) and with improved prefrontal cognition (Sheldrick *et al.*, 2008), as described in table II. No data associating the A52T polymorphism with disease have been published.

Table II. Polymorphisms of COMT and diseases association

	COMT activity allele	**Polymorphism**	**Reference**
Polysubstance abusers	high	108Val	(Vandenbergh *et al.*, 1997)
Anorexia nervosa	high	108Val	(Frisch *et al.*, 2001)
Schizophrenia	high	108Val	(Glatt *et al.*, 2003; Li *et al.*, 1996)
	low	22Ser	(Lee *et al.*, 2005)
Type 1 alcoholism	low	108Met	(Tiihonen *et al.*, 1999)
Bipolar disorder	low	108Met	(Kirov *et al.*, 1998; Papolos *et al.*, 1998)
Obsessive-compulsive disorder	low	108 Met	(Karayiorgou *et al.*, 1997)
Depression	low	108 Met	(Karege *et al.*, 1987)
Breast cancer	low	108 Met	(Lavigne *et al.*, 1997; Yim *et al.*, 2001)
Panic disorder	low	108 Met	(Diatchenko *et al.*, 2006; Woo *et al.*, 2002)
Suicidal behavior	low	108Val	(Rujescu *et al.*, 2003)

In general, the level of COMT enzyme activity is genetically polymorphic in human tissues with a trimodal distribution of low (*COMT*LL), intermediate (*COMT*LH), and high (*COMT*HH) activity (Boudikova *et al.*, 1990; Weinshilboum and Raymond, 1977). The enzyme activity is ubiquitous and these levels vary not only among different species (Ellingson *et al.*, 1999; Schultz *et al.*, 1989), but also in individuals of the same species (Palmatier *et al.*, 1999) as well as in tissues from the same individuals (Ellingson *et al.*, 1999; Guldberg and Marsden, 1975; Mannisto and Kaakkola, 1999).

1.5.1 COMT role in Parkinson disease

Parkinson's disease is a progressive neurodegenerative disorder caused by the loss of dopaminergic nigrostriatal neurons, leading to characteristic motor symptoms. Until now, the most effective treatment for this disease is the dopamine replacement therapy with L-DOPA (dopamine precursor) together with an inhibitor of aromatic amino acid decarboxylase (AADC), as showed in Figure 5. The efficacy of this therapy, however, decreases with time and most patients develop fluctuating responses and dyskinesias (Bene *et al.*, 2009). The last decade showed that the use of COMT inhibitors as adjuvants to the L-DOPA/AADC inhibitor therapy, in order to increase the bioavailability of L-DOPA, improve the clinical benefits of this therapy (Figure 5) (Bene *et al.*, 2009). A historical overview of the discovery and development of COMT inhibitors reveal a special emphasis on nebicapone, presently under clinical development, as well as entacapone and tolcapone, which are already approved as adjuncts in the therapy of PD (Bonifacio *et al.*, 2007). In spite of the association of the COMT alleles with PD has been extensively studied, no association has been found (Hoda *et al.*, 1996; Syvanen *et al.*, 1997; Xie *et al.*, 1997). Nevertheless, some results demonstrated that some Japanese individuals with the low COMT activity allele may have an increased risk for PD (Kunugi *et al.*, 1997; Yoritaka *et al.*, 1997).

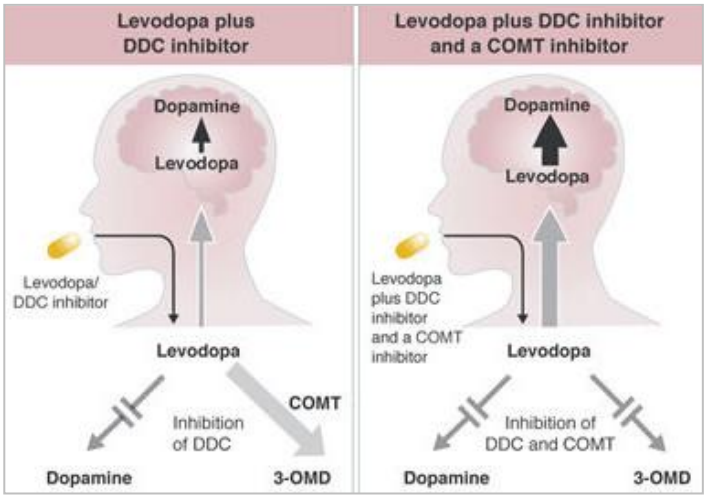


Figure 5. Dopamine replacement in PD therapy with dopamine precursor (L-DOPA). Scheme of the triple combination therapy in PD. (European Parkinson's Disease Association, 2007).

1.6 Analytical methods in COMT assays

Typically, COMT activity analysis has been applied clinically since COMT inhibitors have been introduced as adjuvant drugs in the treatment of PD (Pihlavisto and Reenila, 2002). The discovery of COMT as a drug target and the identification of polymorphic COMT forms (soluble and membrane-bound) have increased the demand for reliable, sensitive and fast analytical activity assays for COMT activity. For example, for the:

-Measurement of the recombinant protein activity;

-Testing *in vitro* the efficacy of new inhibitor candidates;

-Determination of structure–activity relationships;

-Activity measurement in various physiological and pathophysiological states.

In general, there is a great variation in analytical methods of COMT assays. These strategies consist in the sample handling and incubation followed by separation and detection of the reaction products. The major combinations methods are shown in Figure 4, such as separation of the COMT reaction products by solvent extraction and detection by fluorometry (Axelrod *et al.*, 1958) or radiochemical techniques (Bates *et al.*, 1979; Hong *et al.*, 1998; Zurcher and Da Prada, 1982). However, the introduction of High Pressure Liquid Chromatography (HPLC) techniques coupled with UV detection (Pennings and Van Kempen, 1979), fluorometric (Nohta *et al.*, 1984; Smit *et al.*, 1990), radiochemical (Nissinen, 1985) and electrochemical detection (Nissinen and Mannisto, 1984; Passarinha *et al.*, 2006; Schultz *et al.*, 1989) have improved the sensitivity and specificity of the analysis. However, activities between different COMT enzyme sources are not necessarily comparable and the distinct protein assay methods in samples should also be considered (Pihlavisto and Reenila, 2002), in order to access COMT activity.

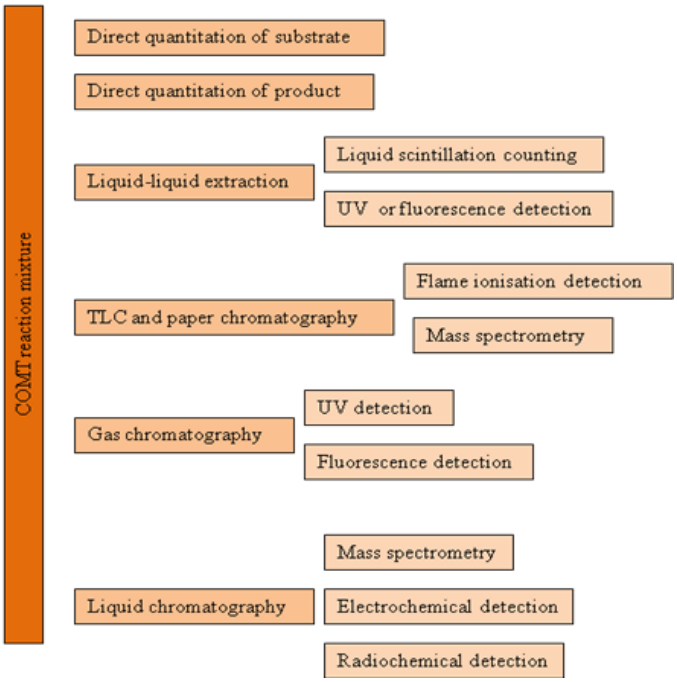


Figure 6. Analytical methods applied to COMT activity analysis (Adapted from (Pihlavisto and Reenila, 2002)).

Chapter II. Aims and Outline

The global aim of this work was the improvement of recovery yields and activity protein results in hSCOMT purification process. Therefore, the work will be oriented according to the following intermediate goals:

Chapter IV. Hydrophobic Interaction Chromatography: Octyl and Epoxy supports

- Octyl-sepharose support and destabilizing elution conditions using L-arginine
  - To optimize new fractionation methods in Octyl-sepharose sorbents by testing destabilizing elution conditions with the incorporation of L-arginine in the mobile phase.

- Epoxy-sepharose support and dual salt system application
  - To analyze the application of dual salt system effects in epoxy-sepharose matrice, in order to improve hSCOMT downstream processes.

Chapter V. Pseudo-Bioaffinity Chromatography: Amino Acids as Immobilized Ligands

- To examine the performance of pseudo-bioaffinity ligands, based on amino acids matrices in the isolation of hSCOMT from *E. coli* lysates. In this point, the intermediate aims will be understanding the selectivity's achieved by these new supports and consequently their influence in the purity and kinetic properties of the target protein. Typical elution methods will be developed based on non-specific desorption profiles supported on pH and ionic strength manipulation.

Chapter III. General methodologies

3.1 Material

Ultrapure reagent-grade water was obtained with a Milli-Q system (Milipore/Waters). Carbenicillin (disodium salt), isopropylthiogalactosidase (IPTG), tryptone, bact-yeast extract, lysozyme, dithiothreitol (DTT), ammonium sulphate (NH_2SO_4), Tris(hydroxymethyl)aminomethane (Tris), sodium citrate ($Na_3C_6H_5O_7$), L-cysteine, L-arginine, AdoMet, CAPS, DNase, RNase, epinephrine (bitartrate salt), disodium ethylenediaminetetraacetic (EDTA), citric acid monohydrate, sodium octyl sulfate, dibutylamine, Bovine serum albumin (BSA) and L-arginine, L-methionine, L-histidine, L-aspartate, L-glutamine and L-leucine agarose supports were obtained from sigma were obtained from Sigma Chemical Co (St Louis, MO, USA). Potassium chloride (KCl), sodium acetate (anhydrous). Sodium chloride (NaCl) were supplied by Fluka (Buchs, Switzerland). Bis-acrylamide 30% and Bio-Rad protein assay reagent was purchased from (Bio-Rad, Hercules, CA).The full range rainbow protein standards used for estimation of subunit molecular weight, anti-rabbit IgG alkaline phosphatase secondary antibody and Octyl-Sepharose 6FF support were purchased by GE Healthcare Biosciences (Uppsalla, Sweden). Polyclonal rabbit anti-COMT antibody was produced in BIAL using purified recombinant rat COMT (Bonifacio *et al.*, 2001). The epoxy-Sepharose CL-6B was prepared by covalent immobilization of 1,4-butanediol diglycidyl ether on Sepharose CL-6B according to the protocol described elsewhere (Sundberg and Porath, 1974). All other reagents were of analytical grade and used without further purification.

3.2 Methods

3.2.1 Recombinant hSCOMT production and recuperation

3.2.1.1 Plasmid and bacterial strain

The Champion pET101 Directional TOPO expression kit (Invitrogen Corporation, Carlsbad, CA, U.S.A.) was used for the expression of hSCOMT in its native form on *E. coli* BL21-Star (Invitrogene, USA) strain. The overexpression of hSCOMT protein is under the control of the IPTG inducible promoter and employing carbenicillin supplementation as a selection marker.

3.2.1.2 Recombinant hSCOMT protein production

The recombinant plasmids pET101, with construct hSCOMT, were transformed into *E. coli* BL21-star cells and grown overnight at 37 ºC in agar plates with LB medium containing carbenicilin disodium salt (50 µg/ml). Next, a single colony was inoculated in 62,5 mL of SOB medium in 250 mL shake flasks and grown at 37 ºC. When they reach an optical density at 600 nm (OD_{600nm}) of 2.6 units, an aliquot was added in 250mL of SOB medium in 1 L shake flasks, since the inoculation volume was fixed to achieve an initial OD_{600nm} of 0.2–0.3 units. As the OD_{600nm} reached 0.6 units, was induced the recombinant hSCOMT production by the addition of IPTG (final concentration of 1 mM). After induction, cells were grown at 37 ºC during 4 h and collected by centrifugation at 4600 rpm for 20 min at 4 ºC and the pellet was stored at -20 ºC.

3.2.1.3 Cell lysis

The bacterial cell pellet (250 mL) was resuspended in 10 mL of Bial buffer, and disrupted by lysozyme treatment (0.5 mg/mL) during 15 minutes at room temperature (RT). After this, six consecutive freeze (-196ºC) / thaw (42ºC) cycles were performed. After, DNase (250 µg/mL) and RNase (500 µg/mL) are added and the lysate was incubated for 10 minutes at 37ºC. Next, the lysate obtained was centrifuged at 16000 g for 20 min at 4 ºC to remove the cell debris. The supernatant was then applied to chromatographic columns and used in stability assays.

3.2.2 Total protein quantification: Bradford micro-assay

The Bio-Rad Protein Assay, based on the Bradford method, is a dye-binding assay in which a differential color change of a dye occurs in response to various proteins concentrations. The maximum absorbance for an acidic solution of Coomassie® Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when the binding to a protein occurs. The Coomassie blue dye binds essentially to basic and aromatic amino acid residues, especially arginine.

3.2.2.1 Procedure

The protein content in samples was measured by the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA), with BSA as standard samples (1.2 to 10.0 µg/ml), according to manufacturer's indications.

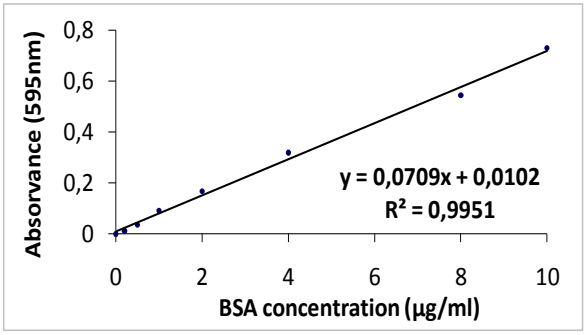


Figure 7. BSA bial buffer calibration curve.

3.2.3 Analytical chromatography: hSCOMT specific activity assay

The activity assay was designed to evaluate the methylation efficiency of recombinant hSCOMT by measuring the amount of *O*-methylated reaction products with electrochemical detectors. These detectors have excellent limits of detection and application of a wide range of analytes, suitable for the assay of catecholamines and phenolic hydroxyls of the *O*-methylated products in an oxidative/reductive mode at inert electrodes. These methods are commonly reported because of the high sensitivity and selectivity achieved (Saxer *et al.*, 2004).

3.2.3.1 Procedure

In hSCOMT activity assay an aliquot of 150 µg/mL (500 µL) of the soluble extract was incubated in reaction mixture, at 37 ºC during 5 min. After, was added 0.1 mL of Epinefrine (1 mM) and the reaction was stopped by adding 200 µL of percloric acid (0.4 M) 10 minutes later. The samples were centrifuged at 6000 rpm for 10 min at 4ºC and after the supernatants were filtered through a 0.22 µm pore size filter to remove precipitated debris. Finally, the injection was performed into the HPLC system (Waters) with an amperometric electrochemical detection.

Chromatographic separation was achieved using a 5 µm particle size XTerra MS C18 ODS reversed-phase analytical column (waters, 250 × 4.6 mm i.d.) connected to a precolumn (Waters, 5 µm, 10 × 4.6 mm i.d.). All buffers pumped in HPLC system were

filtered through a 0.22 µm pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically. The mobile fase was pumped by an isocratic mode through the chromatographic system at 1.0 mL/min. All the injections were made with a rheodyne valve, equipped with a 20 µL sample loop. The electrochemical oxidation of metanephrine to their respective *O*-quinones was performed in a flow single-cell, equipped with a 3 mm diameter glassy carbon working electrode set at +750 mV over an ISAAC reference electrode. The eluent, analytical column and flow single-cell were maintained with a constant temperature (33 ºC) using a preheated module installed in the electrochemical amperometric detector. The method sensitivity was set at 100 nA.

3.2.4 SDS-PAGE and *Western blot*

Typically, the SDS-PAGE (Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis) method is useful for molecular weight and purity analysis of proteins. In the presence of the negatively charged detergent, Sodium Dodecyl Sulfate (SDS) binds to the polypeptides in order to form complexes with constant charge to mass ratios. This results in a means of differentiation based on the molecular weight of the molecules. After the electrophoresis, proteins are immobilized on membrane, usually a polyvinylidene difluoride (PVDF) membrane, and the blot is then probed for a specific protein using primary antibodies directed against the protein of interest. Subsequently, secondary antibody recognizes the primary antibody. The secondary antibody has been conjugated to an enzyme wich in addition of a specific substract produces a colorimetric reaction in membrane site corresponding to protein.

3.2.4.1 Procedure

Were boiled in a loading buffer for 10 min at 100 ºC and then deposit and run, respectively on stacking (4%) and resolving (12,5%) SDS-PAGE, with a running buffer at 150 V for approximately 90 min. Then, one gel was stained by Comassie brilliant blue R-250 and the second was used to perform *Western blot*, for the analysis of purity and immunoreactivity, respectively.

For comassie brilliant blue staining procedure, the gel was placed in the staining solution for 30 min on agitation. After this, for excess staining, the gel was placed in the

distaining solution with the same procedure. The gel could be stored indefinitely in fixing solution.

The second gel was transferred to a PVDF membrane (GE Healthcare Biosciences, 0.45 µm) in blotting buffer, over a 10 min at 750 mA at 4°C. The PVDF membrane were blocked with Tris buffer saline – Tween 20 (TBS-T) containing 5% (w/v) skimmed milk powder for 1 h and incubated overnight at 4 ºC with rabbit anti-ratSCOMT polyclonal antibody (diluted 1:2000 in TBS-T), that cross reacts with the human protein. After washing three times (15 min) with TBS-T, PVDF membrane was incubated with anti-rabbit IgG alkaline phosphatase secondary antibody (diluted 1:10,000 in TBS-T) for 1 h at RT. Posteriorly, the membranes were washed three times again with TBS-T. Revelation reaction were developed using 200 µL of ECF substrate (GE Healthcare Biosciences) and images of blots were captured with the Molecular Imager FX Pro Plus MultiImager system by chemiluminescence's detection

Chapter IV. Hydrophobic Interaction Chromatography:

Octyl and Epoxy supports

4.1 General considerations

Hydrophobic interaction chromatography, a form of multivalent interaction chromatography, complementary to other separation techniques, is a powerful technique in modern biotechnology for the downstream processing of several biomolecules (Mahn *et al.*, 2005). In general, it is a method that can be applied to all proteins, at laboratory-scale applications and industrial processes, since HIC can be adapted to the special hydrophobic needs of each protein. Typically, HIC involves the separation of protein molecules owing to the differential hydrophobic interactions between immobilized hydrophobic ligands on the gel and non polar regions on the surface of proteins (Queiroz *et al.*, 2001). The adsorption (and retention) of proteins is carried out by moderate to high concentrations of anti-chaotropic salts followed by a linear or stepwise decrease in the ionic strength of the eluent. Recovery and resolution levels of HIC are often satisfactory (Lienqueo *et al.*, 2003) if a comprehension of certain parameters governing HIC, such as the stationary phase and the mobile phase (Queiroz *et al.*, 2001), could offer a rational optimization scheme for the purification process as a whole. Four parameters govern the adsorption of a protein to a homologous series of hydrophobic stationary phases: (1) the type of ligand, (2) the chain-length, (3) the surface concentration of immobilized ligand and (4) the type of the matrix or support (Tomaz *et al.*, 2002). The most widely used ligands for HIC are aromatic groups (e.g. phenyl) due to the presence of hydrophobic and aromatic (Π–Π) interactions and linear chains alkanes, such as Octyl (Queiroz *et al.*, 2001). Furthermore, ligands with intermediate hydrophobic character, such as Epoxy-Sepharose, have been useful in HIC processes, since they promote a mild binding strength and one elution by simple decreasing the ionic strength of the eluent (Tomaz *et al.*, 2002).

4.2 Procedure

Chromatographic separations were performed on a Fast Performance Liquid Chromatography (FPLC) system (GE Healthcare Biosciences, Uppsalla, Sweden). All buffers pumped in FLPC system were filtered through a 0.22 μm pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically.

The media were packed according to company guidelines (20 mL of gel volume) into a C16 glass column (GE Healthcare Biosciences). The column was equilibrated

with an appropriate loading buffer as indicated in each experiment. In the Octyl-sepharose assays, the buffer used throughout this study was NH_2SO_4, $Na_3C_6H_5O_7$ and L-arginine in 10 mM Tris-HCl buffer, pH 7.8. Aliquots (10 mL with a protein concentration of ~11.5 mg/mL) of recombinant hSCOMT-containing supernatant were loaded onto the columns and isocratic elution at 1 mL/min was performed. After loading, the column was washed with the same buffer and the bound proteins were eluted with NH_2SO_4, $Na_3C_6H_5O_7$ and L-arginine solutions at different concentrations in 10 mM Tris-HCl buffer, pH 7.8, in an appropriate stepwise gradient as indicated for each experiment. In Epoxy-sepharose assays, the buffer used was NH_2SO_4 and $Na_3C_6H_5O_7$ in 10 mM Tris-HCl buffer, pH 7.8. Aliquots (500 µL with a protein concentration of ~11.5 mg/mL) of recombinant hSCOMT- containing supernatant were loaded onto the columns and isocratic elution at 1 mL/ min was performed. After loading, the column was washed with the same buffer and the bound proteins were eluted with NH_2SO_4 and $Na_3C_6H_5O_7$ solution at different concentrations in 10 mM Tris-HCl buffer, pH 7.8, in an appropriate stepwise gradient as indicated for each experiment. In all separations, the optical density was monitored at 280 nm throughout the entire chromatographic run, while 1 mL fractions were collected and evaluated for hSCOMT detection.

4.3 Octyl-sepharose support and destabilizing elution condition using L-arginine

In earlier purification trials, our group showed that in the presence of dual salt systems the octyl media exhibited satisfactory protein selectivity and purification factor, reducing the denaturation effects of hSCOMT observed with higher salt concentrations. Also, the inclusion of temperature control during the elution step appears advantageous for greater activity recovery without enzyme aggregation. In spite of these results allowing the prediction of more stabilizing conditions for this termolabile enzyme, additional experiments have to be performed, based on new elution strategies in order to improve HIC selectivity and desirable purity on the target fractions. In general, the intense interaction promotes by the hydrophobic binding of proteins under salting-out conditions, compromise the elution strategy, while adsorbed proteins cannot be eluted by simply reduce of salt concentration. A typical example is found in a hSCOMT purification strategy, using HIC with dual salt system that needs low temperature to the

total elution of target protein (Nunes *et al.*, 2009). In this context, the using of amino acids emerge to facilitate purification or to stabilize proteins during purification. Specially, L-arginine is highly effective for the improvement of performance onto several chromatographyc strategies (Arakawa *et al.*, 2007a). Reports showed that target proteins to be purified can bind to HIC resins in the presence of L-arginine and eluted in the presence or not of L-arginine. Indeed, inclusion of L-arginine in chromatographic buffers, increases the protein recoveries from the columns and results in less aggregation (Arakawa *et al.*, 2007a; Arakawa *et al.*, 2007b; Ishibashi *et al.*, 2005).

4.3.1 Results and discussion

4.3.1.1 hSCOMT stability at several L-arginine concentrations

As showed in Figure 8, preliminary control experiments were conducted to analyze the effects of 10 mM Tris-HCl buffer with 0.5, 1, 1.5 and 2 M of L-arginine on hSCOMT stability. The positive control was 10 mM Tris-HCl buffer without L-arginine. These experiments were performed during 3 h at 4 ºC.

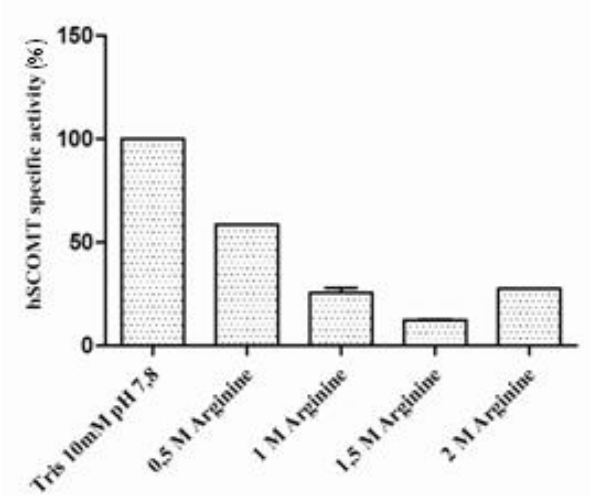


Figure 8. hSCOMT specific activity at L-arginine concentrations range from 0.5 to 2 M in comparison with the positive control achieved with 10 mM Tris-HCl at pH 7.8. The experiments were performed at 4ºC during 3 h.

The results of stability trials showed that L-arginine in concentrations used in these assays decrease hSCOMT specific activity. In particular, L-arginine concentration

above 1 M influences activity assays, and this fact decreases the statistic significance of these results.

As previously described, L-arginine is commonly used in chromatographic procedures. In spite of this amino acid does not affect the native structure of the proteins it does not stabilize them either (Arakawa *et al.*, 2007a). Indeed, L-arginine is not classified as an osmolyte, interfering with enzymatic function (Ishibashi *et al.*, 2005), and probably this fact is crucial for the decreasing of hSCOMT specific activity by L-arginine.

4.3.1.2 Preparative chromatographic assays

These experiments were performed to understand the effect of L-arginine incorporation in binding buffer, using octyl-sepharose with dual salt system. As showed in Figure 9, chromatographic assays were performed in octyl-sepharose with a three stepwise gradients. In the Figure 9 (A) binding buffer has not L-arginine compared with Figure 9 (B) that contains L-arginine in the binding buffer. In order, to compare these two assays, the elution steps were performed in the next strategy; in the first step was performed maintaining salt concentration and increasing L-arginine concentration and the last elution step was conducted simultaneously without salt and L-arginine. Specifically, the Figure 9 (A) and (B) showed that the presence of L-arginine 0.5 M in binding buffer (Figure 9 (B)) interfere in *E. coli* proteins retention. When it was used L-arginine in binding buffer, the first elution step (Figure 9 (B)) do not allow protein species to elute contrarily to what happens when it was not used L-arginine in the binding buffer. These results showed that the protein species eluted in the first elution step, only binding in the absence of L-arginine in the binding buffer.

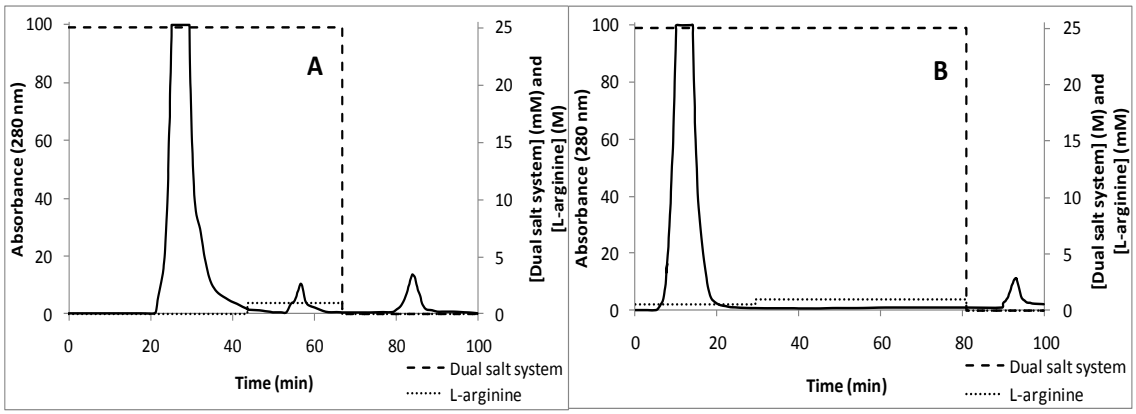


Figure 9. Chromatographic profile of an *E. coli* lysate extract onto the octyl-sepharose support with a dual salt system and stepwise gradients from buffer A, B to C. (A) buffer A: NH_2SO_4 and $Na_3C_6H_5O_7$ at 25 mM in 10 mM Tris–HCl, pH 7.8; buffer B: NH_2SO_4 and $Na_3C_6H_5O_7$ at 25 mM, L-arginine 1 M in 10 mM Tris–HCl, pH 7.8 and buffer C: 10 mM Tris–HCl, pH 7.8. (B) buffer A: NH_2SO_4 and $Na_3C_6H_5O_7$ at 25 mM, L-arginine 0,5 M in 10 mM Tris–HCl, pH 7.8; buffer B: NH_2SO_4 and $Na_3C_6H_5O_7$ at 25 mM, L-arginine 1 M in 10 mM Tris–HCl, pH 7.8 and buffer C: 10 mM Tris–HCl, pH 7.8.

Also, the results demonstrated that L-arginine competes with specific protein species for the binding in the chromatographic support. In addition, hSCOMT was eluted only in the absence of salt concentration. Subsequently, the next approach was to analyze simultaneously the effect of a dual salt system strategy with the presence or absence of L-arginine onto chromatographic buffers. As showed in Figure 10, this chromatographic assay was performed with L-arginine incorporation only in elution buffer with dual salt system, specifically 1 mM (data not show). In these experiments the dual salt concentration was progressively decreases and 1 mM was the minimal concentration that allows hSCOMT retention onto octyl support. In spite of the L-arginine had some effect in the host proteins contaminants elution (Figure 9), its comparison with the Figure 10, proved that the elution was due mostly to salt decreasing.

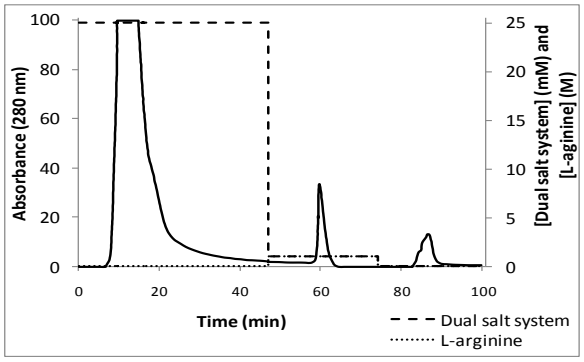


Figure 10. Typical chromatographic profile of an *E. coli* lysate extract onto an octyl support with a dual salt system and stepwise gradient from buffer A, B to C. Buffer A: NH_2SO_4 and $Na_3C_6H_5O_7$ at 25 mM in 10 mM Tris–HCl, pH 7.8; buffer B: NH_2SO_4 and $Na_3C_6H_5O_7$ at 1 mM, L-arginine 1M in 10 mM Tris–HCl, pH 7.8 and buffer C: 10 mM Tris–HCl, pH 7.8.

As show in Figure 8, the synergy between the decreasing of salt concentration and L-arginine incorporation on elution buffer, increasing the host contaminant proteins elution. However, the effect of L-arginine concentrations requirements, lead to a decrease in hSCOMT specific activity (Figure 8). So, this fact compromises the successful of the hSCOMT purification by the use of destabilizing elution conditions strategy with L-arginine incorporation.

4.4 Epoxy-sepharose support and dual salt system

For the purification of termolabile and sensitive proteins, such as hSCOMT, a good alternative to NH_2SO_4 could be $Na_3C_6H_5O_7$, a biodegradable and anti-chaotropic ion, described as a powerful protein stabilizer (Bottomley and Tew, 2000) and used in the isolation of several biomolecules, such as antibodies (Azevedo *et al.*, 2009) and small proteins (Sousa *et al.*, 2008a).

In the butyl support, the adsorption phase at 200 mM of $Na_3C_6H_5O_7$ leaded to preferential hSCOMT inactivation and lowers activity recoveries. In contrast for the octyl support, suitable combinations of NH_2SO_4 and $Na_3C_6H_5O_7$ gradients could be a better option than NH_2SO_4 stepwise strategies, regarding protein contaminant elimination (Nunes *et al.*, 2009). Indeed, studies above others HIC supports with soft hydrophobicity, such epoxy-sepharose, are desirable to promote mild interactions with the proteins, facilitating the posterior elution by decreasing the salt concentration of the mobile phase. So, the aim of these studies was to promote hSCOMT retention in epoxy-

sepharose with a dual salt system, maintaining hSCOMT specific activity and reducing protein aggregation, usually promoted in the presence of higher NH_2SO_4 concentrations (1.2 M).

4.4.1 Results and discussion

The chromatographic assays were performed with a stepwise gradient using a dual salt system in 10 mM Tris-HCl at pH 7.8. As showed in table III, these experiments were performed using crescent dual salt system concentrations, to allow hSCOMT retention. The elution of adsorbed protein species was performed in all assays with 10 mM Tris-HCl buffer at pH 7.8.

Table III. Dual salt system concentrations used in hSCOMT-Epoxy Sepharose retention assays.

Binding buffer		**hSCOMT retention**
NH_2SO_4 (mM)	$Na_3C_6H_5O_7$ (mM)	
50	50	No retention
100	100	No retention
300	150	No retention
600	150	No retention
700	150	No retention
800	150	No retention

The screening of dual salt system concentrations (table III) showed that the using of 800 mM NH_2SO_4 and 150 mM of $Na_3C_6H_5O_7$ not allowed hSCOMT retention (data not show). Technically, in spite of the increase of dual salt system concentration allow hSCOMT retention; $Na_3C_6H_5O_7$ and NH_2SO_4 can promote, respectively, the loss of enzymatic activity and hSCOMT aggregation, with a consequently decrease in hSCOMT specific activity. So, the application of a dual salt system in Epoxy-sepharose support does not achieve the pre-requisites in order to improve hSCOMT purification from complex protein extract.

Chapter V. Pseudo-Bioaffinity Chromatography: Amino Acids as Immobilized Ligands

5.1 General considerations

In the last decades, recombinant hSCOMT was purified as a fusion protein using affinity techniques as the main capture step. In general, removal of tags are desirable for most pharmaceutical and clinical applications in order to prevent artificial influence of the engineered tag region on protein activity measurements and structural studies. Therefore, the application of less selective supports, such as pseudo-bioaffinity ligands, can offer supplementary advantages than other traditional ligands with a less time-consuming multi-step procedures.

The amino acids immobilized ligands (AAIL's) were introduced, in 1989 by Vijayalakshmi and coworker, as "pseudobiospecific affinity ligands" and represent a promising alternative to traditional biospecific resins in affinity chromatography, due to their resistance to harsh chemicals, high temperatures (sterilization conditions) and low cost (Vijayalakshmi, 1989). Typically, AAIL´s act as electron acceptors of NH, SH or OH groups from protein amino acid residues such as histidine, cysteine, tryptophan, and serine. In general, the feasibility of these interactions with the ligand will depend on several conditions such as buffer composition, pH, temperature, ionic strength and support matrix. Nevertheless, the degree of interaction depend specifically on the protein surface amino acids accessibility (el-Kak *et al.*, 1992). These supports have been extensively used in the separation of several molecules such oligouronides (Delattre *et al.*, 2008; Delattre *et al.*, 2005), oligonucleotides (Sousa *et al.*, 2009a), pDNA (Sousa *et al.*, 2009b; Sousa *et al.*, 2008b), RNA (Jones *et al.*, 1976) and proteins (el-Kak and Vijayalakshmi, 1991; Haupt and Vijayalakshmi, 1993; Kanoun *et al.*, 1986; Summaria *et al.*, 1976).

To our best knowledge there are no any known structural evidences or experimental data to preview the behavior of mammalian COMTs on AAIL's. In this context, the global aim of this chapter was to analyze the performance of bio-pseudoaffinity supports for hSCOMT retention and consequently the degree of purification.

5.2 Procedures

5.2.1 Econo-Pac® disposable Chromatography Columns system

Chromatographic separations were performed at RT. The amino acids commercial media were packed according to company guidelines (5 mL of gel volume), into a Econo-Pac® disposable Chromatography Columns (Bio-Rad, Hercules, CA). All chromatographic separations were performed at RT and a 500 µL aliquot (protein concentration of ~11.5 mg/mL) of recombinant hSCOMT-containing supernatant, were loaded onto the columns. In retention using pH manipulation, columns were initially equilibrated with 10 mM Tris-HCl at different pH values and isocratic elution was performed with the same pH value. The elution of the bound proteins was performed with NaCl solutions at different concentrations in 10 mM Tris-HCl buffer at pH indicated in each experiment, in an appropriate stepwise gradient. The same procedure was performed in retention using salt manipulation, the binding buffer was NH_2SO_4 in Tris-HCl 10 mM at pH 7.8 and elution buffer was 10 mM Tris-HCl at pH 7.8. In all separations, 1 mL fractions were collected and the optical density was monitored at 280 nm. These fractions were following evaluated for hSCOMT detection.

5.2.2 FPLC system

The amino acids commercial media were packed according to company guidelines (10 mL) into a C16 glass column (GE Healthcare Biosciences). Aliquots (1 mL with a protein concentration of ~11.5 mg/mL) of recombinant hSCOMT-containing supernatant were loaded onto the columns and isocratic elution at 1 mL/ min was performed. The columns were initially equilibrated with Tris-HCl buffer (10 mM) at pH 5, and isocratic elution was performed with the same pH value. The elution of the bound proteins was performed with NaCl solutions at different concentrations in 10 mM Tris-HCl buffer at pH 7.8, in a stepwise gradient. In all separations, the optical density was monitored at 280 nm throughout the entire chromatographic run, while 1 mL fractions were collected and evaluated for hSCOMT detection.

5.3 Results and discussion

5.3.1 Retention using salt manipulation

In hydrophobic interaction the protein adsorption is carried out by moderate to high concentrations of anti-chaotropic salts, usually NH_2SO_4 (Queiroz *et al.*, 2001). In spite of previous data in the literature highlights the fact that hSCOMT is extremely labile in contact with NH_2SO_4, deactivation and/or modification dependence for this salt would be reduced as a result of the shortening processing time and low ionic strength applied onto typical HIC supports (Passarinha *et al.*, 2008).

The following assays were performed with 5 mL of AAIL's resins using an Econo-Pac® disposable Chromatography Columns system. In this stage was studied the applicability of hydrophobic interactions on AAIL´s supports, using NH_2SO_4 in the binding buffer, for capture of hSCOMT from the cell culture supernatants. These trials were carried out increasing NH_2SO_4 concentrations (0.5; 1; 1.25; 1.5 M). Representative chromatograms using 1.5 M NH_2SO_4 in the binding buffer, for the six commercial supports are showed in Figure 11. The chromatographic results showed a different degree of protein species retention; this fact is essentially due to two factors, the first is that the supports possess different ligands density and the second is the presence/absence of spacer harm. In spite of L-arginine had the lowest protein species adsorption, it had the second most ligands density. This fact is probably due to synergy between density ligands and hydrofobicity of this matrice.

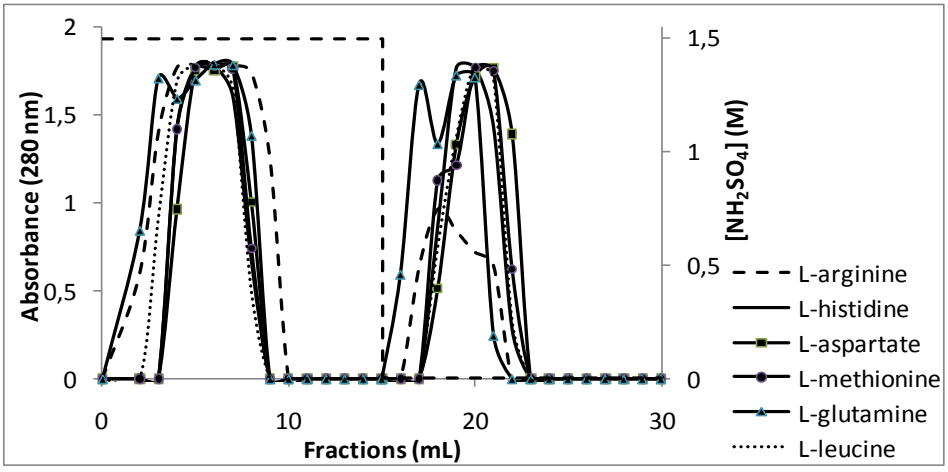


Figure 11. Chromatographic profiles of an *E. coli* lysate extract with recombinant hSCOMT onto the six AAIL's tested with a stepwise gradient from 100 to 0% buffer A (buffer A: 1.5 M NH_2SO_4 in 10 mM Tris–HCl pH 7.8; buffer B: 10 mM Tris–HCl, pH 7.8).

In addition, the SDS-PAGE (Figure 12(A)) and *Western blot* (Figure 12(B)) screening of chromatographic peaks showed that the target protein can be retained (L-arginine, L-aspartate, L-leucine and L-methionine) or not retained (L-glutamine and L-histidine) onto the experimental conditions described below. Also, the results showed that the major salt concentration that allows hSCOMT adsorption, was above 1.25 M in L-arginine, L-aspartate, L-leucine, L-methionine and above 1.5 M NH_2SO_4 in L-glutamine, L-histidine supports.

In spite of these achievements, previous data described by our group demonstrated that NH_2SO_4 at higher levels can reduce desired hSCOMT recovery and activity recuperation (Nunes *et al.*, 2009). Therefore, this strategy can compromise the successful of a bioprocess for hSCOMT isolation.

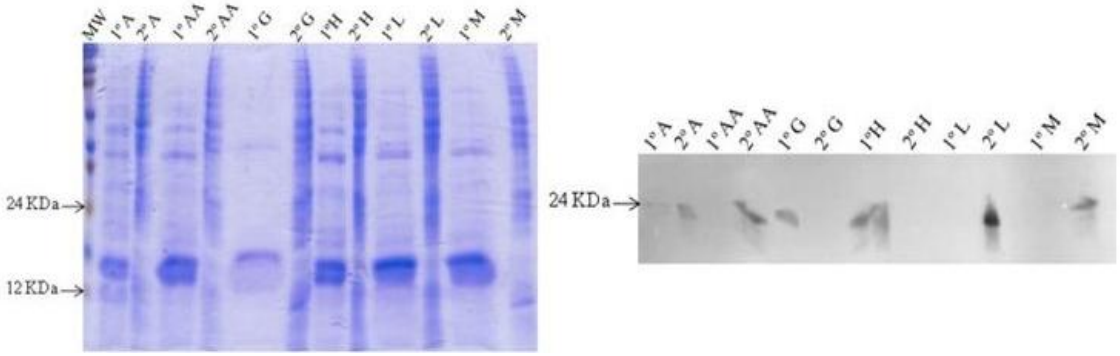


Figure 12. (A) SDS-PAGE analysis of *E. coli* recombinant hSCOMT lysates, loaded onto the several AAIL`s supports: (lanes 1, 2) L-arginine, (lanes 3, 4) L-aspartate, (lanes 5, 6) L-glutamine, (lanes 7, 8) L-histidine, (lanes 9, 10) L-leucine, (lanes 11, 12) L-methionine. The representative first and second lanes corresponded respectively to fractions collected at 100% buffer A and 0% buffer B. (B) *Western blot* from the same fractions obtained from AAIL's chromatographic trials.

According with literature, the ionic strength requirements for proteins binding can be higher and lower, depending on the hydrophobicity of the chromatographic support. Among the supports tested, the hydrofobicity decreases from L-leucine and L-methionine, to neutral L-histidine and L-arginine, and to the hydrophilic amino acids L-glutamine and L-aspartate. So, theoretically, with this hydrophilic scale we can establish that the ionic strength requirements are lower for the first amino acids and higher for the more hydrophilic. However, based on these results, the supports that needed more salt concentration to bind hSCOMT were L-glutamine and L-histidine, while L-aspartate

resin required a lower salt concentration. Therefore, results showed that there it was not possible to establish a linear relationship between AAIL's supports hydrofobicity and ionic strength for hSCOMT retention.

5.3.2 Retention using pH manipulation

5.3.2.1 hSCOMT stability at several pH

In general, the specific retention on AAIL's occurs mainly at pH values at, or around, protein isoelectric point (pI) (el-Kak *et al.*, 1992). So, ideally the adsorption pH for hSCOMT can be selected based early on the knowledge of its pI value (pI 5,5). As described for different proteins, such as yeast carboxypeptidase (pH 3-4) and goat chymosin (pH 5.5), acidic pH can be applied in global purification strategies without changes in their structural integrity (el-Kak *et al.*, 1992). Therefore, preliminary control experiments were conducted to analyze the effects of 10 mM Tris-HCl buffer with pH ranges from 2 to 13, on hSCOMT stability as show in Figure 13.

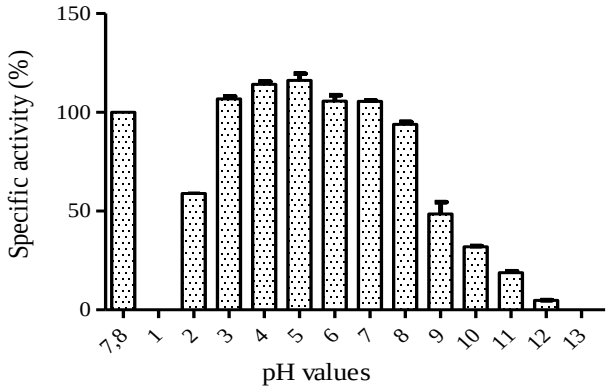


Figure 13. Activity (%) of hSCOMT at pH ranges from 1 to 13 in comparison to specific activity data achieved at pH 7.8, performed at 4 ºC during 12h.

The results of stability trials proved that hSCOMT maintains its activity over pH ranges from 3 to 8. Nevertheless, it was observed a decreasing for hSCOMT specific activity at values outside the range mentioned. These results highlight that hSCOMT was more stable at acidic pH's when compared with than basic ones. This fact is, probably, due to cysteine residues side chain ionization above pH 8 that could increases disulfide bond formation and consequently decreasing the hSCOMT native structural stability. Under this pH the effect is opposite because cysteine residues was protoned

and the probability for disulfide bond formation decreases while consequently hSCOMT specific activity increases.

5.3.2.2 hSCOMT binding studies

For this item, the assays were performed with 5 mL of AAIL's resins using an Econo-Pac® disposable Chromatography Columns system.

Based on preliminary stability assays and in order to maintain the enzymatic activity, the pH set point selection at the binding buffers was based on the activity profile obtained on Figure 13. The binding pH range tested to promote hSCOMT retention was showed in table IV

Table IV. Screening of binding buffer pH in six AAIL's supports.

	Binding buffer pH	hSCOMT retention
L-arginine	4	+
	5	+
	7.8	+
L-leucine	4	-
	5	-
	5.7	+
	7.8	-
L-histidine	4	-
	5	+
	6.5	-
	7.8	-
L-glutamine	4	-
	5	+
	6.5	+
	7.8	-
L-aspartate	4	+
	5	-
	6.5	-
	7.8	-
L-methionine	4	-
	5	+
	7.8	-

The results showed that only L-arginine and L-aspartate binds hSCOMT at pH around 4 and for pH 5 hSCOMT retention is observed onto L-arginine, L-histidine and

L-glutamine supports. In addition, L-leucine and L-glutamine binds hSCOMT respectively, only at pH 5.7 and 6.5. Specifically, it was observed that L-arginine resin allowed a positive adsorption for all the pH range studied. In addition, this support is exclusive for a hSCOMT binding at optimal COMT activity pH nearly 7.8. Indeed, the results described highlight that hSCOMT-AAIL's interaction seems to be dependent in the synergy in pH mobile phase and support under study.

In all chromatographic assays the AAIL's used had the α-carboxyl acid group ionized and the α-amino group protoned (table V). Specifically, L-aspartate support possesses the side chain ionized in all pH tested; in contrast L-glutamine and L-histidine supports had the side chain ionized respectively, above pH 5 and 7.8 in the binding buffer. The comparison between present ionizable groups in supports and pH used in chromatographic assays, showed that probably ionizable groups present could not have relation with hSCOMT majority retention.

Therefore, L-arginine-hSCOMT interaction occurs at pH's, such as pH 7.8, in which the hSCOMT do not interact with other AAIL's supports. This reveals that hSCOMT retention was not hSCOMT behavior-pH dependent.

Table V. Typical pKa's and pI's of amino acids immobilized in the chromatographic supports study.

	pKa α-carboxyl acid group	**pKa α-amino group**	**pKa Side chain**	**pI**
L-arginine	2.01	9.04	12.48	10.8
L-aspartate	2.10	9.82	3.86	2.8
L-glutamine	2.17	9.13	4.07	5.7
L-histidine	1.77	9.18	6.10	7.6
L-leucine	2.33	9.74	-	5.98
L-methionine	2.28	9.21	-	5.7

Nevertheless, the total hSCOMT retention on AAIL's tested is not achieved, independently of the binding conditions used. In addition, injection volumes of *E. coli* lysate was optimized for 250 to 500 µL (data not show), but hSCOMT retention was always partial. Probably, this is due to the small representatively of hSCOMT in *E. coli* lysates. This fact, increase the entropy inside of chromatographic columns leading to a decreasing of hSCOMT retention, that in the absence of this proteic entropy, the retention would be totally.

In spite of all the supports, binding hSCOMT at pH values that maintains protein stability, was decided to make the scale-up and following experimental procedures in L-methionine and L-histidine since these are the only ones commercially available.

5.3.2.3 L-methionine and L-histidine AAIL's

5.3.2.3.1 Elution conditions

In this point, the first approach was to analyze L-methionine and L-histidine selectivity in the presence of complex protein extracts from *E. coli* lysates. Based on previous binding results in the scale-down, the starting point of this stage was to performe two elution strategies. The first with pH manipulation and the second with the incorporation of NaCl onto the mobile phase.

As showed in Figure 14 (A, B) the pH manipulation using 3 elution steps allowed a residual protein elution at pH 7.8. However, when the elution strategy was based on the pH manipulation with one step (pH 7.8), the elution of proteins was significantly increased (Figure 14 (E, F)). In addition, these results showed that the pH manipulation in an only single unit (pH 5 to pH 6, pH 6 to pH 7 and pH7 to pH7.8) (Figure 14 (A, B)), did not allowed a residual proteins elution. However, the pH alteration of pH 5 to pH 7,8 (Figure 14 (E, F)) significantly increased proteins elution. Indeed, total absorbed proteins only eluted with 1 M of NaCl in the binding buffer (Figure 14 (E, F)).

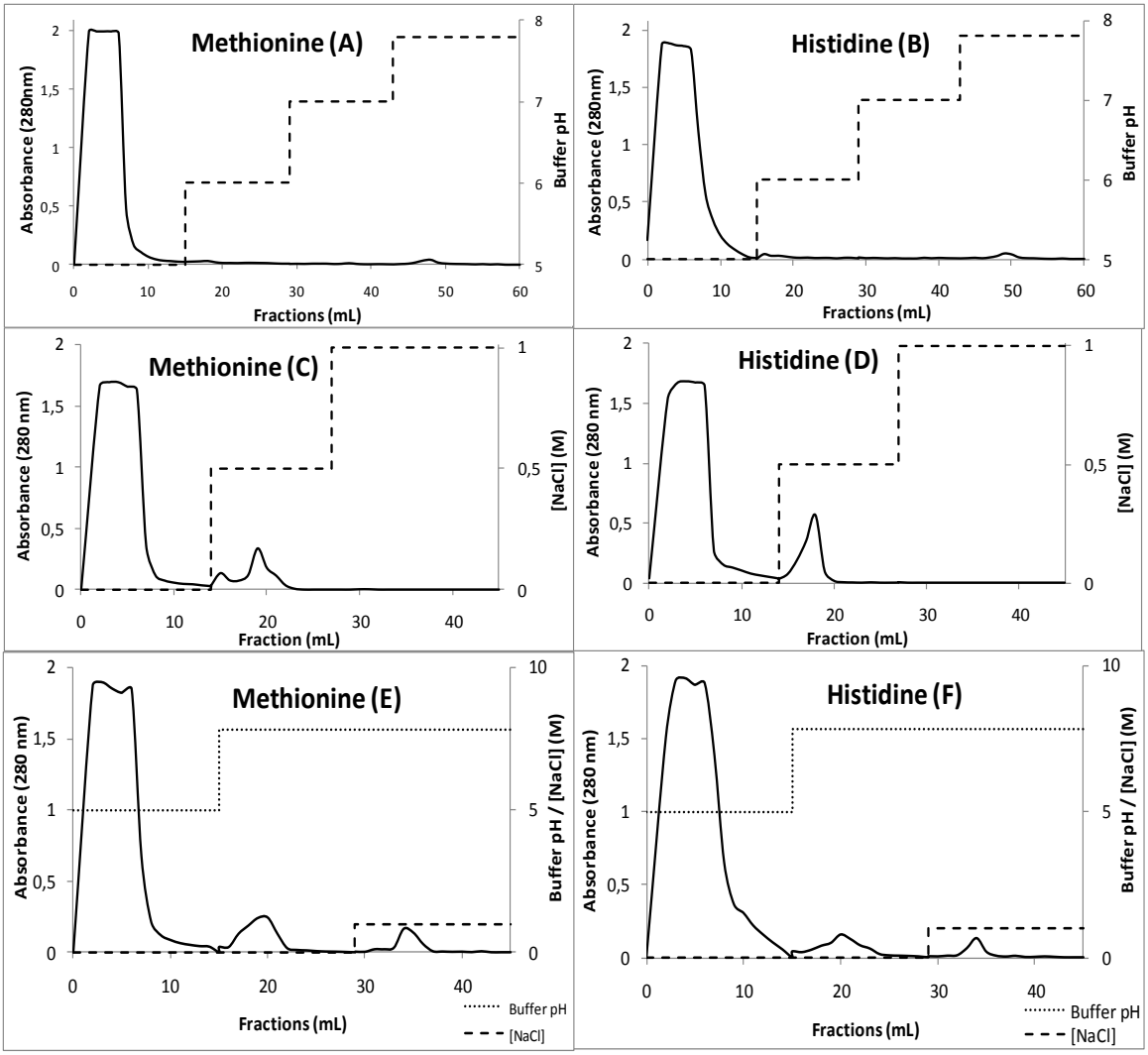


Figure 14. Comparative elution profiles on two AAIL's, L-methionine (A, C, E) and L-histidine (B, D, F), supports. (A, B) binding buffer 10 mM Tris-HCl at pH 5 and elution steps without salt (10 mM Tris-HCl at pH 6, pH 7, pH 7.8). (C, D) binding buffer 10 mM Tris-HCl at pH 5 and elution steps with 0.5 M NaCl and 1 M in 10 mM Tris-HCl at pH 7.8. (E, F) binding buffer 10 mM Tris-HCl at pH 5 and elution steps with 1 M NaCl in 10 mM Tris-HCl at pH 7,8.

For the chromatographic assays showed in Figure 14 (C, D), do not occur pH alteration during the chromatographic process, and total proteins species elution are achieved with NaCl 0.5 M. In addition, the screening fractions (Figure 15 (C, D)) of chromatographic assay, schematically in Figure (14 (E, F)), showed that hSCOMT was not eluted only with pH manipulation but when 1 M NaCl was added. On the other hand, the comparison of L-arginine and L-methionine supports showed that do not had significant differences between chromatographic profiles, Figure 14. In addition, when the screening was based on electrophoresis gels, (Figure 15 (A: 2ºH and 2ºM)) it was

showed that the purification degree achieved between L-histidine and L-methionine supports were similar. However, Figure 15 (C: 3ºH and 3ºM) show that the purification degree achieved in L-methionine was, slightly improved than in L-histidine support.

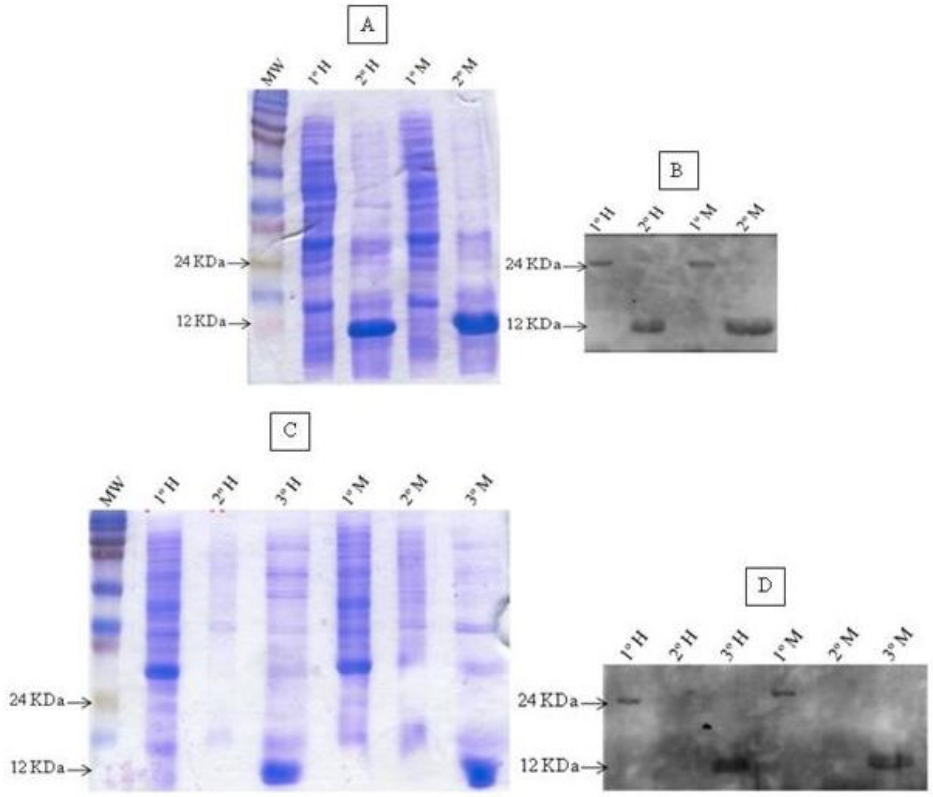


Figure 15. (A, C) SDS-PAGE analysis of *E. coli* recombinant hSCOMT lysates, loaded onto the L-methionine (A) and L-histidine (C) supports. The representative first lanes corresponded to fractions collected at 10 mM Tris-HCl at pH 5 (A, C). (A) second lane corresponded to fractions collected at 1 M NaCl in 10 mM Tris-HCl at pH 7.8 and (C) second and third lanes corresponded to fractions collected at 0.5 and 1 M NaCl in 10 mM Tris-HCl at pH 7.8, respectively. (B, D) *Western blot* of fractions obtained from AAIL's chromatographic trials visualized in (A) and (C).

In both supports, the target protein was eluted with a different molecular weight, near to protein standards of 12 kDa (Figure 15 (B: 2ºH and 2ºM) and (D: 3ºH and 3ºM)). However, complementary specific activity assays are needed to analyze the performance of the delineated strategy. Indeed a process scale-up is necessary to perform activity assays.

Therefore, the studies were performed with 10 mL of L-methionine and L-histidine resins using a FPLC system. The aim of the second approach was to optimize

the salt concentration that allows a total elution of the target protein. Therefore, in the next procedures was used the pH 7.8 in elution buffer with different NaCl concentrations. In this strategy, two elution steps were applied with the modification of NaCl concentration (Table VI).

Table VI. Summary of elution steps used for hSCOMT L-methionine/histidine retention assays.

AAIL	Adsorption	Intermediate step	Elution
L-methionine	pH 5	500 mM NaCl/pH 7.8	1 M NaCl/pH 7.8
	pH 5	250 mM NaCl/pH 7.8	500 mM NaCl/pH 7.8
	pH 5	150 mM NaCl/pH 7.8	250 mM NaCl/pH 7.8
	pH 5	50 mM NaCl/pH 7.8	250 mM NaCl/pH 7.8
	pH 5	65 mM NaCl/pH 7.8	250 mM NaCl/pH 7.8
	pH 5	60 mM NaCl/pH 7.8	250 mM NaCl/pH 7.8
L-histidine	pH 5	500 mM NaCl/pH 7.8	1 M NaCl/pH 7.8
	pH 5	250 mM NaCl/pH 7.8	500 mM NaCl/pH 7.8
	pH 5	150 mM NaCl/pH 7.8	250 mM NaCl/pH 7.8
	pH 5	60 mM NaCl/pH 7.8	250 mM NaCl/pH 7.8
	pH 5	50 mM NaCl/pH 7.8	250 mM NaCl/pH 7.8

Representative chromatographic profiles are showed in Figure 16. Indeed, the major difference in retention capacities between these two supports were essentially due to the different ligands density, 2-10 and 1-2 µmol/mL respectively, in L-methionine and L-histidine supports and probably the presence/absence of the spacer arm in L-histidine and L-methionine, respectively. In general, a greater amount of *E. coli* protein contaminants were eluted with 65 mM and 50 mM of NaCl, respectively in L-methionine and L-histidine supports (Figure 16). However, hSCOMT as well as some

host protein contaminants were only eluted when the ionic strength is established at 250 mM NaCl (Figure 17).

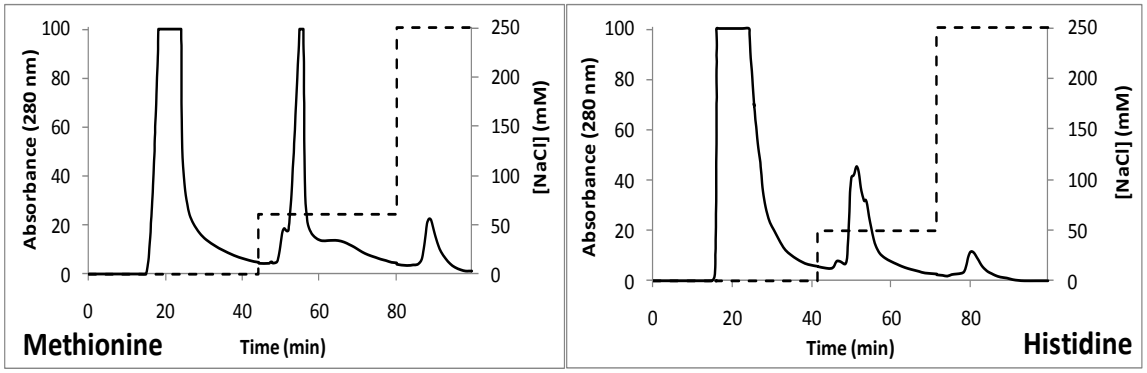


Figure 16. Elution profiles on L-methionine and L-histidine supports. The binding buffer is 10 mM Tris-HCl at pH 5 and the elution steps in L-methionine and L-histidine resins is respectively 65 mM/250 mM NaCl and 50 mM/250 mM NaCl. All these solutions were prepared in 10 mM Tris-HCl at pH 7.8.

As can be observed in SDS-PAGE (Figure 17 (A, C)) and *Western blot* (Figure 17 (B, D)), the analysis of chromatographic fractions showed that target protein were eluted with a considerable purification degree. Nevertheless, the purification degree achieved in L-methionine support was higher than in L-histidine matrice.

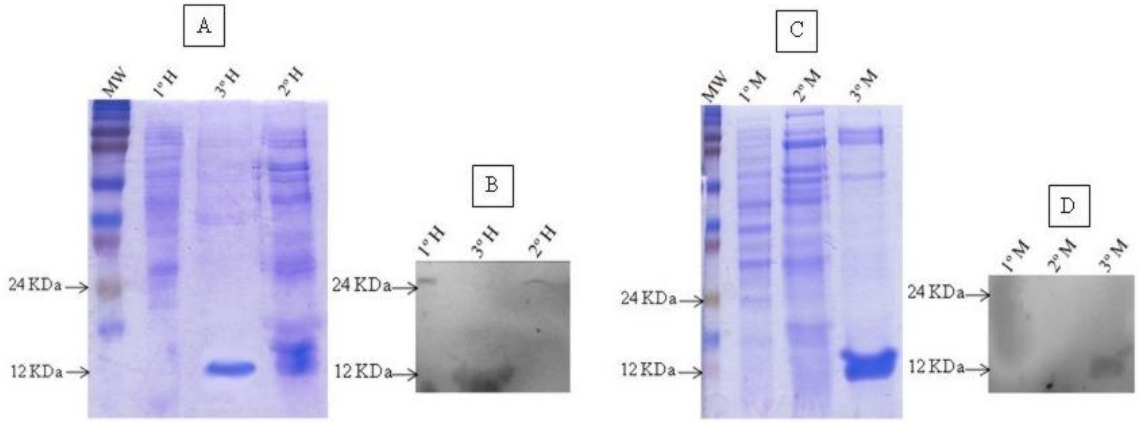


Figure 17. (A, C) SDS-PAGE analysis of *E. coli* recombinant hSCOMT lysates, loaded onto the L-methionine (A) and L-histidine (C) supports. The representative first lanes corresponded to fractions collected at 10 mM Tris-HCl at pH 5, second and third lanes corresponded to fractions collected at 0.5 and 1M NaCl in 10 mM Tris-HCl at pH 7.8, respectively. (B, D) *Western blot* of the same fractions obtained from the AAIL's chromatographic trials.

At this stage and to complement the previous studies, were performed enzymatic assays, to analyze the biological hSCOMT activity in *Western blot* hSCOMT positive fractions with a different molecular weight. These assays showed that hSCOMT does not possess enzymatic activity, onto the positive fractions.

5.3.2.3.2 Specific activity control assays

Some studies described a specific susceptibility for hSCOMT cysteine residues to oxidation and consequent loss of specific activity, essentially due to disulfide bonds formation (Cotton *et al.*, 2004; Mannisto and Kaakkola, 1999). However, cysteine residues oxidation is a reversible interaction with the incorporation of an appropriate reducing agent. At this item the intermediate aim was to understand the behavior of hSCOMT specific activity in L-methionine support, through elution buffer supplementation with reducing agents, as cysteine and DTT (Figure 18) were added in the second elution step. In hSCOMT positive fractions, obtained from this purification strategy, the addition of these reducing agents did not recovered enzyme activity.

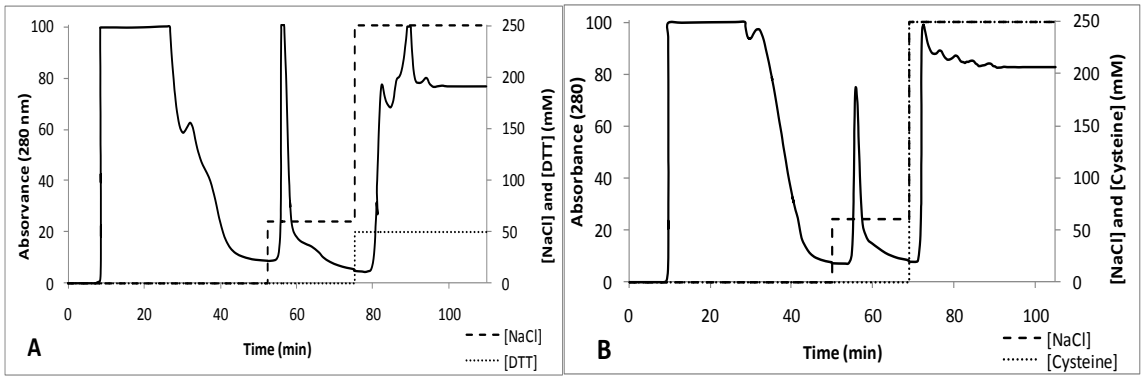
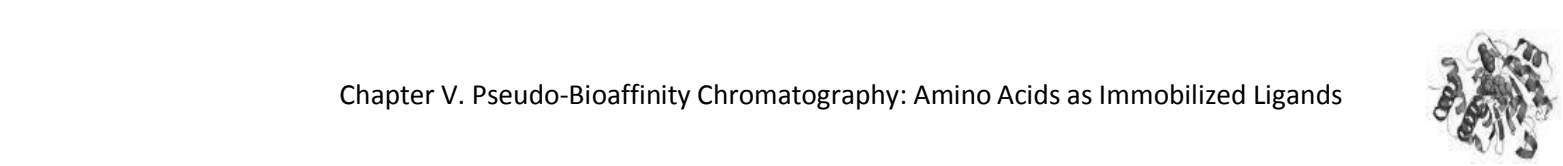


Figure 18. Chromatographic profiles of an *E. coli* lysate extract with recombinant hSCOMT onto the L-methionine resin. The binding buffer was 10 mM Tris-HCl at pH 5. The first elution step with 65 mM in 10 mM Tris-HCl at pH 7.8 and the second elution stage was performed with (A) DTT 50 mM and (B) cysteine 250 mM, both in 10 mM Tris-HCl at pH 7.8.

Therefore, additional studies were performed to understand if the loss on hSCOMT specific activity was due to the chromatographic conditions applied or to hSCOMT-L-methionine support interaction. All these experiments were performed with *E. coli* recombinant hSCOMT lysates as control with times and temperatures conditions analyzed. Indeed, the control experiments, schematized in Figure 19, showed that salt concentration used in the elution step at RT and 4 ºC (Figure 19 (A, B)) did not



influence hSCOMT specific activity. Simultaneously, acidic binding conditions (Figure 19 (C, D)), maintained hSCOMT specific activity, confirming the previous control assays showed in Figure 13. Also, in Figure 19 (E) control experiments were conducted to simulated all chromatographic steps in the methionine support, but even these conditions did not influence hSCOMT specific activity.

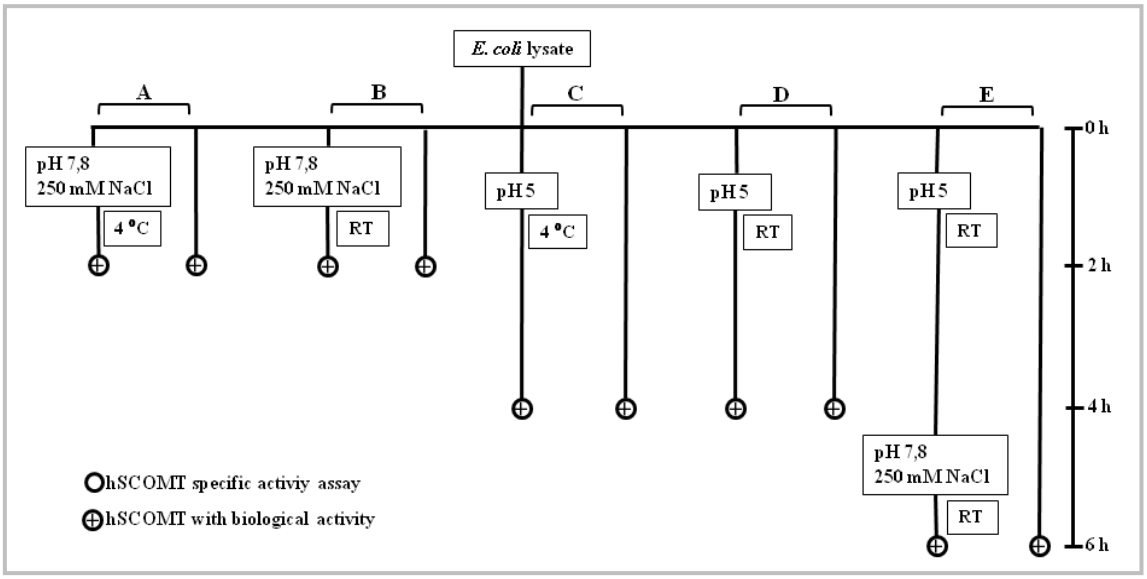


Figure 19. Control of hSCOMT specific activity in a chromatographic process on the L-methionine support. (A, B) *E. coli* lysates was in contact with solution II (250 mM NaCl in 10 mM Tris-HCl, pH 7.8) during 2 h at 4ºC and RT. (C, D) *E. coli* lysates was in contact with solution I (10 mM Tris-HCl, pH 5) during 4 h at 4ºC and RT. (E) *E. coli* lysates was in solution A during 2 h and next in the presence of solution B during 4 h at RT.

5.3.2.4 hSCOMT molecular weight discrepancy: the influence of acidic pH's in biding conditions

The following assays were performed with 5 mL of AAIL's resins using an Econo-Pac® disposable Chromatography Columns system. At this stage, lysate extracts containing hSCOMT were loaded onto six AAIL's and eluted with a stepwise gradient at 1M NaCl concentration. The Figure 20 shows the results of the AAIL's binding and elution profiles with several pH values. In order to maintain the enzymatic activity, the pH selection set point on the binding buffer was based on the activity profile showed in Figure 13. Although the criterion selection did not aim a complete hSCOMT binding, at this stage we tried to understand the protein behavior onto the AAIL´s resins at several pH conditions.

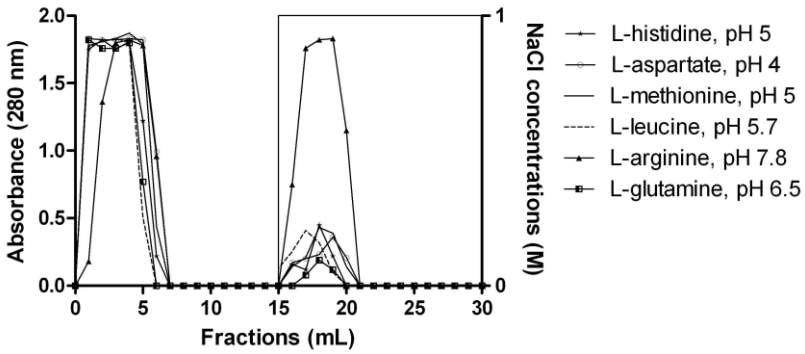


Figure 20. Chromatographic profiles of an *E. coli* lysate extract with recombinant hSCOMT onto the six AAIL's tested with a stepwise gradient from 0 to 100% buffer A (buffer A: 10 mM Tris–HCl, pH 7.8 (L-arginine), 4 (L-aspartate), 6,5 (L-glutamine), 5 (L-methionine and L-histidine), 5,7 (L-leucine); buffer B: NaCl 1M in 10 mM Tris–HCl, pH 7.8).

In the six AAIL's supports, SDS-PAGE (Figure 21 (A)) and *Western blot* (Figure 21 (B)) screening of chromatographic peaks showed that the target protein eluted mostly in the second peaks with 1 M NaCl, with a considerable removal of proteins contaminants. In comparison at standard conditions, L-arginine resin promoted the higher retention observed, as judged by Figure 21 (A).

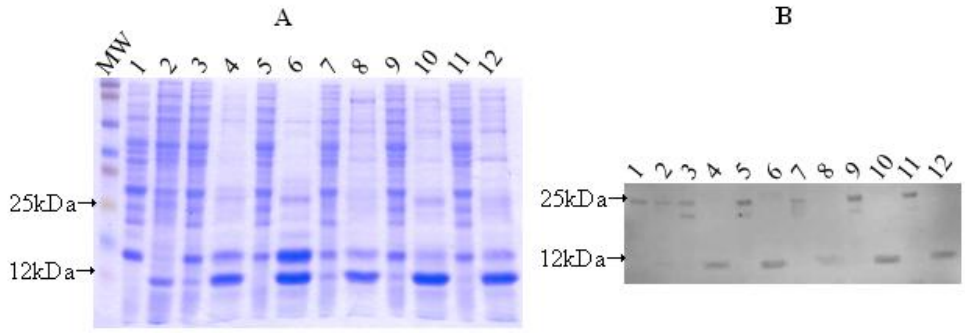


Figure 21. (A) SDS-PAGE analysis of *E. coli* recombinant hSCOMT lysates, loaded onto the several AAIL's supports: (lanes 1, 2) L-arginine, (lanes 3, 4) L-aspartate, (lanes 5, 6) L-glutamine, (lanes 7, 8) L-histidine, (lanes 9, 10) L-leucine, (lanes 11, 12) L-methionine. The representative lanes corresponded to the respective binding pH: 7.8 (1), 4 (3), 6.5 (5), 5 (7), 5.7 (9), 5 (11) and elution at 1 M of NaCl (lanes 2, 4, 6, 8, 10, 12). (B) *Western blot* from the same fractions obtained from AAIL's chromatographic trials.

In all AAIL's tested with acidic pH onto buffer binding, the target protein was elute, with a different molecular weight, near to protein standards of 12 kDa, according to Figure 21. In contrast, the molecular weight alteration was not observed in the L-

arginine AAIL, as promotes partial retention of hSCOMT at pH 7.8. However, if the binding of hSCOMT was performed at pH 4 in this support, it promoted a molecular weight elution close to protein standards of 12 kDa (data not shown). In general, this strategy could have a close relationship with the variety of environmental conditions, probably leading to irreversible and covalent changes in hSCOMT structure. Certainly, these results highlight that the molecular weight disparity does not depends of AAIL applied but probably on the acid pH used in the chromatographic buffers. The molecular weight disparity observed at acidic binding pH's affects negatively the hSCOMT stability with a consequent loss of bioactivity. There are two possible mechanisms to explain this phenomenon; the first and the most commonly is the nonenzymatic deamidation of asparagine residues (Clarke *et al.*, 1992; Wright, 1992). Under acidic conditions, deamidation is thought to proceed by direct hydrolysis, resulting in the formation of α-aspartyl residues alone (Tyler-Cross and Schirch, 1991). Secondly, is the preferential hydrolysis of peptide bonds at aspartic acid residues under acidic conditions, due to the cleavage of a peptide bond that obviously disrupts the linear sequence of amino acid residues within a protein chain (Clarke *et al.*, 1992).

In general. these results may indicate that, perturbation of hSCOMT structure, specifically by acidic pH's, can lead to the exposure of previously buried amino acid residues, facilitating their chemical interaction with AAIL and consequently their degradation. Stability assays showed that the contact of acidic pH buffers with hSCOMT do not interfere in protein activity. Indeed, literature predicts the existence of two classes of denatured states in these conditions: one highly unfolded and the other compact. The latter, designed as *molten globule* is stabilized by a high degree of hydrophobic clustering, but it differs from the native state since not possesses hydrophobic "core" (Stigter *et al.*, 1991). In comparison to these results, using chromatographic strategies with different acidic pH, hSCOMT probably exposes amino acids residues that, in native fold, are in the hydrophobic "core", and these are probably the majority for the promotion of enzyme-AAIL interactions.

Chapter VI. Concluding remarks and Future work

6.1 Concluding remarks

A challenge in biotechnology is the purification of recombinant proteins which demand procedures to endow the purified protein with structural integrity and biological activity without contamination. The isolation of hSCOMT in its functional form is difficult. Although, several previously procedures being suitable for the isolation of homogenous active SCOMT, these processes are time-consuming and suffer from significant enzyme activity losses at earlier stages. Here, we have examined three enzyme purification strategies: two using classic Hydrophobic Interaction Chromatography in Octyl and Epoxy-Sepharose supports and a third using Pseudo Bioaffinity Chromatography in AAIL's supports.

In Octyl-Sepharose support, the incorporation of L-arginine in chromatographic buffers appears as a solution due this strong hSCOMT-support interaction that consequently has a hard elution. However, preliminary activity assays showed that L-arginine decreases hSCOMT specific activity; comparing the described above with the purification fold achieve, was concluded that this strategy do not comprise hSCOMT purification strategy requests.

In general, the less hydrophobic adsorbent, such Epoxy-Sepharose, is considered as a last resort in a global purification scheme, due to the high NH_2SO_4 concentration needed for the hSCOMT binding (1.2 M) and elution (1 M). So, the major goal on this study was to discern new operation conditions, based on dual salt systems, to achieve a new purification strategy using less salt concentrations. Specifically, the high concentrations of dual salt system required, such 0,7 M NH_2SO_4/0,15 M $Na_3C_6H_5O_7$, to allow hSCOMT retention, make this strategy needless considering previous purification strategies described by our group.

This is the first report of AAIL's screening data showing a suitable approach in the application of pseudo-bioaffinity supports for hSCOMT isolation. The first studies, using salt (NH_2SO_4) on retention, showed that high salt concentrations needed to promote hSCOMT retention leads to the decrease of enzymatic activity. However, the conditions applied in the follow studies with pH manipulation, were demonstrated that acid pH in the binding buffers promotes a decrease in the enzyme molecular weight, independently of AAIL used. Probably, the contribution of the different molecular forces derived from acidic adsorption conditions leads to a molecular weight alteration and consequently loss of hSCOMT specific activity. These data suggest that AAIL´s

interactions are not crucial for the discrepancy of the molecular weight, but the conditions used to bind the target protein.

In conclusion, the comparison of these three approaches in this dissertation demonstrated the complexity of hSCOMT purification processes. In spite of the requirement of structural studies to understand the hSCOMT-AAIL binding mechanism, these supports clearly possesses advantages over the earlier methods published due of its simplicity and efficiency in hSCOMT purification.

6.2 Future work

Future work will undoubtedly seek to experimentally test the proposed mechanism and response how hSCOMT binding to AAIL's in acidic conditions and promotes the changing of its native conformation and how this can compromise hSCOMT specific activity. Specifically, in the use of salt to promote hSCOMT retention the dual salt system application can be a solution to high NH_2SO_4 concentration requirements.

The using of pseudo bioaffinity chromatography, specifically nucleotides immobilized ligands for hSCOMT purification, is still being an unexplored area. As well as AAIL's purification strategies show interesting results. Probability it can be useful to understand the selectivity's achieve by nucleotides immobilized ligands. Indeed, analyses of influence in kinetic properties of the hSCOMT based on competitive strategies or non-specific desorption profiles supported on pH and ionic strength manipulation.

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Chapter VIII. Attachments

I. Solutions composition

SOB medium: 2 % w/v tryptone, 0.5 % w/v bacto-yeast extract, 8.56 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$.

Bial buffer: 150 mM NaCl, 10 mM DTT, 50 mM Tris pH 8.0, 5 µg/mL leupeptin and 0.7 µg/mL pepstatin.

Activity assay reaction mixture: 5 mM sodium phosphate buffer (pH 7,8), 0,2 M $MgCl_2$, 2 mM EGTA, 0,250 mM AdoMet.

HPLC mobile phase: 0.145 mM EDTA, 0.1 M sodium acetate, 0.1 mM citric acid, 0.5 mM sodium octyl sulphate, 1 mM dibutylamine and 5% methanol, v/v. Adjusted to pH 3.5 with perchloric acid 70% (v/v).

Loading buffer: 500 mM Tris–HCl (pH 6.8), 10% SDS, 0.02% bromophenol blue (w/v), 0.2% glycerol (v/v) and 0.02% β-mercaptoethanol (v/v).

Stacking gel (4,7%): 1,75 mL acrilamide/bis-acrilamide (30%), 1,25 mL Tris-HCl 1,25 M pH 6,8, 6,9 mL water, 1,0 mL SDS 10 % (0,2 mL PSA 10% and 0,03 mL TEMED)

Resolving gel (12,5%): 8,3 mL bis-acrilamide (30%), 7,5 mL Tris-HCl 1,875 M pH 8,8, 3,7 mL water, 0,2 mL SDS 10 %

Running buffer: 25 mM Tris, 192mM glycine and 0,1%, w/v SDS.

Blotting buffer: 10 mM CAPS and 10% (v/v) of methanol.

TBS-T: 20 mM Tris, 137mM NaCl and 0.1% Tween 20, pH 7,6.

Stain solution: 62,5 mg Comassie brillant blue R – 250, 100 mL methanol, 17,5 mL Glaciar acetic acid and 132,5 mL water.

Distain solution: 80 mL methanol, 14 mL Glaciar acetic acid and 156 mL water.

Fixing solution: 12,5 mL methanol, 17,5 mL Glaciar acetic acid and 220 mL water.

II. Abstract and poster presentation: "6º Encontro Nacional de Cromatografia in Madeira, Portugal - 2009"

Application of amino acids ligands as a new chromatographic strategy for hSCOMT isolation

Costa SR[1],Bonifácio MJ[2], Queiroz JA[1], Passarinha LA[1]
[1]CICS – Centro de Investigação em Ciências da Saúde, 6200-505 Covilhã, Portugal; [2]BIAL – Departamento de Investigação e Desenvolvimento, 4745-457 São Mamede do coronado, Portugal

Catechol-*O*-methyltransferase (COMT; EC2.1.1.6) play an important role in the metabolism and inactivation of biologically active and toxic catechols, since catalyzes the transfer of a methyl group from the donor s-adenosyl-L-methionine to a catechol substrate in the presence of magnesium cation. Nowadays, this enzyme has been a relevant focus of research, due to the normal brain function, especially in the regulation of both the dopaminergic and noradrenergic neurotransmitter systems, which has a close relationship with a variety of mental disorders, such a Parkinson's diseases. As a result, while the development of pharmaceutical human Soluble COMT (hSCOMT) trials for a rational drug design depends on the availability of high purified samples, more suitable purification strategies[1] must be develop and emerge in order to satisfy the requirements of pharmaceutical industry.

The diversity of biomolecules presents in hSCOMT recombinant extracts from *Escherichia coli*, with structural and chemical similarities, are the foremost challenge in order to establish novel and suitable laboratory purification protocols[2]. So, the aim of this study was to develop a selective purification of hSCOMT, using distinct pseudoaffinity supports with amino acid as immobilized ligands. This strategy could be particularly promising while combines a natural biological interaction, improving the selectivity's onto the support and purity achieved in the target protein fractions maintaining the desirable kinetic properties.

In particular, using L-histidine, L-arginine, L-glutamine, L-aspartate and L-leucine as amino acids immobilized ligands, a variety of hSCOMT adsorption and elution methods has been tested and developed, incorporating more than one mode of adsorption, commonly based on hydrophobic and ionic interactions. Particularly, the majority of the group tested, excluding arginine, allowed a total retention of hSCOMT at higher salt concentrations above 1M of ammonium sulphate. Only the arginine ligand allows the binding of the target protein without salt in the mobile phase. Also, additional studies by non-specific desorption profiles based on pH manipulation will be presented since previous stability trials showed that hSCOMT maintains its activity over pH ranges from 3 to 8.

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[1]L.A. Passarinha, M.J. Bonifácio, P. Soares-da-Silva, J.A. Queiroz, *Journal of Chromatography A*, vol. 1177, 2008, pp. 287-296.

[2]V.S. Nunes, M.J. Bonifácio, J.A. Queiroz, L.A. Passarinha, (2009), submitted.

III. Abstract: "8th European Symposium on Biochemical Engineering Science (ESBES) in Bologna, Italy - 2010"

Screening of pseudo affinity supports as a new tool for hSCOMT purification

Costa SR[1], Bonifácio MJ[2], Queiroz JA[1], Passarinha LA[1]

[1]*CICS – Centro de Investigação em Ciências da Saúde, 6200-505 Covilhã, Portugal;*

[2]*BIAL – Departamento de Investigação e Desenvolvimento, 4745-457 São Mamede do coronado, Portugal*

Catechol-*O*-methyltransferase (COMT), play an important role in the metabolism of catecholamines, catecholestrogens and catechol drugs and consequently has close relationship with several mental disorders. As a result, while the development of pharmaceutical Human Soluble COMT (hSCOMT) trials for a rational drug design depends on the availability of high purified samples, more suitable purification strategies[1] must be developed and emerged in order to fulfil the requirements of pharmaceutical industry.

The diversity of biomolecules presents in hSCOMT recombinant extracts from *Escherichia coli*, with structural and chemical similarities, are the foremost challenge in order to establish novel and suitable laboratory purification protocols[2]. So, the central aim of the current study is to investigate the application of distinct pseudoaffinity support with aminoacids as immobilized ligands in order to: (1) study ligand performance in terms of selectivity, binding and elution conditions, (2) reduce the main protein interferents release in COMT primary isolation from the recombinant host and (3) analyse the recovery activity levels in all the strategies performed. This strategy could be particularly promising while combines a natural biological interaction, improving the selectivity's onto the support and purity achieved in the target protein fractions maintaining the desirable kinetic properties.

The majority of the amino acid tested (L-histidine, L-leucine, L-aspartate, L-glutamine), allowed a total retention of hSCOMT at higher salt concentrations above 1M. Nevertheless, this salt concentration can reduce desired product recovery and activity levels, compromising the applicability of a specific purification method. In addition, L-methionine as immobilized ligand, a variety of hSCOMT non-specific adsorption profiles based on pH manipulation has been tested and developed. In order to allow the total binding of the target protein without a decrease in the activity levels, stability trials had been performed. The results show that hSCOMT maintains its activity over pH ranges from 3 to 8. Specifically, this support appears to be a mimic ionic interaction, eluting the target protein with a residual salt concentration. Nevertheless, desorption of most interferent proteins of recombinant host was performed only with suitable adjusts in pH setpoint, revealing a higher specificity.

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IV. Manuscript

Separation of hSCOMT by pseudo-bioaffinity chromatography with immobilized amino acids: The influence of pH and salt on retention

Sara R. Costa [1], Maria J. Bonifácio [2], João A. Queiroz [1], Luís A. Passarinha [1*]
[1]*Centro de Investigação em Ciências da Saúde, Covilhã, Portugal*
[2]*BIAL – Departamento de Investigação e Desenvolvimento, São Mamede do Coronado, Portugal*

***correspondence to:** L. A. Passarinha, CICS – Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, 6201-001 Covilhã, Portugal.
E-mail: lpassarinha@fcsaude.ubi.pt
Av. Infante D. Henrique
6200-506 Covilhã
Tel: +351 275 329 002/3
Fax: +351 275 329 099

Abbreviations used
AAIL's - amino acids immobilized ligands
hSCOMT – human soluble catechol-*O*-methyltransferase
AS – ammonium sulphate

Key-words: Amino acids immobilized ligands, human soluble catechol-*O*-methyltransferase, purification, pH, pseudo-bioaffinity interactions

Abstract

Amino acids immobilized ligands (AAIL's) were been used for isolation of several biomolecules, such as proteins. In this context and based on specific structural properties of human soluble Cathecol-*O*-methyltransferase (hSCOMT), we screened and analyzed the effect of experimental conditions, such as pH and salt gradients, over six AAIL´s commercial supports (L-arginine, L-methionine, L-histidine, L-aspartate, L-glutamine and L-leucine) on hSCOMT retention. In general and independently of the resin tested, hSCOMT retention is around their isoelectric point, and sometimes nearly acid pH's. In spite of these harsh conditions, stability assays showed that the effect of lower pH alone, do not interfere with the enzymatic activity. Nevertheless, SDS-PAGE and immunoreactivity assays showed a decrease in hSCOMT typical molecular weight (25 kDa), probably due to the rate and the extent of covalent interactions over the AAIL´s surface at acidic pH. Indeed, the interaction mechanism is highly dependent on the binding pH revealing that AAIL´s chromatographic operating windows are more unwieldy than typical hydrophobic supports This work also allow to understanding if the interactions observed in the several supports, lead to similar hSCOMT changes and infer about the performance and application of a specific AAIL support for hSCOMT isolation from complex extracts. Overall, the purification degree achieved with pH

manipulation, specifically in methionine AAIL, is remarkably acceptable and higher than in routine hydrophobic assays.

1. Introduction

The amino acids immobilized ligands (AAIL's) were introduced, in 1989 by Vijayalakshmi and coworkers, with the designation of "pseudo-biospecific affinity ligands". Nowadays, these supports represent promising alternatives to traditional biospecific resins in affinity chromatography, due to their resistance to harsh chemicals, high temperatures (sterilization conditions) and low cost (Vijayalakshmi, 1989). Unless, these resins have been extensively used in separation of several molecules such as oligouronides (Delattre *et al.*, 2008; Delattre *et al.*, 2005), oligonucleotides (Sousa *et al.*, 2009a), pDNA (Sousa *et al.*, 2009b; Sousa *et al.*, 2008b), RNA (Jones *et al.*, 1976) and proteins (el-Kak and Vijayalakshmi, 1991; Haupt and Vijayalakshmi, 1993; Kanoun *et al.*, 1986; Summaria *et al.*, 1976).

Typically, AAIL's act as electron acceptors of NH, SH or OH groups from protein amino acid residues such as histidine, cysteine, tryptophan, and serine. In general, the feasibility of these interactions with the ligand will depend on several conditions such as buffer composition, pH, temperature, ionic strength and support matrix. Nevertheless, the degree of interaction depend more specifically on the protein surface amino acids accessibility (el-Kak *et al.*, 1992). Also, the three-dimensional structure of the target protein can be a relevant factor in these systems, affecting the rate and extent of the interactions. In general, the native conformation is directly affected by environmental conditions such as pH and salt concentration variation used, in order to promote ionic and hydrophobic interactions onto these supports (Vijayalakshmi) .

To our best knowledge there are not yet known structural evidences or experimental data to preview the behavior of mammalian COMTs on AAIL's. This enzyme catalyzes the *O*-methylation in catecholamines and other catechols and is a significant target in protein engineering due to its role not only in normal brain function but also its possible involvement in some human disorders (Axelrod *et al.*, 1958). Specifically, the protein is composed of a seven-stranded β-sheet core, wiched between two sets of α-helices. Its active site consists of the *S*-adenosyl-L-methionine binding domains and the actual catalytic site (Veerapandian, 1997) that is formed by a few amino acids extremely relevant for binding substrate, water, and Mg^{+2}, that bound to hSCOMT after AdoMet binding. For instance, amino acids residues such as Lys144 that accepts the proton from the hydroxyl, and the "gatekeeper" residues Trp38, Trp143, and Pro174 that form the hydrophobic "walls" which define the COMT substrate selectivity (Mannisto and Kaakkola, 1999). In this context, the chromatographic strategies applied for highly sensitive proteins, such as hSCOMT, should be careful designed since it can compromise the native intrinsic kinetic properties of the target protein.

In this work recombinant hSCOMT obtained from *Escherichia coli (E. coli)* lysates was chosen as a model enzyme, in order to study the incorporation of AAIL´s chromatographic supports in a hSCOMT isolation process and explore the problematic selection of experimental conditions, such as salt concentration and pH range. This prompted us to undertake the present study; principally investigating the effects of

introducing gradient pH´s in chromatographic strategies with the aim of ascertaining whether pseudoaffinity interaction mechanism could affect native hSCOMT conformation and consequently biological activity.

2. Experimental

2.1 Material. Ultrapure reagent-grade water for FPLC was obtained with a Milli-Q system (Milipore/Waters). Carbenicillin disodium salt, isopropylthiogalactosidase (IPTG), tryptone, yeast extract, lysozyme, dithiothreitol (DTT), ammonium sulfate (AS), Tris(hydroxymethyl)aminomethane were obtained from Sigma Chemical Co (St Louis, MO, USA). Potassium chloride and sodium chloride were supplied by Fluka (Buchs, Switzerland). The full range rainbow protein standards used for estimation of subunit masses were purchased by GE Healthcare Biosciences (Uppsalla, Sweden). Polyclonal rabbit anti-COMT antibody affinity purified was produced in BIAL using purified recombinant rat COMT (Bonifacio *et al.*, 2001). L-arginine, L-methionine, L-histidine, L-aspartate, L-glutamine and L-leucine supports were obtained from sigma (St. Louis, MO, USA). All reagents were of analytical grade and used without further purification.

2.2 Recombinant hSCOMT Expression and Recuperation. Plasmid pET101/D-hSCOMT was used as the expression construct. Commercial *E. coli* BL21-star (DE3) (Invitrogene, USA) was used as the recombinant strain for hSCOMT over expression under the control of the IPTG inducible promoter and employing carbanecillin supplementation as a selection marker. The fermentation and disruption conditions were previously described by our group (Passarinha *et al.*, 2008).

2.3 AAIL's chromatography. Chromatographic separations were performed at room temperature. The AAIL's media were packed according to company guidelines (5 mL of gel volume), into a Econo-Pac® disposable Chromatography Columns (Bio-Rad, Hercules, CA). Aliquots (500 µL with an estimated protein concentration of ~11.5 mg/mL) of recombinant hSCOMT-containing supernatant were loaded onto the columns with two different strategies. In retention using pH manipulation, columns were initially equilibrated with Tris-HCl buffer (10 mM) at different pH values, and isocratic elution was performed with the same pH value. After elution of unretained species, the bound proteins were eluted in a stepwise gradient with sodium chloride 1 M in 10 mM Tris-HCl buffer, pH 7.8. The same procedure was performed in retention using salt manipulation, were the binding buffer was 1,5/2 M AS in Tris-HCl 10 mM, pH 7.8 and elution buffer was Tris-HCl 10 mM, pH 7.8. In all separations, the optical density was monitored at 280 nm throughout the entire chromatographic run, while 1 mL fractions were collected and evaluated for hSCOMT detection.

2.4 Analytical methods. The protein content in samples was measured by the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA), with bovine serum albumin as the standards and calibration control samples (1.2 to 10.0 µg/ml), according to

manufacturer's indications. The fractions from chromatographic procedures were further analyzed, respectively by SDS-PAGE and *Western blot* (Laemmli, 1970; Passarinha *et al.*, 2008). The experiments of activity were designed to evaluate the methylation efficiency of recombinant hSCOMT, by measuring the amount of metanephrine, using epinephrine as substrate as previously described (Passarinha *et al.*, 2006).

3. Results and discussion

In hydrophobic interaction the protein adsorption is carried out by moderate to high concentrations of anti-chaotropic salts, usually AS (Queiroz *et al.*, 2001). In spite of previous data in the literature highlights that hSCOMT is extremely labile in contact with AS, deactivation and/or modification dependence for this salt concentration would be reduced as a result of the shortening processing time and low ionic strength on HIC (Passarinha *et al.*, 2008). In this stage we studied the applicability of AS in binding buffer on AAIL´s supports, for the capture of hSCOMT from cell culture supernatants. The results showed that a complete binding of hSCOMT, above 1,5 M of AS concentration, was achieved in the six AAIL´s tested (data not shown). Previous data described by our group demonstrated that AS at higher levels can reduce desired hSCOMT recovery and activity levels (Nunes *et al.*, 2009), so this strategy can compromise the applicability of hSCOMT isolation.

In general, not only high salt concentrations are deleterious to proteins. Also, several pH ranges can promote unfolding and even aggregation of proteins. These effects may be attributed to electrostatic repulsion failing to overcome the interactions favouring folding, such as hydrophobic forces, disulfide bonds, salt bridges, and metal ion-protein interactions. Also, acidic pH can lead to hydrolysis of polypeptide chains (especially at Asp-Pro sequences), whereas alkaline pH can catalyze cystine destruction and asparagine deamidation (Volkin *et al.*, 1997). However, the specific retention on the AAIL occurred mainly at pH values at, or around, their isoelectric points (pI) (el-Kak *et al.*, 1992). As described for different proteins, such as yeast carboxypeptidase (pH 3-4), goat chymosin (pH 5.5), acidic pH can be applied in purification strategies without changes in their structural integrity (el-Kak *et al.*, 1992). So ideally, the adsorption pH for hSCOMT can be selected based early on the knowledge of its pI value (pI 5.5). So, preliminary control experiments were conducted to analyze the effects of 10 mM Tris-HCl buffer with pH ranges from 2 to 13 on hSCOMT stability (Figure 1). The results of stability trials showed that hSCOMT maintains its activity over pH ranges from 3 to 7. In spite of we observed a decreasing for hSCOMT specific activity, at values outside the range mentioned. The same control assays were performed at room temperature, in the absence or presence of sodium chloride at 1 M (data not shown). In particular, these experiments do not evidence significant alteration to data described in Figure 1.

At this stage, lysate extracts containing hSCOMT were loaded onto six AAIL's and eluted with a stepwise gradient at 1M NaCl concentration. Figure 2 shows the results of the AAIL's binding and elution profiles with several pH values. In order to maintain the enzymatic activity, the pH selection setpoint on the binding buffer was based on the activity profile (Figure 1). Although the criteria selection didn´t aim for a

complete hSCOMT binding, at this stage we tried to understand the protein behavior onto the AAIL´s resins at several pH conditions. In the six AAIL's supports, SDS-PAGE (Figure 3(A)) and *Western blot* (Figure 3(B)) screening of chromatographic peaks show that the target protein eluted mostly in the second peaks with 1M sodium chloride, with a considerable removal of host proteins contaminants. In comparison at standard conditions, L-arginine resin promotes the higher retention observed, as judged by Figure 3(A). In all AAIL's tested with acidic pH onto buffer binding, the target protein is elute, with a different molecular weight, near to protein standards of 12 kDa, according to Figure 3. In contrast, the molecular weight alteration was not observed in the L-arginine AAIL, as promotes partial retention of hSCOMT at pH 7.8. However, if the binding of hSCOMT was performed at pH 4 in this support, it promotes a molecular weight elution close to protein standards of 12 kDa (data not shown). In general, this strategy could have a close relationship with the variety of environmental conditions leading to structural changes in hSCOMT. Definitely, our results highlight that the molecular weight disparity does not depends of AAIL applied but probably on the acid pH used in the chromatographic buffers. Indeed, lower binding pH affects adversely hSCOMT conformation, stability, and bioactivity with a loss of activity (data not shown). There are three possible mechanisms to this phenomenon; the first and the most commonly encountered in chemical modifications of proteins is the nonenzymatic deamidation of asparagine residues (Clarke; Wright, 1991). Under acidic conditions, deamidation is thought to proceed by direct hydrolysis, resulting in the formation of α-aspartyl residues alone (Tyler-Cross and Schirch, 1991). Secondly, preferential hydrolysis of peptide bonds at aspartic acid residues under acidic conditions, due to the cleavage of a peptide bond that obviously disrupts the linear sequence of amino acid residues within a protein chain. Finally, cysteine residues oxidation or the disulfide bonds cleavage, that is most common in extracellular proteins (Volkin *et al.*, 1997). Published studies described the susceptibility of hSCOMT cysteine residues to oxidation and consequent rapid loss of its specific activity, due to the formation of a disulfide bond (Cotton *et al.*, 2004). Also, methionine residues oxidation as been associated with a loss of protein activity (Volkin *et al.*, 1997). However, both amino acid residues oxidation is a reversible interaction and consequently does not appear to be a determinative factor for hSCOMT loss of activity. Previous studies showed that DTT, with further addition of cysteine in buffers applied on purification experiments, not only maintains but increases the protein activity (data not shown). Nevertheless, the addition of these reducing agents in fractions obtained from AAIL's purification strategies tested with pH gradients does not recover enzyme activity (data not shown).

In general, stability assays showed that the contact of acidic pH buffers with hSCOMT do not interfere in protein activity. Nevertheless, our AAIL´s experiments may indicate that, perturbation of hSCOMT structure, specifically by acidic pH, can lead to the exposure of previously buried amino acid residues, facilitating their chemical interaction with AAIL's and consequently their degradation. The literature predicts the existence of two classes of denatured states in these conditions: one highly unfolded and the other compact. The latter, designed as *molten globule* is stabilized by a high degree of hydrophobic clustering, but it differs from the native state since not possesses

hydrophobic "core" (Stigter *et al.*, 1991). According to our results, using chromatographic strategies with different acidic pH, the enzyme probably exposes amino acids residues that, in native fold, are in the hydrophobic "core", and these are probably the majority for the promotion of hSCOMT-AAIL´s interactions.

4. Concluding remarks

This is the first report of AAIL screening data show a suitable approach of using pseudo-bioaffinity supports for hSCOMT isolation. Overall, the experiments provided a new insight of pH and salt strategies in hSCOMT-AAIL´s interaction mechanism. Specifically, strategies based on high salt gradients in AAIL´s maintain the typical hSCOMT molecular weight. However, acidic pH in the binding buffers promotes a decrease in the enzyme molecular weight. These data could be interpreted as meaning that AAIL´s interactions are not crucial for the discrepancy of the molecular weight, but the conditions used to bind the target protein. Despite the higher pseudo-bioaffinity interactions can change hSCOMT molecular weight, the purification degree achieved with pH manipulation, specifically in methionine AAIL, is remarkably acceptable and higher than in routine hydrophobic assays. So, in AAIL´s resins the careful selection of experimental conditions, based on the particular properties of recombinant hSCOMT is extremely relevant, since it can minimize or even eliminate the occurrence of structural adjustments that compromise the intrinsic kinetic properties of the enzyme. The hSCOMT-AAIL´s interaction mechanism is not firmly established; however it seems to be highly dependent on the binding pH. Future work will undoubtedly seek to experimentally test the proposed mechanism and response how hSCOMT binding to AAIL's in acidic conditions and promotes the changing of its native conformation and how this can compromise hSCOMT specific activity.

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7. **Figures**

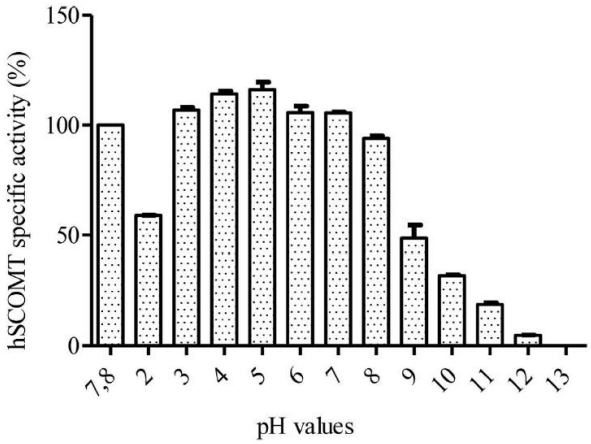

Figure 1. hSCOMT specific activity at pH ranges from 2 to 13 in comparison with the positive control of specific activity data achieved at pH 7.8. The experiments were performed at 4ºC during 12 h. This experiment was repeated, independently, three times.

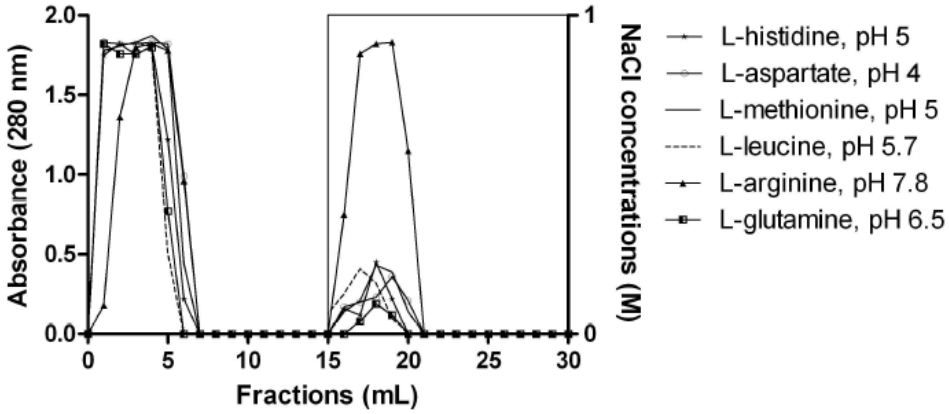

Figure 2. Chromatographic profiles of an *E. coli* lysate extract with recombinant hSCOMT onto the six AAIL tested with a stepwise gradient from 0 to 100% buffer A (buffer A: 10 mM Tris–HCl, pH 7.8 (L-arginine), 4 (L-aspartate), 6,5 (L-glutamine), 5 (L-methionine and L-histidine), 5,7 (L-leucine); buffer B: sodium chloride 1M in 10 mM Tris–HCl, pH 7.8).

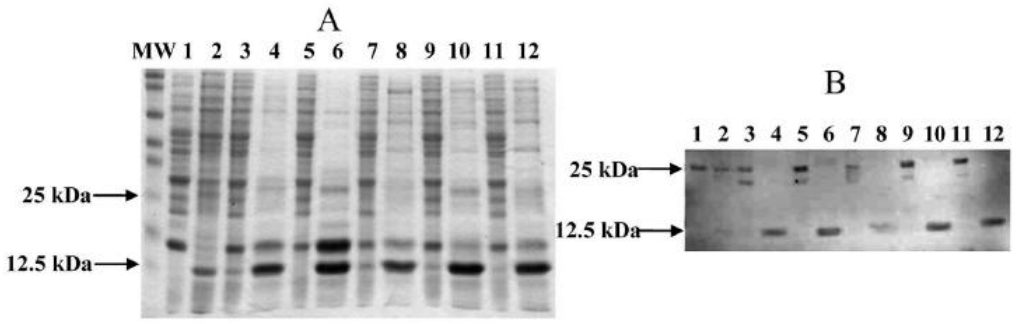

Figure 3. (A) SDS-PAGE analysis of *E. coli* recombinant hSCOMT lysates, loaded onto the several AAIL`s supports: (lanes 1, 2) L-arginine, (lanes 3, 4) L-aspartate, (lanes 5, 6) L-glutamine, (lanes 7, 8) L-histidine, (lanes 9, 10) L-leucine, (lanes 11, 12) L-methionine. The representative lanes corresponded to the respective binding pH: 7.8 (1), 4 (3), 6.5 (5), 5 (7), 5.7 (9), 5 (11) and elution at 1M of sodium chloride (lanes 2, 4, 6, 8, 10, 12). (B) *Western blot* from the same fractions obtained from AAIL's chromatographic trials.