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Ciências

Lyophilization and a preliminary thermodynamic characterization of recombinant SCOMT_His$_6$

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“When we are no longer able to change a situation,
we are challenged to change ourselves”

Viktor Frankl

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Resumo

A proteína catecol-O-metiltransferase (COMT, CE 2.1.1.6) foi descrita pela primeira vez em 1958. A COMT é uma enzima monomérica, dependente de magnésio que está envolvida na inativação de substratos com estrutura catecólica como a dopamina, a epinefrina e a norepinefrina. A COMT é um alvo importante na engenharia de proteínas devido à sua função na atividade normal do cérebro e ao seu possível papel em desordens neurológicas humanas, como a doença de Parkinson, esquizofrenia, depressão e Alzheimer. Atualmente, o tratamento mais eficaz para a doença de Parkinson consiste numa tripla profilaxia que consiste na reposição de dopamina com levodopa, um inibidor da enzima descarboxilase dos aminoácidos aromáticos e um inibidor da COMT. Nos últimos anos têm vindo a ser desenvolvidos vários inibidores desta enzima para o tratamento da doença de Parkinson, e há um grande interesse em desenvolver novos inibidores mais eficazes. Para tal, dois pontos devem ser tidos em consideração. Em primeiro lugar, apesar de atualmente ser possível produzir grandes quantidades de COMT por processos biotecnológicos, ainda é difícil manter a proteína num estado estável e ativo, principalmente num estado mais puro, como aquele em que se encontra após um passo cromatográfico. Não há uma forma rápida de obter a proteína a menos que todo o processo desde a produção ou (no caso de células congeladas) desde a lise seja feito. Para ultrapassar essa limitação, neste trabalho, um processo de liofilização aplicado a uma fração purificada por cromatografia de afinidade com metal imobilizado (IMAC) de COMT foi estudado com o objetivo de armazenar ou transportar estas frações mais facilmente e por períodos de tempo maiores. O segundo aspeto a ter em conta diz respeito aos inibidores produzidos para a COMT. Com o número de novos inibidores a serem produzidos, é importante estudar as suas interações com a proteína. As técnicas de microcalorimetria surgem então como um bom meio para caracterizar cineticamente essas interações. Portanto, também neste trabalho, é discutida uma abordagem inicial para estabelecer um método de calorimetria de titulação isotérmica (ITC) para estudar as interações entre a enzima e o inibidor 3,5-dinitrocatecol (3,5-DNC).

Palavras-chave

Calorimetria de titulação isotérmica (ITC), catecol-O-metiltransferase, doença de Parkinson, liofilização, 3,5-dinitrocatecol

Abstract

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) was first described in 1958. COMT is a monomeric enzyme magnesium dependent that is involved in the inactivation of catechol substrates such as dopamine, epinephrine and norepinephrine. COMT is an important target in protein engineering due to their role in normal brain and their possible role in human neurological disorders such as Parkinson's, schizophrenia, depression and Alzheimer's disease. Actually, the most effective treatment for Parkinson’s disease is a triple prophylaxis consisting of the dopamine replacement with levodopa together with an inhibitor of aromatic amino acid decarboxylase and an inhibitor of COMT. In recent years, several COMT inhibitors have been developed for Parkinson's disease treatment, and there is considerable interest in developing new and more effective inhibitors. To do so, two points should be taken into consideration. Firstly, although large quantities of COMT can nowadays be produced through biotechnological processes, is still difficult to maintain the protein in a stable and active state mainly in a more pure state, like that achieved after a chromatographic step. There is no fast way to get active protein unless all the process since the production or (in case of frozen cells) since the lyses was done. To overcome this limitation, in this work, a lyophilization process applied to a purified immobilized-metal affinity chromatograph (IMAC) COMT fraction was study with the objective to store or transporting this fractions more easily and for longer periods. The second aspect to take into account concerns the inhibitors of COMT produced. With the number of new inhibitors being produced, is important to study their interactions with the protein. The microcalorimetric technics appears as a good means to kinetically characterize these interactions. So, also in this work, is discussed an initially approach to establish an isothermal titration calorimetry (ITC) method to study interactions between the COMT enzyme and the 3,5-dinitrocatechol (3,5-DNC) inhibitor.

Keywords

Catechol-*O*-methyltransferase; Isothermal titration calorimetry; Lyophilization; Parkinson's disease; 3,5-dinitrocatechol.

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List of Acronyms

AADC	Aromatic L-amino acid decarboxylase
COMT	Catechol-*O*-methyltransferase
DMSO	Dimethylsulfoxide
3,5-DNC	3,5-Dinitrocatechol
E. coli	Escherichia coli
EGTA	Ethylene glycol tetraacetic acid
ΔH_{app}	Total molar enthalpy
hSCOMT	Human soluble catechol-*O*-methyltransferase
hSCOMT_His$_6$	human hexa-histidine tagged soluble catechol-*O*-methyltransferase
IMAC	Immobilized-Metal Affinity Chromatograph
IMC	Isothermal microcalorimetry
ITC	Isothermal titration calorimetry
K_m	Michaelis constant
MWCO	Molecular weight cutoff
PD	Parkinson's disease
PMSF	Phenylmethanesulfonyl fluoride
P. pastoris	*Pichia pastoris*
SAM	S-adenosyl-$_{L}$-methionine
S. *cerevisiae*	*Saccharomyces cerevisiae*
SCOMT	Soluble catechol-*O*-methyltransferase
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol
V_{max}	Maximum velocity

Chapter I – Introduction

1.1. General characterization of catechol-*O*-methyltransferase

Axelrod and coworkers [1] described catechol-*O*-methyltransferase (COMT; EC 2.1.1.6) for the first time in 1958. This is a magnesium-dependent enzyme that catalyzes the methylation of catechol substrates using S-adenosyl-$_L$-methionine (SAM) as a methyl donor and yielding, as reaction products, the *O*-methylated catechol and S-adenosyl-$_L$-homocysteine [2–4].

COMT exists in two isoforms, one is the soluble protein (SCOMT) that contains 221 amino acid residues and 24.4 KDa and the other is a membrane-bound protein [4,5]. The membrane-bound isoform has an additional 50 hydrophobic residues at the N-terminus [4]. Both the isoforms are coded by the same gene (located in chromosome 22), from two promoters [5]. Nevertheless, in humans, whereas most of the peripheral tissues express both COMT mRNA transcripts, membrane-bound protein is the dominant isoforms in brain, with only low amounts of the SCOMT present [5].

At a physiological level, the main role of COMT is the elimination of biologically active or toxic catechols and other metabolites [2]. Indeed, it acts as a detoxifying barrier between the blood and other tissues, being particularly active in the liver, kidneys and gastrointestinal tract [3].

1.2. The catechol-*O*-methyltransferase valine$^{108/158}$methionine polymorphism

The COMT valine$^{108/158}$methionine polymorphism involves a substitution of a valine to a methionine in the polypeptide chain at position 108/158 (for soluble and membrane-bound COMT, respectively) [6]. Nevertheless, methionine homozygotes have decreased COMT levels that presents lower structural stability, being more susceptible to oxidation and unfolding [3,4,6–8]. Indeed, the valine/methionine genetic polymorphism has been the subject of intense molecular epidemiologic studies and has been reported to be a risk factor not only for a number of psychiatric disorders including schizophrenia [9–12], obsessive-compulsive disorder [13] and psychosis susceptibility in Alzheimer disease [12], but it has also been linked to increased risk for breast cancer [14], increased sensitivity to pain [15], and improved prefrontal cognitive function [16]. The functional effects of the polymorphism for neurotransmission in the brain have been previously documented, being suggested that the low enzyme activity allele (methionine) increases susceptibility to a range of psychiatric symptoms [17–19]. The valine allele has been linked to poor prefrontal function and neuronal signal to noise ratio, having a compensatory advantage in terms of self-regulation and emotion processing, whereas methionine allele may contribute to poorer regulation and susceptibility to emotion related psychopathology [6,20–22].

Moreover, since this polymorphism hasn't been found in any other species, the $^{108/158}$methionine variant may be specific to humans. Nevertheless, the fact that COMT activity has decreased during evolution, could reflect the beneficial effect of lowered COMT activity on prefrontal function. However, the existence of only one COMT gene without any known tissue-specific variants, originates that the valine$^{108/158}$methionine polymorphism leads to functional alterations of COMT in all tissues, highlighting its importance when studying this enzyme [3].

1.3. Three-Dimensional Structure of catechol-*O*-methyltransferase

A large number of crystal structures are available not only for human, but also for rat COMTs [23]. Actually, the rat and human COMTs share 81% sequence identity and both belong to the highly structurally conserved SAM-dependent methyltransferase fold family (class I) [24]. The crystal structures for COMT have provided rationales why this protein could accept a range of structurally variable substrates with the only strict requirement that the substrate must have a catechol structure. Indeed, this aspect is of great importance for the search and design of COMT inhibitors that would enhance the levodopa treatment of Parkinson's disease (PD) [23].

The COMT enzyme is composed of a seven-stranded ß-sheet core (arranged in an order of 3214576) sandwiched between two sets of α-helices (helices α1–α5 on one side and helices α6–α8 on the other side) (Figure 1). In the ß-sheet, strand 7 is antiparallel to the others [4,23]. The crystal structures of the human ^{108}valine COMT and ^{108}methionine COMT with bound SAM and 3,5-dinitrocatechol (3,5-DNC) were described by Rutherford and coworkers in 2008 [4]. In fact, the structure previously referred is characteristic of the SAM-dependent methyltransferase fold family [24]. The differences between the polymorphisms are only observed in the presence of an extended loop between ß-strands 6 and 7, which forms part of the catechol-binding site [4].

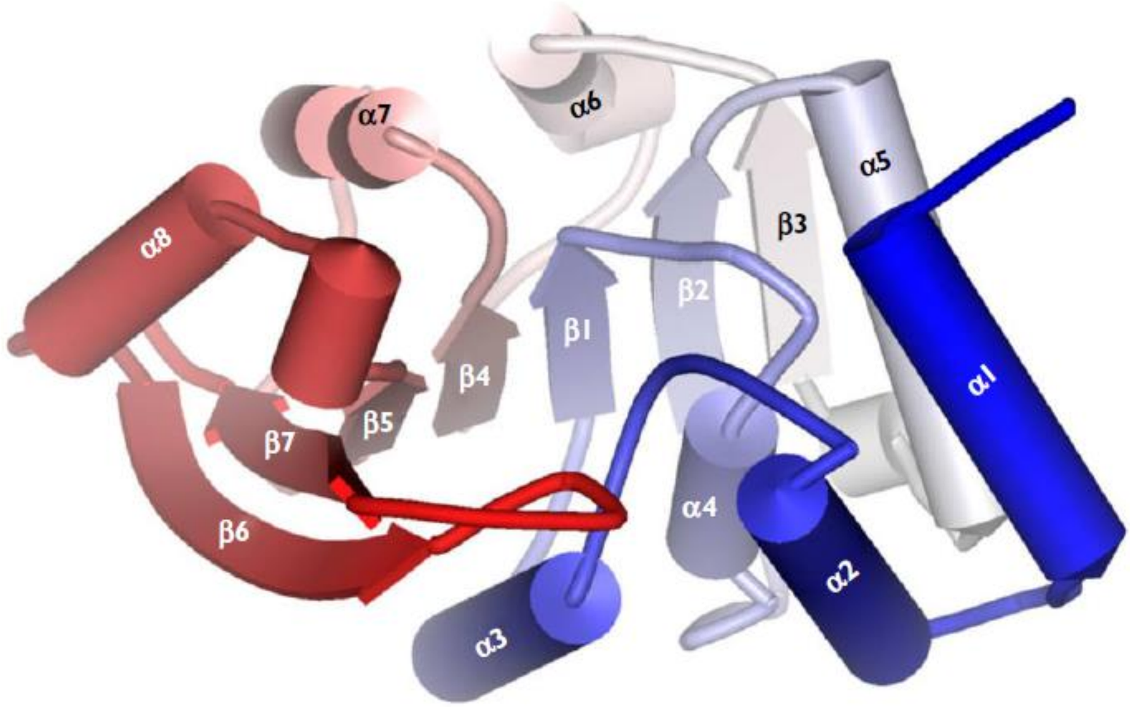


Figure 1 - The tertiary structure of the catechol-O methyl transferase enzyme, depicting the overall fold of a catechol-O methyl transferase structure [23].

The cofactors SAM and the magnesium ion (Mg^{2+}) as well as the inhibitor 3,5-DNC bind in shallow clefts on the protein surface. Additionally, the cofactor SAM interacts with conserved motifs along the first half of the core ß-sheet (Figure1) [4,23]. There are described specific interactions: the E90 (ß2) forms hydrogen bonds with the ribose hydroxyl groups of SAM; the adenine ring of SAM forms hydrogen bonds with S119 and Q120 (α6) and van der Waals interactions with residues I91 (ß2), A118 (ß3–α6 loop) and W143 (ß4–α7 loop); the methionine portion of SAM is coordinated through the hydrogen bonds with residues V42 (α2-α3 loop), S72 (α4), D141 (ß4) and the hydrophobic interactions with M40,V42, and Y68; and the methyl group (CH_3) attached to the methionine sulfur atom in SAM is oriented toward the substrate binding site and specifically towards the catechol oxygen atom to be methylated (Figure 1) [23].

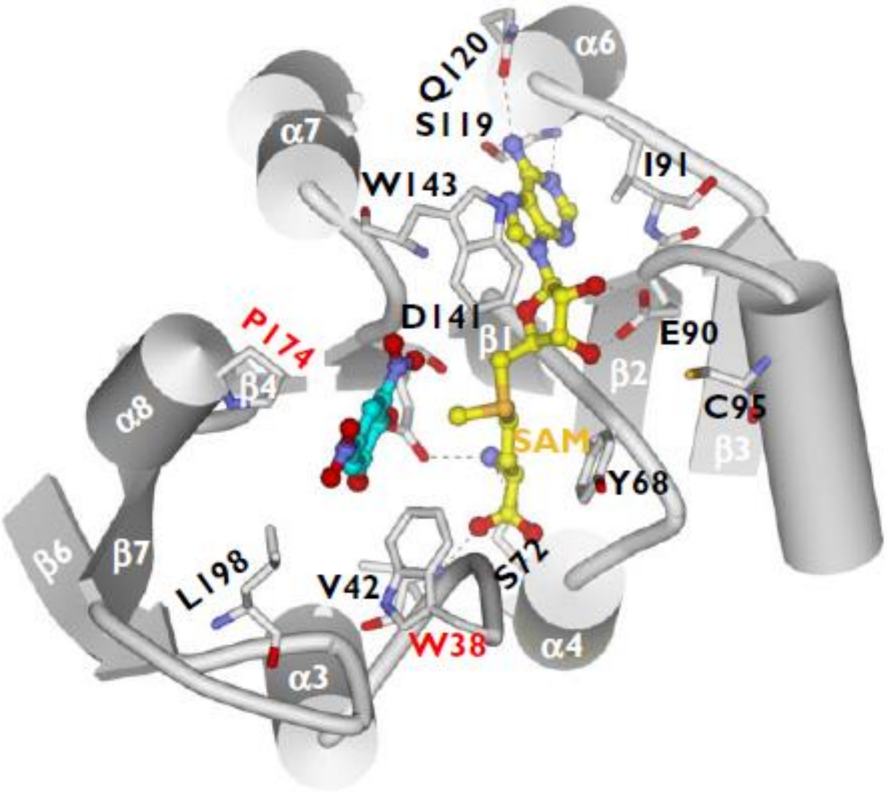


Figure 2 - The substrate-binding and S-adenosylmethionine-binding sites in the catechol-O methyl transferase protein: molecular interactions of S-adenosylmethionine and 3,5-dinitrocatechol with the binding site residues. The dashed lines indicate hydrogen bonds [23].

The substrate (catechol)-binding site is a shallow pocket defined by five residues, M40, L198, W143 and 'gatekeeper' residues W38 and P174, which hold the substrate in the correct orientation for methylation, according to Figure 2 . The van der Waals contacts are recognised as the main forces for ligand binding because all these residues are hydrophobic. The Mg^{2+}, a necessary cofactor for the methylation, is present in all the COMT crystals complexed with a ligand [4,24–33]. This ion is octahedrally coordinated in the active site by the side chains of three residues, the two hydroxyl groups of the catechol substrate, and a water molecule [4,23]. This fact evidences that the Mg^{2+} participates in the enzymatic reaction by assisting substrate binding, explaining why the ion is required for COMT mediated catalysis [23].

Human SCOMT contains seven cysteine residues and three of these residues, namely is C95, C173, C188, are not conserved in the rat enzyme. Moreover, whereas C95 is located in the catechol binding site, theC173 is located next to the P174 "gatekeeper" residue. Additionally the crystal structure reveals that four of the seven residues are located in surface loops with their sulfur atoms exposed to solvent. This fact, make this residues potential sites for intermolecular disulfide bond formation [4].

1.4. Catechol-*O*-methyltransferase in catecholamine metabolism

COMT is, along with monoamine oxidase A, the major enzyme responsible for catecholamine catabolism, namely dopamine, norepinephrine and epinephrine, in the brain [34,35]. Dopamine, for example, is converted into 3-methoxytyramine (Figure 3) through the *O*-methylation of dopamine by COMT [5,12,35,36]. It has been recognized that COMT may be unexpectedly important for the breakdown of dopamine, particularly in prefrontal cortex [5]. Nevertheless, the dopamine *O*-methylation reaction was recognized to be a prominent pathway in this region due to the fact that this compound is the major metabolite of released dopamine in rat in prefrontal cortex [5]. Studies revealed that besides there are no changes in other brain regions or in noradrenaline levels, the COMT knockout mice revealed an almost 3-fold increase of dopamine levels in the frontal cortex of males [3,5,35]. In fact, those results were supported by the finding that the administration of the specific and brain-penetrant COMT inhibitor tolcapone greatly increases extracellular dopamine, but not noradrenaline, in rat prefrontal cortex [37].

Nevertheless, the exact mechanism by which COMT modulates dopamine but not noradrenaline remains unclear [5]. Moreover, residual homovanillic acid levels were detected in several brain areas, despite the complete lack of COMT gene and protein. This aspects point to the possibility that there is still an unidentified methylation pathway in the brain [5,37]. Additionally to prefrontal cortex, the striatum is the only other brain region in which the importance of COMT for modulating catecholamine function has been extensively studied, being demonstrated its minor role in the removal of dopamine. Actually, in spite of the COMT activity is highest in excretory organs such as liver and kidney, it is also present in the central nervous system where it is most abundant in microglia cells. This enzyme is less prevalent in neurons and astrocytes and was not at all detected in human dopaminergic nigro-striatal neurons [38]. It has been recognized that COMT activity is probably under hormonal control being identified a significantly lower COMT activity in females [35].

As previously referred, the human COMT gene polymorphism valine$^{108/158}$methionine influences the enzyme activity [39]. Accordingly, when this residue is a valine, an increase in COMT activity was observed, resulting in reduced prefrontal dopamine levels and leading to an increased regulation of striatal dopamine activity; on the other side, when the residue is a methionine, a significant reduction in COMT's enzymatic activity was observed [3,5,8,12].

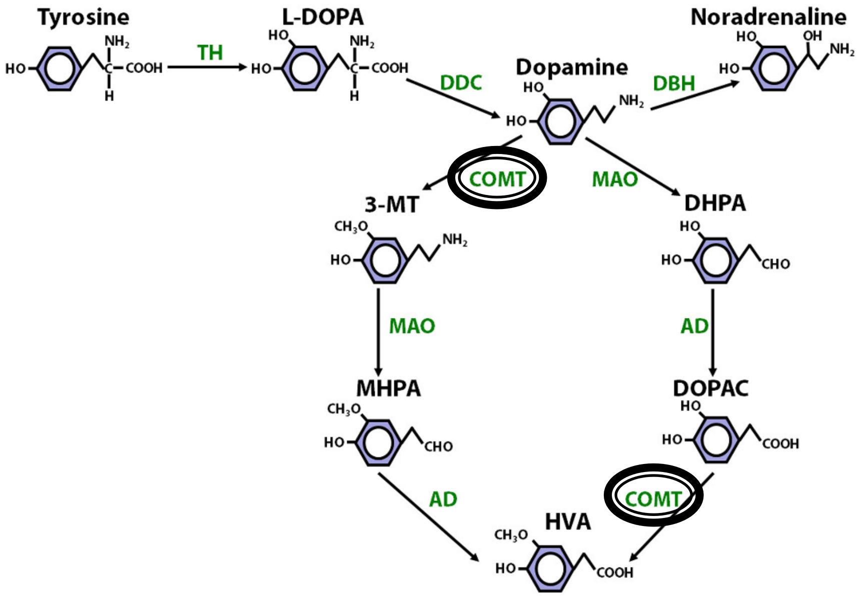


Figure 3 - The synthesis and degradation of dopamine. AH, aldehyde dehydrogenase; COMT, catechol-O methyl transferase; DBH, dopamine β-hydroxylase; DDC dopamine decarboxylase; DHPA; 3,4 dihydrophenylacetaldehyde; MAO, monoamine oxidase; MHPA, 3-methoxy-4-hydroxyphenylacetaldehyde; 3-MT, 3-methoxytyramine; TH, tyrosine hydroxylase [39].

1.5. Catechol-*O*-methyltransferase and neurodegenerative diseases

Neurodegenerative diseases result from the gradual and progressive loss of neural cells, which leads to nervous system dysfunction [40]. Indeed, there is as a deterioration (often irreversible) in the intellectual and cognitive faculties [41]. There are recognized more than 600 neurologic disorders [40]. Pathologically, the accumulation and aggregation of abnormal or misfolded proteins is a frequent characteristic of these diseases, namely beta-amyloid in Alzheimer's disease, huntingtin protein in Huntington's disease, transactive response DNA-binding protein 43 in frontotemporal dementia and amyotrophic lateral sclerosis, and α-synuclein in PD [42]. It is known that the etiology of neurodegenerative diseases is multifactorial. Moreover, there is evidence that potential external factors including lifestyle and chemical exposures are linked with the risk of the onset of these diseases [43]. Nevertheless, some genetic polymorphisms, and also the increasing age are also primary risk factors [12,40,41,44].

1.6. Catechol-*O*-methyltransferase and Parkinson's disease

PD is the second most common neurodegenerative disorder after Alzheimer's disease [45–47], affecting 1% of the elderly population with a higher prevalence in men [46]. Although fragments of Parkinsonism can be found in earlier descriptions, this disease was first medically described as a neurological syndrome by James Parkinson 1817 [48].

The clinical characterization of PD includes severe and progressing motor symptoms, namely bradykinesia, resting tremor, rigidity, and postural instability [2,46,47]. Slowness, stiffness, and the inability to initiate movements are other primary symptoms [2]. Those features are mainly linked to the degeneration of dopaminergic neurons in the substantia nigra pars compacta, as well as the presence of Lewy body or Lewy neurite intracellular inclusions largely composed of the protein α-synuclein in surviving neurons [45–47]. Notwithstanding, and although the PD is incurable, there are some therapies that can improve quality of life for many years [49]. The treatment of PD has focused almost exclusively on the replacement of dopamine [50,51]. Accordingly, levodopa or dopamine agonists are usually the drugs of choice when a significant symptomatic effect needs to be achieved [50]. On one hand, levodopa is the metabolic precursor of dopamine and its administration leads to an increment in dopamine levels [50,51]. Clearly, this is the single most effective agent for treating PD [51] but other chemicals may also be applied such as, dopamine agonists that mimic dopamine action [50].

Levodopa, unlike dopamine has the capacity to cross the blood–brain barrier. Then, it is converted to dopamine by aromatic L-amino acid decarboxylase (AADC), which is responsible for the therapeutic effectiveness of the drug in PD [2,51]. However, if levodopa is administered alone, relatively little unchanged drug reaches the cerebral circulation and probably less than 1% penetrates the central nervous system. Consequently, in clinical practice, levodopa is mostly given in combination with a peripherally acting inhibitor of AADC, the inhibitor does not penetrate well into the central nervous system [2,51]. Thus, due to the limitations presented by levodopa in some cases, alternatively, many physicians actually use dopamine-receptor agonists [51]. Indeed, their symptomatic effect and because they have been shown to reduce the risk of motor complications when compared with levodopa, dopamine agonists are widely used as initial therapy. Nevertheless, clinical trials have shown that about 50% of patients initiated on dopamine agonist monotherapy required also supplementary levodopa in order to maintain the symptoms control. Additionally, these drugs could also increase the risk of develop disabling motor complications [50,52,53].

Finally, in addition to an AADC peripheral inhibitor, it was also demonstrated that COMT-inhibitors have a significant effect on the systemic availability and elimination of levodopa [50,51]. In fact, in patients with advanced PD, the use of these drugs or monoamine oxidase B inhibitors plus levodopa was recognized to be superior at reducing PD symptoms, compared to using levodopa alone.

1.6.1. Catechol-*O*-methyltransferase inhibitors in Parkinson's disease

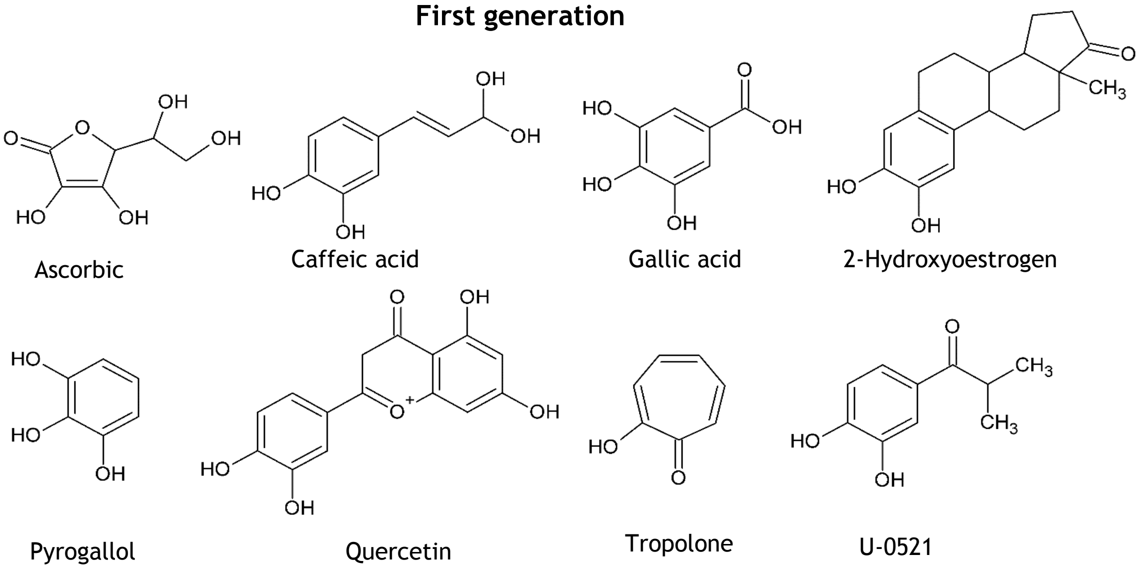
COMT inhibitors act by extending the duration of action of levodopa, thus improving the amount of time a patient can experience benefit from levodopa, ameliorating the wearing-off symptoms [54]. The use of COMT inhibitors in PD as an adjunct therapy to levodopa has the purpose of increase levodopa and consequently dopamine levels in the brain [55]. The design of inhibitors has been in practice since the discovery of COMT in 1958 [56].

The first generation of COMT inhibitors contain a catechol structure or some related bioisosteric moiety, being typically competitive substrates of COMT [2,55] This generation includes not only derivatives of pyrogallol and catechols like gallic acid, but also caffeic acid, U-0521, 2-hydroxyoestrogens and flavonoids like quercetin or rutin. and other non-catecholic compounds such as ascorbic acid and tropolones (Figure 4)[2,3,55,57]. These compounds were tested *in vitro* and *in vivo* and their activity in COMT inhibition was found to be weak or non-selective. Additionally, and although their acute toxicity was not high, these compounds were found to induce convulsions in experimental animals at high doses and showed signs indicative of hepatotoxicity [55,58,59].

The interest in COMT as a therapeutic was triggered by the discovery of a new generation of potent and selective COMT inhibitors in the late 1980s [2,3,55]. [55]. In fact, these "second-generation" inhibitors (Figure 4) were selective and orally active and endowed with improved pharmacokinetic profiles compared with those of the first-generation predecessors [57]. Their enhanced potency was attributed to the substitution of electron-withdrawing groups at a position *ortho* to a hydroxyl group of the catechol moiety and the best results were obtained with the nitro group, giving rise to this new class of nitrocatecholic COMT inhibitors [55].

They are reversible tight-binding and although these inhibitors share the same nitrocatechol pharmacophore, subtle differences do exist in the mode of COMT inhibition, being these characteristic properties of relevance in terms of the downstream clinical efficacy and safety of PD treatment [57,60]. In fact, this generation of COMT inhibitors could be divided into three groups: 1) mainly peripherally acting compounds, 2) broad-spectrum compounds working in both the periphery and the brain, and 3) atypical compounds, probably acting preferably in the brain [3].

First generation



Ascorbic

Caffeic acid

Gallic acid

2-Hydroxyoestrogen

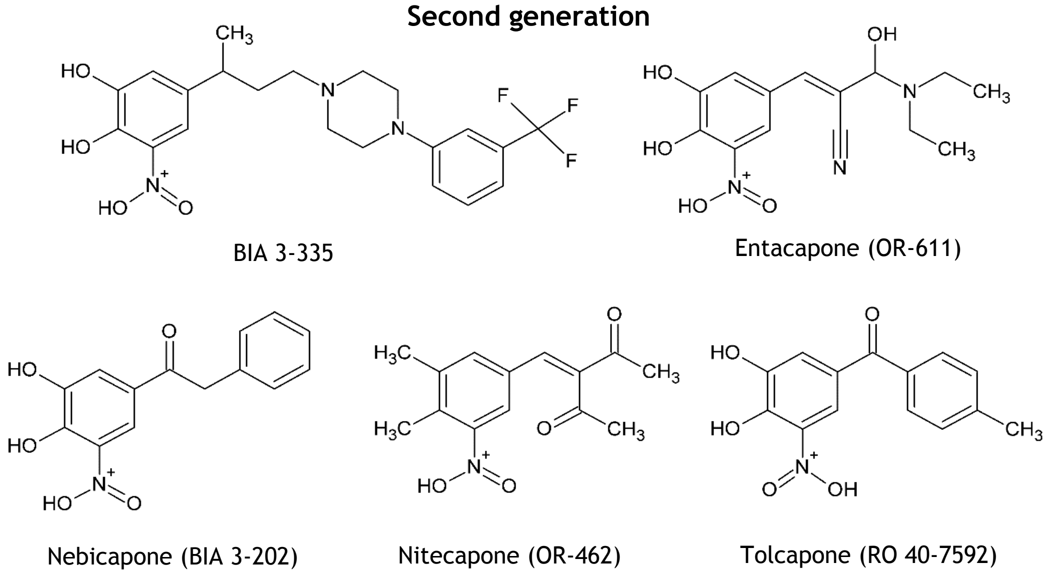
Pyrogallol

Quercetin

Tropolone

U-0521

Second generation



BIA 3-335

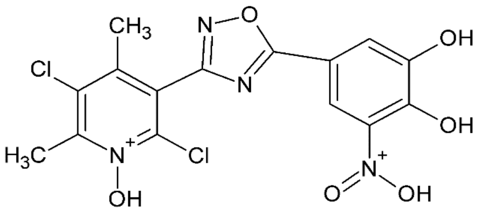
Entacapone (OR-611)

Nebicapone (BIA 3-202)

Nitecapone (OR-462)

Tolcapone (RO 40-7592)

Third generation



Opicapone

Figure 4 - Chemical structures of first, second and third generation of catechol-O methyl transferase inhibitors [2,55,57,62].

Entacapone action is mainly peripheral, being a short-acting selective inhibitor which is taken concomitantly with every dose of levodopa [55,60]. However, albeit it is most widely marketed COMT inhibitor, its clinical efficacy has been questioned [60]. On the other hand, tolcapone is a more potent, longer acting but non selective inhibitor of both cerebral and peripheral COMT, crossing the blood–brain barrier [55]. Nevertheless, unlike entacapone, its clinical use of is severely restricted due to its elevated hepatotoxicity risk [60,61]. Moreover, although other molecules such as nebicapone, BIA 3-335 or CGP 28014 were also developed and tested, all failed for diverse reasons, either to concerns regarding their safety or due their weak activity [2,55,57]. In fact, these inhibitors have been tested in clinical trials, but only entacapone and tolcapone are in clinical use [55].

The need for the development of further COMT inhibitors with novel and better pharmacodynamic profiles capable to deliver more sustained levodopa levels with lower frequency of drug administration in patients with PD led to the development of opicapone (Figure 4) [58]. This purely peripheral third-generation nitrocatechol COMT inhibitor is in the Phase III of development for the treatment of PD, and is a short lived and very long acting novel COMT inhibitor following multiple dose administration in healthy subjects [58,62].

1.7. Human soluble catechol-*O*-methyltransferase biosynthesis and purification

In general, the development of improved COMT inhibitors with enhanced selectivity and potency depends may be based in structure-based drug design studies. Therefore, relatively high quantities of enzymatically active COMT is required not only for crystallization studies but also for *in vitro* studies that are usually performed in the first stage of drug development [23].

The need for considerable amounts of human proteins for structural, functional and pharmacological studies or crystallization lead to the necessity of using the recombinant technology.
For the production of recombinant human SCOMT (hSCOMT), several expressions systems were used based on eukaryotic cells such as transfected mammalian cells [63], insect cells (via baculovirus vectors) [64], vector-infected plants (via a potyvirus) [65] or prokaryotic like *Escherichia coli* (*E. coli*) [66–69].

Tilgmann and coworkers, in 1992 produced active human recombinant soluble and membrane-bound COMT proteins in transfected mammalian cells as well as in recombinant baculovirus infected insect cells [64]. In transfected *Spodoptera frugiperda* (Sf9) cells the amount of recombinant COMT produced is much higher than that obtained in human myelogenous leukemia (K-562) cells [64].

In 1996, Ulmanen and collaborators aiming to determinate the subcellular location of SCOMT and membrane-bound COMT overexpressed this proteins in mammalian (human HeLa and hamster BHK-21) cell cultures [63]. The principal drawback of this expression system for the application in large scale recombinant protein production is the high costs of the media components necessary for cell growth.

More recently, plant cells (*N. benthamiana* and *N. tabacum cv*. Samsun) infected with potato virus A (PVA) based vectors were applied for recombinant SCOMT production, achieving high amounts of active SCOMT in plant tissues [65]. The cost-effective, large-scale production of substantial amounts of plant biomass would allow large quantities of SCOMT to be achieved. However, the recombinant protein obtained with this expression system had a methylation activity substantively lower than that measured from *E. coli* lysates and the purification of SCOMT from plant extracts wasn't an option and the necessity of use the leaf extracts appear as a drawback [65].

Throughout the years, the main expression system for hSCOMT production has been *E. coli* [67-69]. The ability of this host system to grow rapidly at high cell density on inexpensive substrates, the well know genetics and the number of cloning vectors plus mutant host strains make them the most commonly used [66,70,71]. Yet, a disadvantage is the possibility presence of toxic cell walls pyrogens (endotoxins) [72].

However, due to the necessity of post-translational modifications, such as glycosylation, phosphorylation and disulfide bridges formation for the precise folding and correct activity of the proteins, more complex expression systems are required. In particular, as eukaryotic cells, yeasts like *Pichia pastoris* (*P. pastoris*) and *Saccharomyces cerevisiae* (*S. cerevisiae)*, offer the ability of performing many of the aforementioned post-translational modifications in recombinant protein production. The use of yeasts shows some advantages when compared to conventional systems, like mammalian cells, once they are faster, easier and less expensive to use [73,74]. Also, fermentations can be readily scaled up to give answer to greater demands, and the production parameters such as pH, carbon source feed rate and aeration may be controlled [73]. These two yeasts show a propensity for homologous recombination between genomic and artificially introduced DNAs. The introduction can occur either via gene insertion or gene replacement [73]. When we compare these two yeasts, *P. pastoris* gain some advantage, by its capacity to perform a less extensive glycosylation, more similar to the native human form in which the oligosaccharide chains attached to the proteins are shorter [75]. Also, *P. pastoris* does not need a defined culture media to grow like *S. cerevisiae*, thus becoming the production less expensive. In *S. cerevisiae* some limitations have been detected like low product yield and inefficient secretion with many proteins retained in the periplasmic space or associated with the cell wall rather than found free in in the culture medium [72]. Additionally, *P. pastoris* is an organism that is easy to manipulate and secrete low levels of native proteins making the subsequent purification process much simpler than in other systems [73].

In the pursuit to achieve better isolation and purification methodologies, several purifications procedures were applied to human SCOMT over the years, such as hydrophobic interaction chromatography [76–78], cation and anion exchange chromatography [79], bioaffinity chromatography with immobilized amino acids [80] or size exclusion chromatography [81]. However, it is necessary to take into account the protein activity, as well as the enzyme recovery, that some of these techniques present as downside.

One of the most diverse and powerful chromatographic methods for purification of a specific molecule or a group of molecules from complex mixtures is affinity chromatography. It is based on highly specific biological interactions between two molecules, namely interactions between enzyme and substrate, receptor and ligand, or antibody and antigen. These typically reversible interactions are used for purification by placing one of the interacting molecules, referred to as affinity ligand, onto a solid matrix to create a stationary phase while the target molecule is in the mobile phase. Specifically, Immobilized-Metal Ion Affinity chromatography (IMAC) is based on the specific coordinate covalent bond of amino acids, particularly histidine, to metals [82]. In fact, one of the most successful purification processes is accomplished by the fusion of poly-His tags to the C-terminal of the protein, with a special affinity to metal ions like Ni^{2+} making affinity chromatography the main tool to capture the tagged proteins [73].

1.8. Lyophilization

Lyophilization, also known as freeze-drying is commonly used to prepare proteins products for long term storage when the stability in aqueous solutions is insufficient or, for more appropriate transportation [83,84]. This technique appeared during the World War II when the demand for human blood plasma reached serious proportions and more recently was recognized as an important technique for the preservation of biological materials [85]. Actually, it is widely used to manufacture labile drugs such as enzymes and therapeutic proteins [86].

The lyophilization process consists in removing the water or other solvent from a frozen solution by sublimation and desorption, generally under reduced pressure, leaving the solutes in their anhydrous, or almost anhydrous state [85,87]. The resulting material usually acquires a porous and friable structure that can be easily rehydrated [88].

In aqueous solutions, substances like proteins, are exposed to a number of chemical reactions that can compromise their activity, quality, or safety [85]. Some of these reactions are hydrolysis, cross-linking, disulfide rearrangements, and the most problematic, aggregation [85,89]. The major problem with aggregation is that it can occur at almost any stage of production, processing and storage [89]. However, it’s well known that in the dry state these reactions can be substantially retarded enhancing the chemical stability [85]. Lyophilization is adavantageous over traditional drying processes since as it occurs at low temperatures, it is less likely to induce damages to the labile products than drying at ambient or higher temperatures [85,88].

Nevertheless, common limitations associated to lyophilization include the slow drying rate, very low temperatures, use of vacuum and, generally, the high investment and operation cost. Probably, the greater stress that a protein is potentially under during lyophilization, is the freeze concentration of solutes that can reach concentrations as high as 20-50 times their initial concentration, in the remaining liquid during the formation of pure ice [83,88]. The lyophilization experiments are time consuming and usually expensive to be carried out in every possible operating range. To overcome these disadvantages, efforts have been made to facilitate the lyophilization process optimization, such as computer modeling to predict the optimum freeze-drying conditions [87]. Nevertheless, computer modeling requires input real data collected from experiments and is dependent of the accuracy of the input data and the model chosen. Also, the confirmation requires a number of laboratory experiments [86,87].

1.8.1. Lyophilization steps and cooling methods

The lyophilization process can be divided in three stages: freezing, primary drying, and secondary drying [84,87].

In the first stage, the material is cooled down to a temperature below the solidification. All the porous characteristics like shape, size distribution and pore connectivity of the porous network of the dried layer formed during the primary drying depend on the ice crystals formed during the freezing stage. The product can be dried quickly if large dendritic ice crystals are formed in a homogeneous dispersion due to the high mass transfer rate of water vapor [87]. The solvent is removed by sublimation under vacuum and some heat addition in the primary drying. In this step heat is transferred from the shelf to the frozen solution, the ice sublimes and the water vapor formed passes through the dried portion of the product to the surface of the sample. Then the water vapor is transferred from the surface of the product through the chamber to the condenser where it will condensate. At the end of sublimation step a porous plug is formed. Its pores correspond to the spaces that were occupied by ice crystals [84,87,90,91].

The last step is the secondary drying, where the adsorbed water which did not separate out as ice during the freezing, and did not sublimate off is removed. The bound water is removed by slightly heating the product. Nevertheless, the temperature in this step can be as lower as -50°C and vacuum pumps are connected to the drying chamber to maintain pressures in the range of 4 to 40 Pa [84,87,92].

The samples for lyophilization can be cooled by a diversity of methods. Examples for each method will be mentioned below. In “Nucleated and frozen” method the samples are nucleated and frozen at −1°C subsequently dipped in liquid nitrogen just long enough to initiate ice crystallization. They are then transferred to a shelf precooled to −1°C and allowed to sit at −1°C for 15 min before being cooled slowly (~1°C/min) to −40°C. In the course of this ‘‘cooling’’ process the samples never melt completely. It is expected that ice crystals will be large and fairly localized, leaving large domains of concentrated liquid solution, once the removal of heat from the sample was slowly. In opposition, “Supercooled” samples are placed on an ambient temperature shelf without nucleation and are cooled slowly (~1°C/min) until approximately 10°C of supercooling before ice nucleation occurs. Nucleation and growth of ice crystals then happens extremely rapidly and nearly homogeneously throughout the sample. The result is an even distribution of small ice crystals and interstitial solution domains. “Quench frozen" involves dipping sample vials in either a dry ice and acetone slurry (ca. −80°C) or in liquid nitrogen (ca. −200°C) just the time needed for the sample to freeze completely and reach thermal equilibrium (1 min) and then placing the vials on a shelf precooled to −25°C. Both methods rapidly drop the sample temperature below expected glass transition temperatures after ice is formed. “Spray-frozen” method provide the fastest cooling rate by sprayed the samples as a fine mist directly into liquid nitrogen. In this method cooling rates are dependent on many factors and are thus difficult to estimate, [93] still the maximum cooling rates achievable with liquid nitrogen are of the order of 300 K s−1, which could be considered an upper bound for the cooling rate.

1.8.2. Sugars and Surfactants in the lyophilization process

Protein molecules are naturally constructed to be correctly folded and active in an aqueous ambient like the inner of the cell. Because of the deletion of these water molecules that surround the protein during the lyophilization process, frequently this results in the loss of the intact conformation of the protein, even in low temperatures [94]. The two major processes of the lyophilization, freezing and dehydration, can often cause irreversible damage to the protein seen as structural denaturation, aggregation upon rehydration, and loss of biological efficacy [95,96]. Moreover, the pathways responsible for protein denaturation are different for freezing and drying. The typical approach to protect a protein against these stresses are the addition of excipients such as sugars, polyols, amino acids and polymers to the lyophilization formulation to act like stabilizers [83,84].

As the pathways for denaturation, also the excipient added to the formulation show different effectivenesses. In general, sugars exert protein stabilization effects during dehydration (lyoprotectant) but are ineffective in stabilization against the freezing-induced denaturation whereas the effectiveness of a polymer additive was limited to stabilization during freezing (cryoprotectant)[83,89,94,97]. In addition, the combination of a cryoprotectant with a lyoprotectant has been shown to be a good strategy in protecting some proteins during the lyophilization process [95,98]. However, depending on the type of the protein, the stabilization characteristics of individual surfactants may possible be altered, that is, different proteins may be sensitive to different denaturing factors [94].

In table 1 some examples of the excipients commonly applied in lyophilization process of pharmaceutical products are displayed with the reference to their purpose [84].

Table 1 - Examples of normally used excipients in lyophilization of pharmaceutical products (adapted from [84]).

Type	***Function***	***Substance***
Bulking agents	Provide bulk to the formulation especially when the concentration of product to freeze dry is very low.	Hydroxyethyl starch, trehalose, mannitol, lactose, and glycine.
Buffers	Adjust pH changes during freezing.	Phosphate, TRIS-HCl, citrate, and histidine.
Stabilizers	Protect the product during freeze-drying against the freezing and the drying stresses.	Sucrose, lactose, glucose, trehalose, glycerol, mannitol, sorbitol, glycine, alanine, lysine, polyethylene glycol, dextran, and polyvinylpyrrolidone.
Tonicity adjusters	Yield an isotonic solution and control osmotic pressure.	Mannitol, sucrose, glycine, glycerol, and sodium chloride.
Collapse temperature modifiers	Increase collapse temperature of the product to get higher drying temperatures.	Dextran, hydroxypropyl-ß-cyclodextrin, polyethylene glycol, and polyvinylpyrrolidone.

The most usual cryoprotectants described in literature to use in lyophilization are sugars such as trehalose, sucrose, glucose e mannitol. The trehalose seems to have some advantages when compared with the others sugars once is less hygroscopic, the absence of internal hydrogen bounds allows the formation of more flexible hydrogen bonds, it very low chemical reactivity and finally, the higher glass transition temperature [99]. Normally, the level of stabilization given by sugars, are directly related with their concentrations. However, increasing cryoprotectant concentration to a certain level may eventually reach a limit of stabilization or even destabilize proteins. During the dehydration steps the unfrozen water remains dissolved or adsorbed on the solid phase [84]. These conditions may destabilize unprotected proteins. To avoid that, some excipients are added to the formulation to function as lyoprotectants. A suggested stabilization mechanism by lyoprotectants during the drying steps is the water replacement hypothesis that assumes the formation of hydrogen bonds between a lyoprotectant and the protein [100,101].

1.9. Isothermal microcalorimetry and isothermal titration calorimetry

Our current understanding of the mechanism of regulation and control of biological structures have been greatly improved by calorimetric techniques [102]. Indeed, several advances have been made not only in the development of highly sensitive microcalorimetric instrumentation, but also in the development of analytical procedures to extract thermodynamic information about biological systems [102].

Calorimetry is the science of measuring the heat changes of chemical or physical reactions in real time. The measurement of the heat variations is continuous (flow heat rate in µJ/sec=µW) and cumulative (given the total amount of heat consumed or produced (in Joules)) at a constant temperature, by a sample placed in an isothermal microcalorimetry (IMC) instrument. The heat flow is proportional to the aggregate rate of changes taking place at a given time. The aggregate heat produced during a given time interval is proportional to the cumulative amount of aggregate changes which have taken place. Calorimetry is performed with a calorimeter and the simplest use of IMC is detecting that one or more rate processes are taking place in a specimen because heat is being produced or consumed at a rate that is greater than the detection limit of the instrument used.

Isothermal titration calorimetry (ITC) is a technique used to measure the heat exchange associated with molecular interactions at constant temperature. Most often, ITC is used as a direct method to determine thermodynamic parameters associated with complex formation [103,104]. Indeed, ITC has emerged as a powerful tool for determining the thermodynamic properties of chemical or physical equilibria such as protein-protein, ligand-receptor, and protein–DNA binding interactions [105], where the binding mechanism, forces involved in binding and strength of the interaction can be analyzed [106–108].ITC is becoming a major tool in drug discovery studies. Actually, ITC is one of the latest techniques to be used in characterizing the binding affinities of ligands to proteins, being typically used as a secondary screening technique in high throughput screening and valuable tool in the drug discovery [109–111].

ITC could also be used to identify and isolate unknown target proteins including receptors and cell/tissue lysates when conjugated with chromatography [112,113]. Additionally, ITC could provide imperative information to the drug design and lead optimization, namely insights into the structure-activity relationship for ligand interaction with the target [114,115]. Actually, lead compounds can be further optimized with the use of structural and thermodynamic information of binding [116–118]. This technique is also gaining popularity as one of the methods for studying enzyme kinetics, providing invaluable data on the kinetics and thermodynamics of enzyme catalysis, the occurrence of inhibition and its corresponding mechanism [111,119,120].

Whereas calorimetry as a method for simultaneous determination of equilibrium constant and enthalpy was first described by Christensen and coworkers in 1965 [121], the first commercial titration calorimeters was only made available in 1989 [103,122].

An isothermal titration calorimeter (Figure 5) consists of a pair of identical cells, a sample cell and a reference cell, made up of efficient thermal conducting material, located within an adiabatic jacket. Both the cells contain identical buffer systems, and the latter also contains the protein of interest [123]. Moreover, a constant power is applied to the reference cell to maintain a specific temperature, being this the power applied to the sample cell (feedback power) variable and equal to the power needed to maintain a constant temperature difference between the reference and sample cell. A syringe is used to titrate the ligand into the sample cell containing the biomolecule. Indeed, when a precise amount of ligand is titrated to the sample cell via the syringe. Heat is released or absorbed owing to binding and is reflected as a change in the feedback power. Consequently, this differential power is proportional to the temperature differences between the reference and sample cell [124]. To the detection of temperature differences between the two cells are used sensitive thermopile or thermocouple circuits, while maintaining isothermal conditions between them (ΔT=0) (Figure 5). Exothermic and endothermic interactions are characterized by negative and positive change in feedback power, respectively [125].

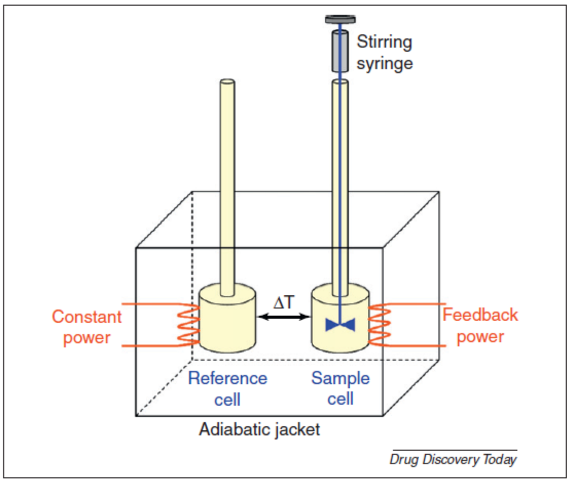

Stirring syringe
ΔT
Constant power
Feedback power
Reference cell
Sample cell
Adiabatic jacket
Drug Discovery Today

Figure 5 - Basic configuration of an isothermal titration calorimetry instrument [125].

Additionally, heat exchange in the sample cell is observed during the titration process, being the power required to maintain isothermal conditions between the two cells measured by a calorimeter. Series of peaks of heat flow (power) are shown by a plot of power (μcals-1) against time (min) (Figure 6A) [125]. Indeed, the enthalpy (ΔH) of binding is given by the area under the peaks [104,126]. The information about the enthalpy (ΔH), the association constant (Ka) and the stoichiometry (n) of binding, which could be used to calculate changes in entropy (ΔS) and Gibbs Free Energy (ΔG) is given by a binding isotherm obtained from the plot of the formation of heat against the molar ratio of ligand to biomolecule (Figure 6B) [125,126]. The K_a values between molecules that could be measured by ITC ranges from millimolar to nanomolar range [127].

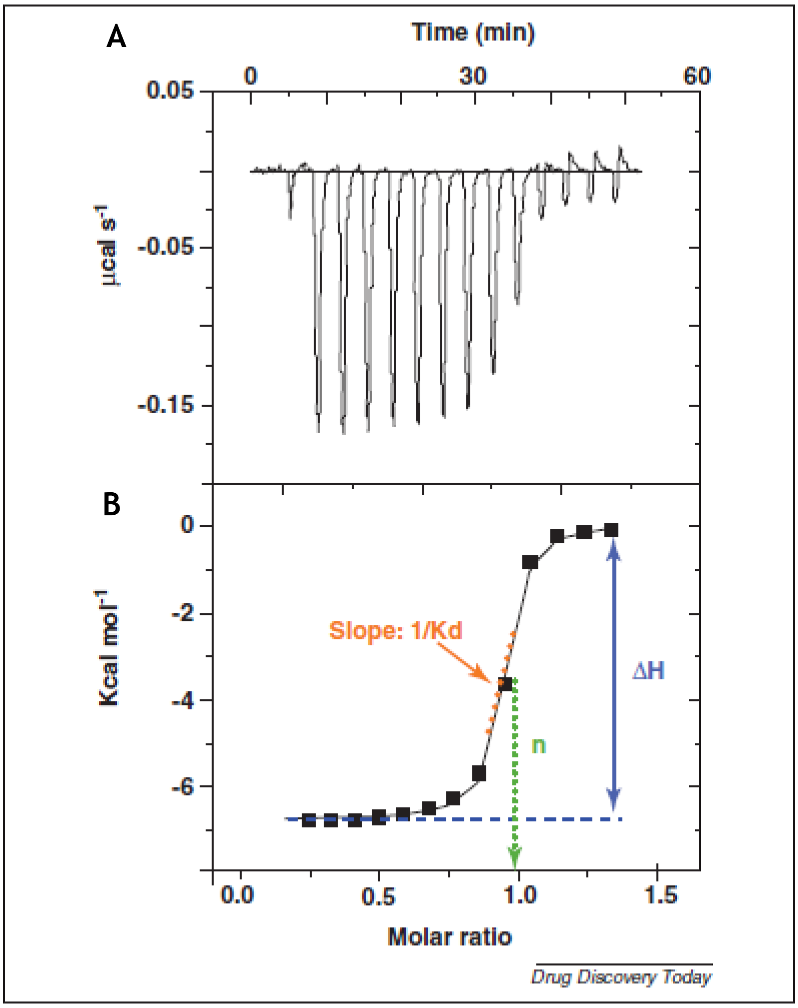


Figure 6 - (A) Raw data captured an isothermal titration calorimetry experiment. (B) Binding isotherm of heat of formation against molar ratio of ligand/biomolecule [125].

Comparing to other techniques such as fluorescence assays, Nuclear Magnetic Resonance and Surface Plasmon Resonance, ITC has advantages for studying complex formation in terms of ease of use and cost. In fact, this technique does not require any fluorescent probes or radioactive tags for data analysis, as well as immobilization and chemical modification of protein [117,127]. Additionally, ITC does not have limitations associated with clarity of the solution, molecular weight, temperature or pH. Actually, ITC it is one of the best methods for determining the thermodynamic parameters of ligand binding and several improvements, namely in the sensitivity of the instrument, decrease in the sample quantity required for titration or increases in the high-throughput capacity, could greatly increase the value of this technique in drug discovery [106].

1.9.1. Isothermal titration calorimetry and Enzyme Kinetics

The right characterization of an enzyme that follows the Michaelis-Menten mechanism is accomplished by the determination of the Michaelis constant, K_m, and the enzymatic conversion rate constant, or turnover number (k_{cat}). Moreover, the k_{cat}/K_m ratio is referred as the catalytic efficiency of an enzyme [128]. The conventional approach to determine these kinetic parameters is to follow the enzyme-catalyzed reaction by optical or electrochemical measurements. Nevertheless, most substrates and/or products properties are not suitable to this kind of experiments. On the other hand, in these approaches, the product accumulation and inhibition are often a problem (making the data analyses more complicate or by masking the results), and can lead to experimental errors in the determination of K_m an k_{cat} [128,129].

In order to avoid these drawbacks, and as virtually all chemical reactions such as enzyme-catalyzed reactions, consume or produce heat, calorimetric techniques are suitable for following, in real time, such reactions. In particular, calorimetric techniques are specially applied to those reactions where no observable change by other probes can be detected, such as proteases and peptidases catalyst hydrolytic reactions, in which no obviously spectroscopic properties or pH changes occur [128,129]. The general principle in using ITC is to measure the heat-flow associated with a given chemical reaction. The rate of the enzymatic reaction is proportional to this heat-flow, and thus measured as the experimental data. Furthermore, the measurement by ITC is independent of the composition of the reaction solvent, and has also been used to measure enzyme activity in crude tissue extracts, non-ideal solutions and in insoluble substrates. This confirms the promising potential of calorimetry towards measurements of enzyme kinetics in complex solutions [130]. The main advantages of the ITC are their sensitivity (nanomolar range of material consumption for experiment), the stable work at various fixed temperatures and fast equilibration at reloading the sample and changing the temperature at which measurements are carried out [131].

1.9.2. Injections method and modes of operating

The determination of enzyme kinetic constants using isothermal titration microcalorimetry may be achieved using two methods. Both methods are based in the proportionality between the rate of a reaction and thermal power (heat/time) generated [119]. Indeed, an enzyme can be titrated with increasing amounts of substrate maintaining the pseudo-fist-order conditions. Alternatively, by following a single injection, the change in thermal power as substrate is depleted can be continuously monitored. Both methods allow highly precise kinetic characterizations in a single experiment and can be used to measure enzyme inhibition [119].

In the pseudo-first-order approach, multiple injections of substrate can be made providing multiple rates determinations on a single experiment. The amount of heat involved with converting *n* moles of substrate to product is given by the Equation 1.

$$Q = n\Delta H_{app} = (P)_{total} V \Delta H_{app} \quad \textit{Equation 1}$$

In this equation, ΔH_{app} is the total molar enthalpy for the reaction, determined experimentally, P is the concentration of product generated while V is the volume of the reaction solution in the cell. It can be easily seen from Equation 2 that measuring the thermal power generated by the enzyme as it catalyzes conversion of substrate gives a measure of the reaction rate since:

$$Power = \frac{dQ}{dt} = \frac{d(P)}{dt} V \Delta H_{app} \quad \textit{Equation 2}$$

d[P]/dt is equal to the rate of product formation, that is the rate of reaction, therefore Equation 2 can be rearranged to give:

$$Rate = \frac{1}{V\Delta H_{app}} \times \frac{dQ}{dt} \quad \textit{Equation 3}$$

By the analysis of Equation 2, in order to obtain a Michaelis-Menten plot, two parameters need to be measured using the calorimeter. The first one is the ΔH_{app} and the cell calorimeter should contain sufficient enzyme to convert all the injected substrate into product in a given time period. As show in Figure 7, these experiments give rise to peaks where the baseline response returns to the same value after the substrate injection as it was before the injection. Integration of these peaks with respect to time yields, the total heat produced by the reaction and dividing this total heat by the amount of substrate converted gives the total enthalpy change. The second parameter to calculate is the power generated (dQ/dt). This must be determined at different substrate concentrations [119].

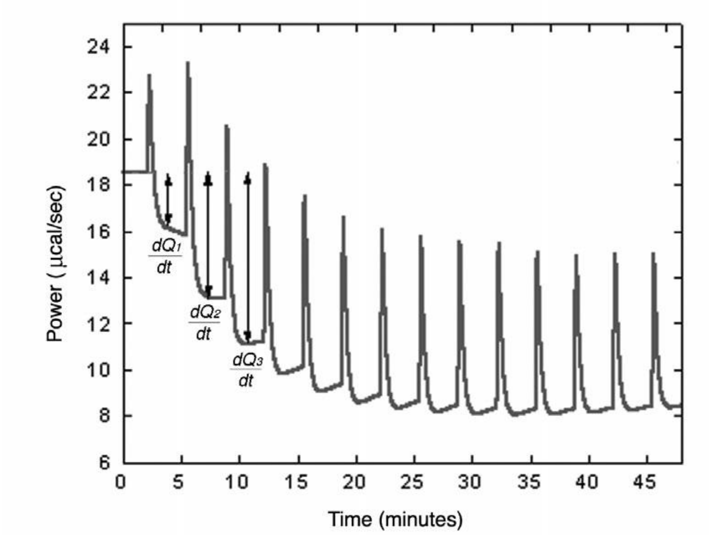


Figure 7 - Example of raw isothermal titration calorimetry data for multiple injections method [132].

As an alternative strategy for obtaining enzyme kinetic parameters, a continuous rate measurement after a single injection of substrate could be done. The concentration in this approach must be higher than K_m. In these experiments, thermal power is monitored as the substrate is completely depleted (Figure 8) [119].

At any given time, the reaction rate can be determined using Equation 2 and the concentration of substrate at any given time can be determined from the integral of the heat evolved. ΔH_{app} is determined by integration of area under peak. Therefore, by plotting the rate and substrate concentration, a continuous kinetic curve is obtained [119,132].

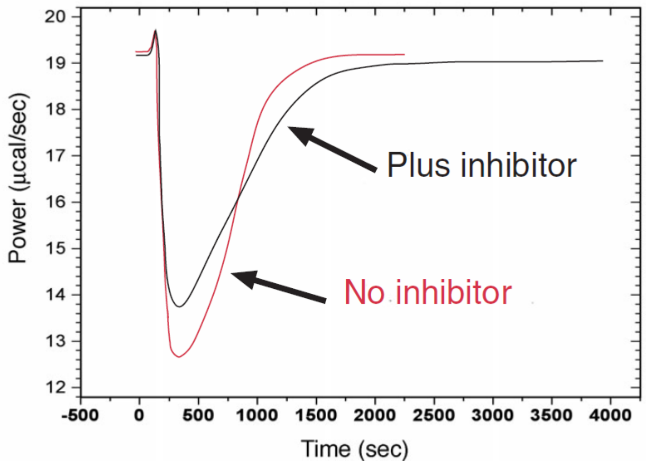


Figure 8 - Example of raw isothermal titration calorimetric data for continuous assay. Presence and absence of inhibitor. The area under each curve was identical [132].

Commercial instruments can operate up to three different methods of calorimetric measurements. The three methods of measurement are, temperature change (either adiabatic or isoperibol), power compensation (regularly called isothermal), and heat conduction [105,133].

In a temperature change instrument, the heat variation occurred during the reaction results in a variation of the calorimeter measuring cell. The raw calorimetric signal is given by temperature of the calorimeter cell as function of the time. With appropriate electrical or chemical calibration, the energy equivalent of the adiabatic (or isoperibol) calorimeter measuring cell can be determined. The measured temperature change is then converted to a heat change. This value for heat change is obtain multiplying the energy of the calorimeter (E_C, in cal/°C) for the measured temperature change (ΔT, in °C) [103].

In a power compensation instrument, the calorimeter measurement cell is maintained at a constant temperature (isothermal). The temperature are controlled by applying constant cooling to the cell. Moreover, a temperature controller and a heater adjust the cooling applied to keep the cell temperature constant. As a chemical reaction occurs, any input from the chemical reaction is sensed and the power applied to the control heater reduced so that again the temperature remains constant. The heating power from the two sources, reaction and controlled heater, are kept at a constant level so that a heat input from the reaction is compensated by a drop in the heat input from the controlled heater. The raw signal in the power compensation calorimeter is the power (µcal/sec or µJ/sec) applied to the control heater that is required to keep the calorimeter cell from changing temperature as a function of time. The heat change is then simply calculated by integrating the heater power over the time (sec) of the measurement (specifically the time required for the control heater power to return to a baseline value) [103,128,129].

In a heat conduction calorimeter, the calorimeter measurement cell is passively maintained at a constant temperature by being coupled with heat flow sensors to a heat sink that is actively controlled at a constant temperature. The raw signal in the heat conduction calorimeter is typically a small voltage that is proportional to the very small ΔT that is temporarily developed across the heat flow sensors as a result of the heat produced by the chemical reaction [103].

Chapter II - Aims

COMT catalyzes the transfer of the methyl group from SAM to one of the methyl groups of a catechol substrate and, consequently, has an important role in the metabolism of dopamine. Thus, this enzyme is directly related with PD and is one of the therapeutic targets for the treatment of this disease. Indeed, there is a great interest in the development and study of COMT inhibitors for administration in patients with PD. Therefore, the main purpose of this work, is the formulation of a cryo/lyoprotectant solution capable of stabilizing COMT during the lyophilization process, and the implementation of a microcalorimetry-based technique for the determination of the kinetic parameters of COMT alone or with an inhibitor bound.

In order to achieve the main objective of this work, intermediate aims were established:

1. hSCOMT biosynthesis and recuperation from *P. pastoris* methanol-induced cultures and purification using IMAC in an active and catalytically active state.
2. Implementation and development of a methodology based in lyophilization to ensure the stability of the target protein through transportation, or simple to increase the time that the protein may be stored without changes in their activity.
3. Implementation and development of an ITC methodology to determine the kinetic parameters of COMT in the absence or presence of commercially available inhibitors.

Chapter III - Material and Methods

3.1. Materials

Ultrapure reagent-grade water for high-performance liquid chromatography (HPLC) was obtained with a Mili-Q system from Milipore/Waters (Billerica, MA, USA). Zeocin was obtained from Invitrogen (Carlsbad, Ca, USA). Glass bleads, SAM, ethylene glycol tetraacetic acid (EGTA), epinephrine, 3,5-DNC, antifoam agent antifoam A, dimethylsulfoxide (DMSO), citric acid monohydrate, sodium octil sulfate (OSA), manose, glucose, cysteine (L-), sucrose and trehalose were obtained from Sigma (St. Louis, MO). Yeast nitrogen base and yeast extract were obtained from Himedia (Mumbai, India). Peptone was obtained from Becton, Dickinson and Company (Sparks, MD). Dipotassium phosphate (K_2HPO_4), perchloric acid and sodium acetate anhydrous (NaH_2PO_4) were obtained from Panreac (Barcelona, Spain). Monopotassium phosphate (KH_2PO_4) and magnesium chloride ($MgCl_2$) were obtained from Chem-Lab (Zedelgem, Belgium). Biotin was obtained from Roche (Basileia, Swiss). Vivaspin concentrators were provided by Vivaproducts, (Littleton, MA, USA). Methanol, glycerol and acetonitrile were obtained from VWR (Carnaxide, Portugal). Pierce BCA Protein Assay Kit and Phenylmethanesulfonyl fluoride (PMSF) were obtained from Thermo Scientific (Rockford, USA). Agar was obtained from Pronadisa (Basel, Switzerland). Imidazole and 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS) were obtained from Fisher Chemicals (Zurich, Switzerland) and from Merk (Darmstadt, Germany), respectively.

All buffer solutions were filtered through a 0.20 µm pore size membrane (Schleicher Schuell, Dassel, Germany) and ultrasonically degassed. All chemicals used were of analytical grade, commercially available and used without further purification.

3.2. Methods

3.2.1. Expression vector and host strain

Easy select expression kit for expression of recombinant proteins using pPICZα in *P. pastoris* X33 cells (Invitrogen, Carlsbad, CA, USA) was used for the expression of human hexa-histidine tagged soluble COMT ($SCOMT\_His_6$) in its native form and the process was carried out according to manufacturer's instructions.

3.2.2. *Pichia pastoris* pre-cultivation, batch and fed-batch conditions

These experiments were carried out according to Pedro *et al.* (2015) [134]. Specifically, *P. pastoris* cells transformed with the gene of interest were subcultured from a cell bank and grown for 72 hours at 30ºC in YPD medium (1% yeast extract, 2% peptone, 2% glucose and 2% agar) plates containing 200 µg/mL Zeocin. From the plate a single colony was pre-cultivated in 100 mL of BMGY (1.34% YNB, $4x10^{-5}$% biotin, 1% glycerol in potassium phosphate buffer [200mM] at pH 6) in 500 mL shake-flasks. Cells were grown over-night at 30ºC and 250 rpm to a cell density at 600 nm (OD_{600}) between 5 and 6.

Batch and fed-batch processes were carried out in 750 mL bench-top parallel mini-bioreactors (Infors HT, Switzerland) with 250 mL of the BMGY medium. The bioreactors were inoculated from the pre-cultivation to obtain a starting OD_{600} of approximately 1 in BMGY medium containing 4.35 mL/L SMT[134]. The batch phase was carry out for approximately 18-20 hours. After that the fed-batch was divided in two phases. In the first, a constant feed of glycerol at 50% were maintained for 3 hours. After 2 hours of the end of batch phase, the second feed of methanol at 25% begins and initiate the induction phase were the protein of interest is produced. The bioreactors were operated with strictly controlled parameters including pH, temperature, airflow, agitation and dissolved oxygen. The temperature and pH were kept constant throughout the batch and first step of fed-batch phases at 28°C and 4.7 (previously optimized by our research group [134]) respectively, with the pH value controlled by the automatic addition of 0.75 M sulfuric acid and 12.5% (v/v) ammonium hydroxide. The pO2 (dissolved oxygen percentage) was controlled by a two-level cascade of stirring (between 500 and 950 rpm) and air flow (between 0.2 and 2). At the induction point, the temperature was raised to 30°C and DMSO 6% (v/v) was added. Foaming was controlled manually by the addition of the antifoam agent antifoam A. The induction phase was maintained during additional 13 h using methanol as sole carbon and energy source. Afterwards cells were harvested by centrifugation (1500xg, 10 min, 4°C) and storage at -20°C until further use.

The whole system was controlled by IRIS software (Infors HT, Switzerland) and, in particular, the addition of feed medium was achieved using peristaltic pumps that were automatically controlled through a feeding profile previously programmed.

3.2.3. Cell lysis

The pellet was ressuspended in 2 mL per gram of cells, of an appropriate Buffer (500 mM NaCl, 50 mM TRIS, 1 mM $MgCl_2$, pH 7.8), supplemented with protease inhibitor (10 µL 100mM PMSF /mL buffer), disrupted by mechanical treatment with glass beads (seven cycles of 1 minute with 1 minute of interval on ice). The resultant supernatant, after centrifugation (500xG, 5min, 4ºC), was discarded and the pellet was ressuspended for a second time in 5 mL per gram of cells in the same buffer for forthcoming assays. These procedures were performed according to what was described by Pedro *et al.* (2015) [134].

3.2.4. Quantification of total protein using BCA assay

The protein quantitation assay was carried out using a Pierce BCA Protein Assay kit (Thermo Scientific, USA) on a 96 well plate, according to the manufacturer's instructions.

For the calibration curves, several solutions of different BSA concentrations (25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/mL) were prepared in triplicates using a standard buffer (10mM TRIS-HCl, pH 7.8). BSA solutions and standard buffer (working as blank) were subjected to the quantitation assay as mentioned above, and their absorbance measured at 562 nm. The results of the three replicates for each BSA concentration and the blank were averaged and the calibration curve were plotted, according to figure 1.

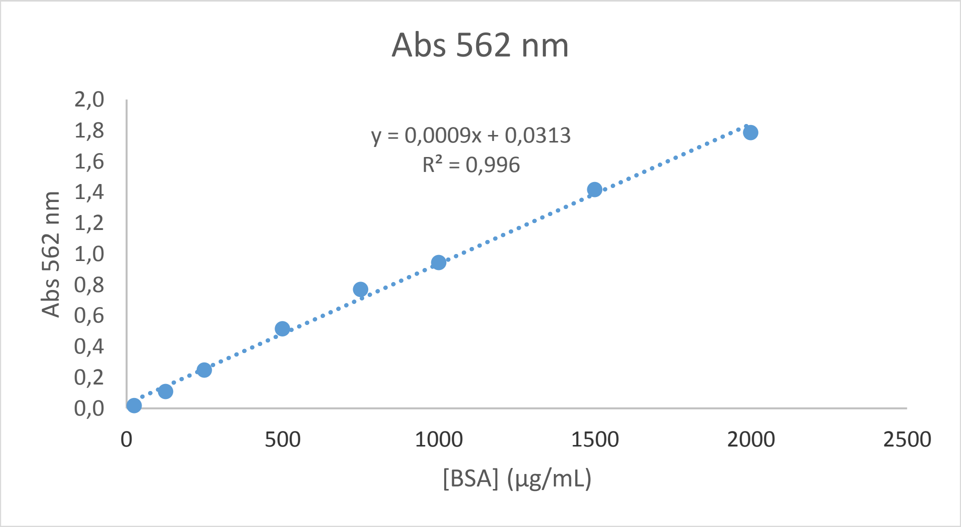


Figure 9 - Calibration curve for BSA solutions absorbance at 562 nm.

3.2.5. Immobilized-metal affinity chromatography

The chromatographic assays were performed at room temperature (25ºC) in an ÄKTA Avant system with UNICORN 6 Software (GE Healthcare, Uppsala, Sweden) equipped with a 10 mL injection loop. All buffers pumped into the system were prepared with Mili-Q system water, filtered through a 0.20 µm pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically.

A HisTrap FF crude (5mL) ready-to-use column, prepacked with precharged Ni Sepharose 6 Fast Flow was used. This prepacked column is intended for purification of histidine-tagged recombinant proteins by IMAC.

The column was initially equilibrated with binding buffer (500 mM NaCl, 50 mM TRIS, 1 mM $MgCl_2$, 5 mM Imidazole pH 7.8). Then, 10mL aliquots of recombinant hSCOMT ressuspended pellet were injected into the column at 0.5 mL in the binding step. Elution was performed at 1 ml/min using different imidazole stepwise gradients (50, 70, 300, and 500 mM. In all chromatographic runs, conductivity was constantly monitored, as well as absorbance at 280 nm. The target protein eluted with 300mM imidazole step and 0.5 mL fractions were collected with 0.5 mL of a suitable stabilizer solution composed by 100 mM trehalose, 150 mM of cysteine, and 10% of glycerol at 4°C. Finally, these samples were concentrated and desalted using Vivaspin 10.000 MWCO (molecular weight cutoff) concentrators and conserved at 4ºC until further analysis.

3.2.6. Enzymatic activity assay

The experiments of enzyme activity were designed to evaluate the methylation efficiency of recombinant hSCOMT, through the measure of the amount of metanephrine, and using epinephrine as substrate, as previously described [135].

The evaluation of enzyme activity was carried out in refrigerated, foil covered test tubes. These experiments were performed through the addition of 500 µL of the sample and 400 µL of incubation solution [0.272 mg/mL SAM, 1 µL/ML MgCl2 (0.2 M), 40 µL/mL EGTA (50 mM) and 959 µL/mL phosphate buffer (10mM pH 7.8)]. This procedure was followed by an incubation of 5 min in a water bath at 37°C and 83 rpm. After this period, 100 µL of epinephrine solution (1mM), dissolved in incubation solution, were added. Then, the tubes were transferred to ice and 200 µL of perchloric acid (2 M) were added in order to stop the enzymatic reaction.

After this step, the tubes were kept at 4°C for 1 hour, and then the resulting samples were transferred to eppendorfs and centrifuged for 10 min at 4°C and 6000 rpm. Finally, the samples were filtered through a 0.22 µm pore size filter to remove precipitated biomaterial and injected to a HPLC system. These samples were analyzed in two different HPLC systems (described in the section High-performance liquid chromatography analysis). Indeed, the activity of hSCOMT was calculated from the peak area or peak height, depending on the HPLC system used, using a calibration curve. The units of total activity provide the metanephrine produced in nmol/h while the units of specific activity provide the metanephrine produced in nmol/h/mg protein. The amount of protein produced was previously determined by using the micro-assay BCA (described in the section *3.6. Quantification of total protein using BCA assay*).

3.2.7. High-performance liquid chromatography analysis

For the analysis of the samples obtained were used two different HPLC systems. One was a HPLC model Agilent 1260 system (Agilent, Santa Clara, CA, USA) equipped with an autosampler and a quaternary pump coupled to an ESA Coulochem III (Milford, MA, USA) coulometric detector [136]. An analytical column Zorbax 300SB C18 reverse phase analytical column (250 x 4.6 mm i.d. 5 µm; Agilent, Santa Clara, CA, USA) allowed the chromatographic separation of the compounds. The mobile phase used in this system, 0.1 M sodium dihydrogen phosphate, 0.024M citric acid monohydrate, 0.5 mM OSA and 9% acetonitrile, v/v pH 2.9, was filtered under vacuum (0.2 µm hydrophilic polypropylene filter; Schleicher & Schuell, Dassel, Germany), and degassed in ultrasonic bath before use. An electrochemical detector with a coulometric mode, which was equipped with a 5011 high sensitivity dual electrode analytical cell (electrodes I and II) and using a procedure of oxidation/reduction (analytical cell #1: +410 mV; analytical cell #2: -350 mV), permitted the monitoring of the column effluent. The column temperature was optimized to 30ºC and applied a flow rate of 1 mL/min. The chromatograms were obtained by monitoring the reduction signal of the working electrode II. The sensitivity of the method was 1 µA.

The other system was a Waters HPLC which includes a quaternary pump with controller (600), a manual injector (Rheodyne 7725), an in-line degasser (AF) installed between the eluent reservoir and the draw-off valve inlet to the pump and an electrochemical amperometric detector (ECD-2465) [137]. The instrumental parts were controlled automatically by Empower software, supplied from Waters Corporation (Milford, MA, USA), and the Faraday box were grounded in order to minimize noise. Chromatographic separation was achieved using a 5 µm particle size XTerra MS C18 ODS reversed-phase analytical column (250 x 4.6 mm i.d.) connected to a precolumn (5 µm, 10 x 4.6 mm i.d.), both from Waters. In this case the mobile phase was constituted by 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), which was also filtered and degassed ultrasonically. The mobile phase was pumped isocratically at 1.0 mL/min as previously described [138]. A rheodyne valve, equipped with a 50 µL sample loop was used to perform all the injections into the system. The electrochemical oxidation of metanephrine to their 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. All the system, this is, the eluent, the analytical column and the flow single-cell, were maintained at a constant temperature using a preheated module installed in the electrochemical amperometric detector. In this case, the method sensitivity was set at 100 nA.

3.2.8. Human soluble catechol-*O*-methyltransferase lyophilization experiments

In this study, SCOMT was submitted to a lyophilization process. Initially, the purified samples were concentrated and the buffer exchanged to TRIS-HCl 10 mM, pH 7.8. Thereafter, the sample was aliquoted and a stabilizing solution (trehalose [200mM] and cysteine [300mM]) as a cryoprotectant solution (mannose 20% (w/v) trehalose 4% (w/v) sucrose 6% (w/v)) are added.

Prior to freeze-dry the samples, these must be frozen. So, two different methods was applied. In the first, samples where frozen at -80°C over-night. The second approach was frost the samples in liquid nitrogen for about 2 minutes.

Once frozen, the samples were placed in the lyophilizer at -110°C and 0.013 hPa during different periods (24, 48, 72 and 96h). Finally, the samples were ressuspended in an appropriate buffer (TRIS-HCl pH 7.8) and SCOMT enzymatic activity evaluated.

3.2.9. Microcalorimetry assay

The calorimetry experiments was carried out on a Microscal FMC 4 Vi (Microscal Limited, London, UK) with a 170μL cell operated in the titration mode. In order to mimic the physiological temperature, it was set at 37 ºC and the experiments performed within a range between 37°C and 38°C.

The cell was filled with 100μL of the protein dissolved in the incubation solution. The incubation solution comprises SAM, EGTA and $MgCl_2$ dissolved in phosphate buffer at pH 7.8. For the blanks, the same solution, in the absence of the protein, was used. In the titration syringe, epinephrine was also dissolved in the incubation solution. Taking into account that the compounds dissociation constants were in the micromolar range, different concentrations of 3,5-DNC were used. Also, different hSCOMT concentrations in the cell were evaluated in demand of the best results. Finally, both approaches of injection (Multiple and Single Injections) were tested. Specifically, in single injection method, after the calibration step, 25 μL of the substrate were injected in the cell, and the variation of energy was measured for 35 minutes. On the other hand, for the multiple injection approach, 10 to 14 injections (of 5 or 3 μL of substrate solution respectively) were made in the cell with intervals of 180 seconds with constant measurement of the energy variations. CALDOS 4 software (Microscal Limited, London, UK) was used to acquire, store, and process all the ITC data.

Chapter IV - Results and Discussion

In recent years, several attempts have been performed to obtain a large quantity of enzymatically active and pure hSCOMT. *P. pastoris* has been successfully used in the production of various proteins[73,74,139] .In particular, this expression system is cultivated at high cell densities in cheap media of easy preparation, thus enabling the high-level expression of proteins [139]. According to some authors, COMT is an extremely unstable enzyme that can lose up to 50-70% of its biological activity in less than 24 hours at 4 °C, and as for other proteins is affected by physicochemical parameters such as pH and temperature changes, or ionic strength of the solution. Therefore, in order to maintain this target protein in an active, native, correctly-folded state, a suitable preservation methodology based on the use of lyophilization was implemented and developed.

On the other hand, COMT is an important enzyme responsible for the inactivation of catechol substrates, making them a therapeutic target for some neurodegenerative disease like Parkinson's [47,54]. In particular, understanding the molecular basis of hSCOMT/inhibitors interactions is crucial to design novel drug technologies. Therefore, in this thesis, microcalorimetric experiments were performed and the thermodynamic information acquired used to support the characterization of the interactions between COMT-inhibitors.

4.1. Recombinant human soluble catechol-*O*-methyltransferase biosynthesis by *Pichia pastoris* X33 cells

Recombinant hSCOMT biosynthesis by *P. pastoris* X33 methanol-induced cultures was carried out in a batch and fed-batch regime in 750 mL bench-top parallel mini-bioreactors (Infors HT, Switzerland) containing Basal Salts Medium (BSM). Initially, cells were grown in glycerol (30 g/L) in a batch mode to ensure accumulation of biomass prior the induction phase that ended when the glycerol was depleted (approximately 20 h), indicated by a sharp increase in the dissolved oxygen. Then, a fed-batch stage was carried out during 3 hours with glycerol 50 % (v/v) at 18.54 mL/L/H followed by induction with methanol 50 % (v/v) during 12 h. In order to promote the depression of the alcohol oxidase promoter prior to induction, 1 h before starting the induction phase, methanol was added to the reaction vessel at the flow-rate later employed in the methanol fed-batch phase. Previously (Pedro AQ et al., 2015, Unpublished Results), subcellular fractionation experiments demonstrated that enzymatically active hSCOMT preferentially accumulates in the ressuspended pellet in comparison with the extracellular medium and the cell lysis supernatant. Therefore, after the application of an appropriate procedure for *P. pastoris* lysis including glass beads (described in the subsection 3.5), catalytically active hSCOMT was successfully obtained in the ressuspended pellet.

4.2. Epinephrine methylation by *Pichia pastoris* X33 endogenous proteins

In order to confirm that do not occur side reactions of methylation to the epinephrine and that the metanephrine obtained in the COMT enzymatic assays is not derived from *P. pastoris* endogenous proteins, some studies with native or recombinant P. pastoris X33 cells were made. The strains used were *P. pastoris* X33 with and without the pPICZα plasmid. The pre-fermentation was made with glycerol as the carbon source that suppress the expression promotor. In the fermentation step, was used methanol which acts as an inducer of the expression in order to evaluate if there is production of proteins capable of epinephrine methylation. In the strains with the vector, the induction has not been applied maintaining the carbon source, with the objective of study if there is a basal production in its absence. Therefore, the pre-fermentation and fermentation were conducted in 500 mL shake-flasks containing 100mL of BMGY medium or BMGH with glycerol or methanol as the main carbon sources, respectively. The pre-fermentations were grown over-night at 30°C and 250 rpm, an aliquot removed for determining COMT enzymatic activity and the fermentation was initiated in fresh BMGY using methanol as carbon source for the cells without the plasmid. Later, the cells from the fermentation were harvested, lysed and subjected to activity assay. Finally, the samples were analyzed by HPLC like described in section 3. The typical chromatograms obtained in this study are shown in Figure 2, respectively, for the metanephrine pattern (2A) and one representative sample (2B) while the obtained values for each sample (metanephrine concentration, COMT total and specific activity) are reported in Table X. Indeed, mainly for the specific activity, the measured quantity of metanephrine is almost zero, independently of the presence of the plasmid for the expression of SCOMT. Therefore, no significant side reactions promote the methylation of epinephrine to metanephrine. In addition, can also be observed that the cells in the fermentation step show a slightly increase in the quantity of metanephrine produced although in residual values. The “mask effect” of the results can thus be neglected. Therefore, the results are according to what was expected that would be the nonexistence of a characteristic signal for the metanephrine in the chromatogram and, thus no *P. pastoris* X33 endogenous proteins are able to methylate epinephrine.

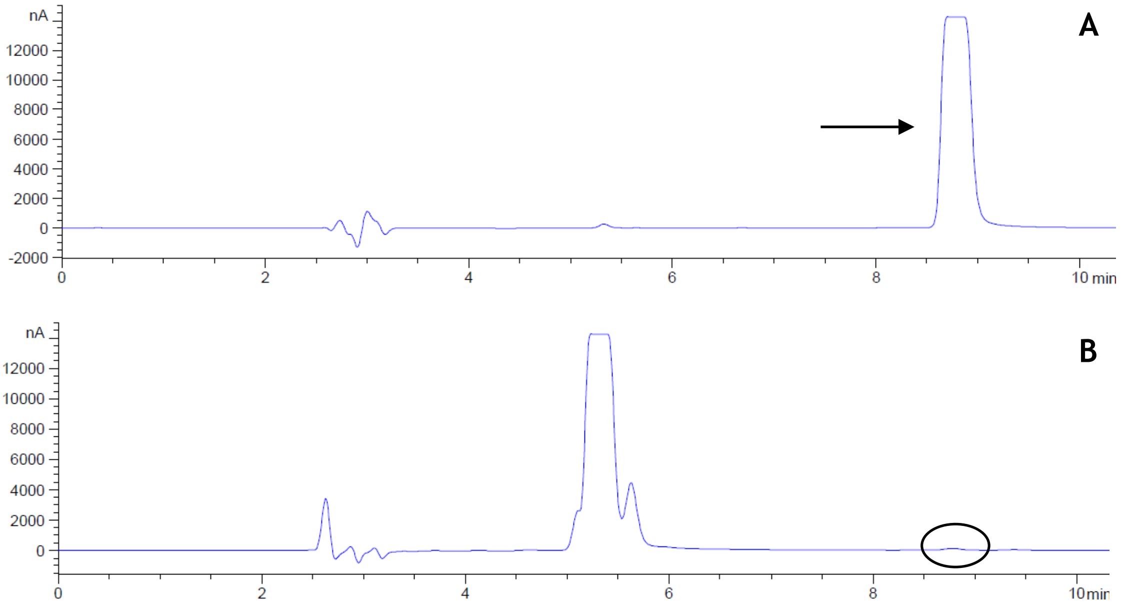


Figure 10 - Agilent high-performance liquid chromatography system chromatogram corresponding to a pattern of metanephrine. Metanephrine has an elution time of approximately 9 min (black arrow) (A) and Agilent high-performance liquid chromatography system chromatogram of one run (black ellipse shows the vestigial peak) (B).

Table 1 - Results of enzymatic activity for each condition tested for the endogenous proteins methylation.

		Protein (mg)	*[Metanephrine] (nmol/mL)*	*Total activity (nmol/h)*	*Specific activity (nmol/h/mg)*
Pre-fermentation (non-inducted)	without plasmid	6.362	0.173	1.041	0.164
	with plasmid	4.099	0.157	0.944	0.230
Fermentation (inducted)	without plasmid	3.494	0.292	1.751	0.501
	with plasmid	4.375	0.369	2.211	0.505

4.3. Recombinant human soluble catechol-*O*-methyltransferase purification using Immobilized-Metal Affinity Chromatography

Preparative chromatographic experiments were performed in an IMAC matrix using as binding buffer: (500 mM NaCl, 50 mM TRIS, 1 mM $MgCl_2$, 5 mM Imidazole pH 7.8). Elution was performed with binding buffer supplemented with 500 mM imidazol since it is described that competes with the proteins for the binding sites of the column and by that way, protein is eluted in a specific imidazole concentration [140]. In addition, elution was performed with several stepwise gradients and it was observed that hSCOMT was eluted at 300 mM imidazole in a highly purified and biologically active form, according to Figure 11. Fractions were collection in a stabilization solution (Final concentrations: trehalose [50mM], cysteine [75mM], glycerol, [5% (v/v)]) is added to maintain the protein in a stable and active conformation until further used.

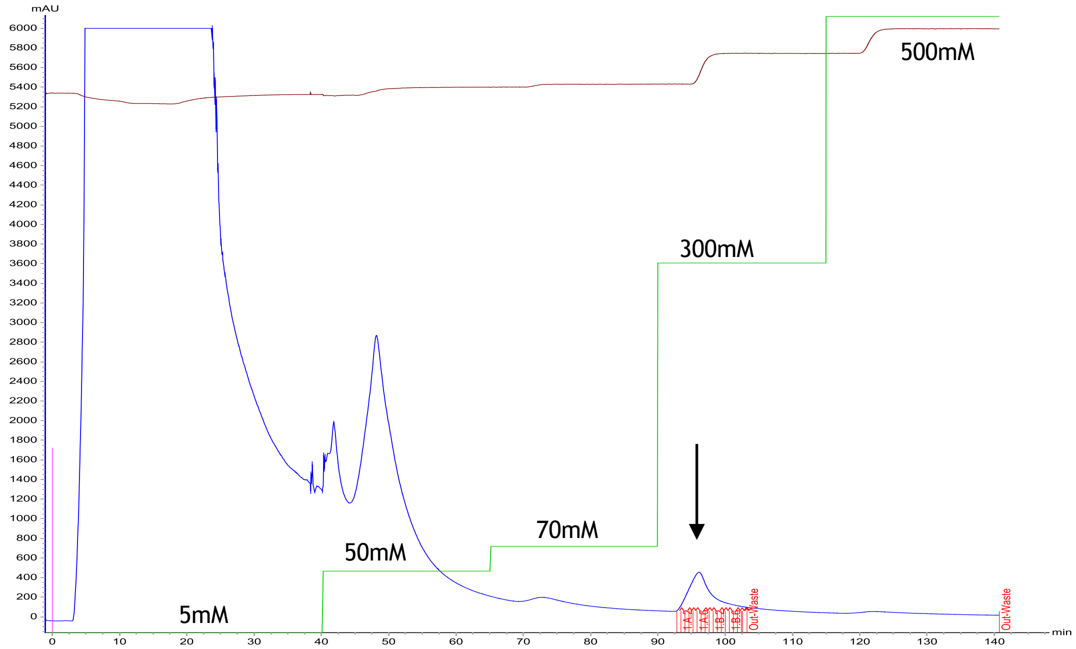


Figure 11 - Typical chromatographic profile of human soluble catechol-O-methyltransferase purification onto HisTrap FF crude, an immobilized metal ion affinity chromatography matrix after injection of 10 mL sample of ressuspended pellet obtained after Pichia pastoris lysis (concentrations showed refered to imidazol).

The main objective of applying the lyophilization process to a protein, is to promote the enzyme storage in a correct, stable and active folded form for a longer period than that obtain in a aqueous solution [89]. Indeed, it is usually applied to increase the preservation time, to be more suitable for transportation, to make the protein more robust to temperature variations or minimize the loss of activity between different, long experiments in order to decrease the variability between different assays [141].

In an initial approach, after the application of an IMAC purification step, the collected fraction was desalted and concentrated in Vivaspin 10.000 MWCO. After that, the samples were aliquoted, cryoprotective and/or stabilizing solutions added and they were frozen at -80°C where were kept for approximately 16 hours. Then, the samples were lyophilized and collected in several different hours (24h, 48h, 72h and 96h) with the objective to visually analyze them. All the samples look alike, not being noticeable alterations between different times. Thus, regarding this fact, the time of 24 hours was chosen in order to minimize the stress that the protein will be submitted.

For the next trials, the experimental conditions including the sample preparation and frozen at - 80ºC were kept constant Hereafter, half the aliquots were lyophilized for 24 hours and the other half unfrozen and kept for the same time at 4°C. This way it is possible estimating the enzymatic activity losses during the lyophilization process. For the aliquots maintained at 4°C, activity assay were made at two different times (0 hours and 24 hours). The lyophilized aliquots were then ressuspended in TRIS buffer. All the samples were submitted to enzymatic assay and the product injected in the Waters system HPLC.

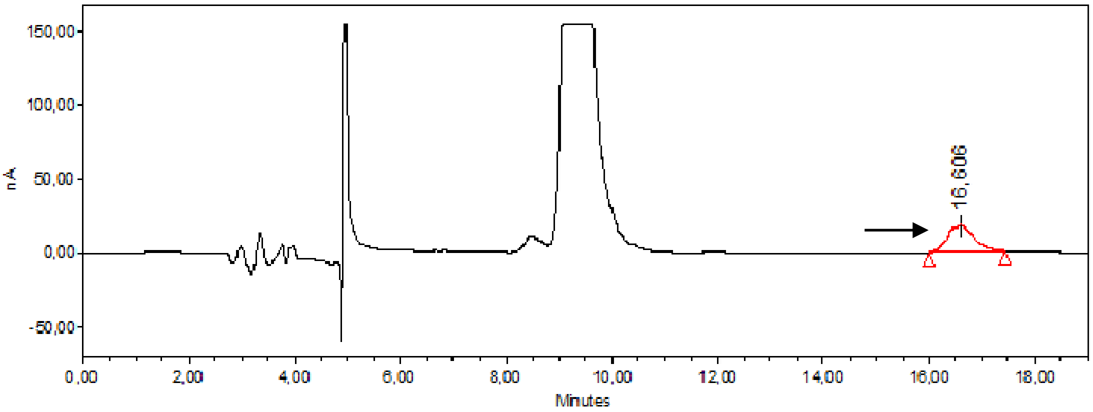


Figure 12 - Waters high-performance liquid chromatography system chromatogram corresponding to a pattern of metanephrine. Metanephrine has an elution time of approximately 16,6 min (black arrow).

The peak correspondent to metanephrine was manually integrated (Figure 15), and the height value used to calculate the total and specific SCOMT activity. The same procedure was applied to all samples, whose results are depicted in Table 2.

Table 2 - Results of enzymatic activity for the lyophilized assay.

Sample	*[Metanephrine] (nmol/ml)*	*Total activity (nmol/ml/h)*	*Bioactivity recuperation (%)*
Lysate	5.46	262.18	
Frozen 15h	93.26	1119.07	100*
Lyophilized for 24h	4.79	57.51	5.14
Frozen and kept at 4°C for 24h	7.76	93.15	8.32

**The values of bioactivity recuperation in the "lyophilized for 24h"and "frozen and kept at 4°C for 24h" samples were calculated assuming that the value of total activity of frozen 15h was 100%.*

Unfortunately, in the injection of the concentrated samples, the metanephrine concentration was too high leading to saturation of the cell (Figure 15). Therefore, the result obtained for those samples couldn't be taken into account, giving just an idea of the impact that has freezing the sample. For that, and assuming the **frozen** sample as the total of activity for the following steps, we can see that the loss of total activity promoted by the lyophilization process was significant with a bioactivity recuperation of only 5%. On the other hand, the aliquot that was kept at 4ºC for the period that the lyophilization was carried out (frozen and kept at 4°C for 24h), the in hSCOMT biological activity recovery is also low but is slightly higher - 8%. Indeed, this led us to conclude that the cryoprotectant solution need to be improved once it wasn't able to totally protect and stabilize the protein through the adverse conditions inflicted during the lyophilization process.

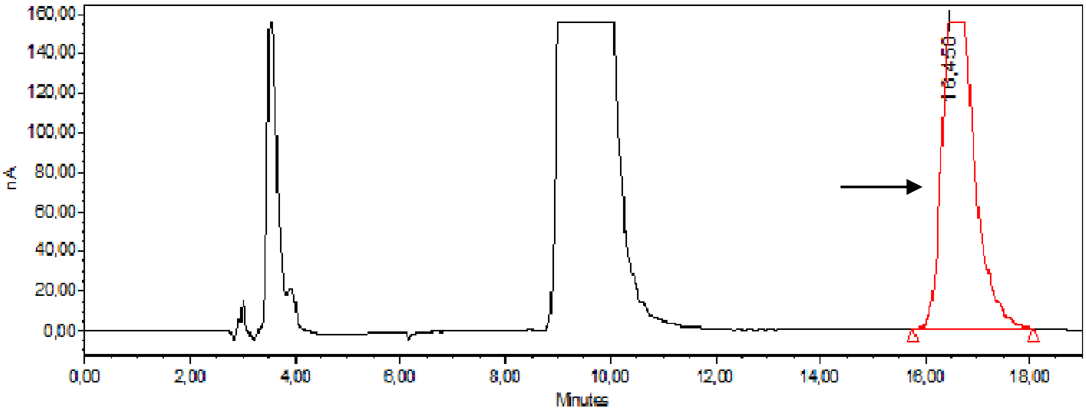


Figure 13 - Waters high-performance liquid chromatography system chromatogram of one run with the cell saturated (black arrow).

A parallel experiment was carried out to investigate the influence of imidazol in solution, as well as the addition of stabilizing solution to the samples. All the samples were concentrated, and those in which the imidazole was removed, the buffer was substituted for TRIS buffer. To all of them, cryoprotective solution was added prior to freezing at -80°C as we can see in table 3.

Table 3 - Design of experiments to study the influence of imidazol and stabilizers in the solution.

Aliquot	*Imidazol*	*Stabilizing solution*	*Cryoprotective solution*	*State*
1	Yes	No	Yes	Lyophilized 76h
2	Yes	No	Yes	Frozen -80°C
3	Yes	Yes	Yes	Lyophilized 76h
4	Yes	Yes	Yes	Frozen -80°C
5	Removed	No	Yes	Lyophilized 76h
6	Removed	No	Yes	Frozen -80°C
7	Removed	Yes	Yes	Lyophilized 76h
8	Removed	Yes	Yes	Frozen -80°C

To all the aliquots of 500 µL was added 100 µL of cryoprotective solution, and for the aliquots with stabilizers, 100 µL of stabilizing solution was also added (final volumes of 600 µL and 700 µL respectively). The aliquots 1, 3, 5 and 7 were then lyophilized and kept at room temperature for 3 weeks. Unfortunately, all of them, do not show any trace of activity when it was evaluated the enzyme biological activity. In future experiments may be important reduce the store period of the samples, or reduce the temperature (for example 4°C) that they are maintained prior to enzymatic assay. Finally, in the conditions here described, hSCOMT was recovered after the lyophilization process with 5.14 %, lower than that obtained when an identical sample was kept at 4ºC during the period of the lyophilization. Indeed, although this value is very low, this is the first report concerning hSCOMT lyophilization and subsequently solubilization with biological activity.

4.5. Microcalorimetry tests

4.5.1. Multi Injection

Microcalorimetric measures, in real time, the heat of chemical reactions at a constant temperature, even in complex solutions. This capacity make microcalorimetry a major tool in the analyses of biological reactions both for the determination of kinetic parameters as well as to study interactions of proteins with drugs. In this study, both these aspects were covered. In an initial approach, the multi injection method was tested following some guidelines described in the literature [130,132], and taking into account the COMT enzymatic assay pre-established [142]. The concentrations used of EGTA, $MgCl_2$ and phosphate buffer (respectively EGTA (50 mM), 0.2 M, and 5mM pH 7.8)) were maintained the same in all the microcalorimetric tests, in accordance to what previously optimized for the COMT enzymatic assay. The $MgCl_2$ give the metallic cofactor of the reaction, the phosphate buffer maintained the value constant of pH, and the EGTA enhance the activity of COMT. On the other hand, different concentrations of the target IMAC-purified enzyme, SAM (cofactor) and epinephrine (substrate) were tested in the pursuit of the best combination. In order to maintain a pseudo-first-order enzyme kinetics, there are some experimental conditions that we must respect, especially the following: after several injections, the substrate concentration must be lower than the K_m value and should not be converted more than 5% of substrate before the next injection. As the volume of the reaction cell is limited, the number, as well as the volume of each injection must be optimized. In this case, the cell only have 170 µL of working volume so were limited to these final volume. The time between injections must also be adjusted to obtain better results, but normally, 3 minutes are suggested between injections [130]. A summary of all conditions tested in the microcalorimetry multi-injection approach is depicted in the table 3.

Table 3 - Combinations of conditions tested in the multi injection approach.

Experiment	*[SAM] (mM)*	*Epinephrine ((mM)*	*Protein (concentration) (µM)*	*Number of injections*	*Volume of injections*
1	1	1	5	10	5
2	1	1	5	10	5
3	1	1	5	10	5
4	1	1	5	10	5
5	1.25	1	5	10	5
6	1.25	10	5	10	5
7	1.25	10	50	14	4
8	10	10	50	14	4

Unfortunately, the results were not as expected. Indeed, even when higher concentrations of SAM, epinephrine and enzyme were applied (please see Figure 17), the heat signal was always too low and no mensurable changes between peaks was noted. Therefore, the regularity of the peaks may be due to the heat of dilution of the solution at the injection point, and this may mask the signal of the reaction (Figure 17).

Another disadvantage of this method is the necessity to perform a single injection experiment in order to calculate the ΔH_{app} once, with the multi injection approach only maximum velocity (V_{max}) and K_m is possible to calculate. On the other hand, by adopting the single injection method only one experience is needed to determinate the V_{max}, K_m and ΔH_{app}. Also, the equipment used, Microscal FMC 4 Vi, offers better results when working in a single injection method. For all those reasons, the continuous assay was adopted as alternative.

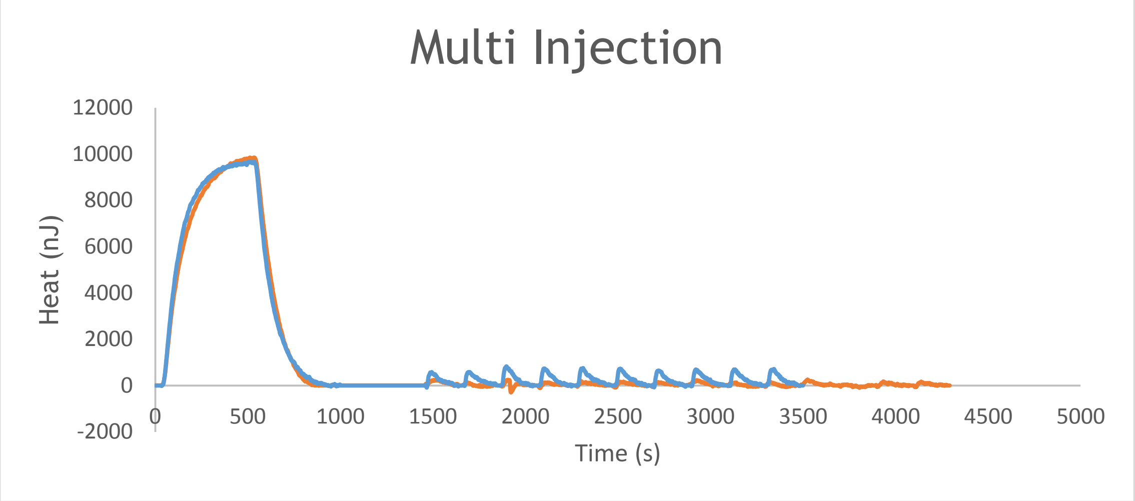


Figure 14 - Isothermal titration calorimetry data for measurement of reaction rate for methylation of epinephrine. 14 injections of 4 μL of epinephrine each for orange line (experiment 8) and 10 injections of 5 μL of epinephrine each for blue line (experiment 1). The interval between the injections is 3 minutes in both experiments.

4.5.2. Single Injection

The alternative strategy for obtaining enzyme kinetics parameters through ITC is the single injection method that involves continuous rate measurements after a single injection of substrate. When substrate is completely depleted, thermal power returns to pre-injection baseline and the ΔH_{app} is determined by integration of the area under peak. At any given time the concentration of substrate is calculated and rate vs. substrate concentration is plotted to give a continuous kinetic curve.

To determinate ΔH_{app}, the integrate area under peak was divided by the amount of substrate depleted.

For calculate the enzyme rate at a given time (R_t) the equation X is used:

$$R_t = \frac{1}{V_0 \times \Delta H_{app}} \times \frac{dQ}{dt}$$

And the instantaneous substrate concentration are determined by:

$$[S]_t = [S]_{t=0} - \frac{\int_0^t (Power)dt}{V_0 \times \Delta H_{app}}$$

With these data, Michaelis-Menten and Lineweaver-Burk graphs are drawn and the kinetic parameters K_m and V_{max} values calculated.

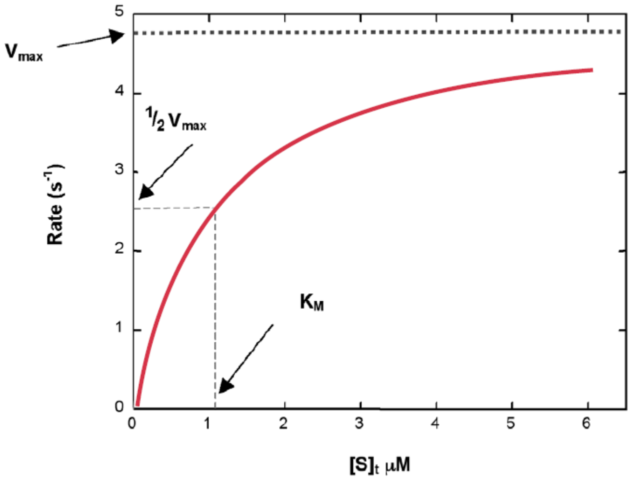


*Figure 15 - Example of a Michaelis-Menten curve (*adapted from [132]).

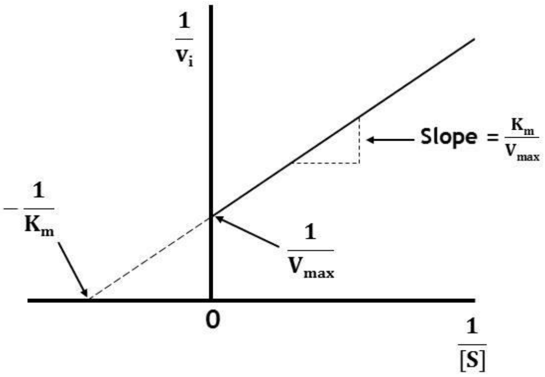


Figure 16 - Example of a Lineweaver-Burk plot.

In the first experiments, we tried to establish the best conditions in order to maximize the obtained signal in the system. In this approach, while the concentrations of EGTA, $MgCl_2$ and phosphate buffer were kept the same (like described above) and the concentrations of SAM, COMT and epinephrine varied.

Since the methylation reaction of epinephrine occurs in a proportion of 1:1 for de substrate and the cofactor, both SAM as epinephrine concentrations were maintained approximately the same in the reaction chamber. Indeed, different ranges of concentrations were tested, namely 40 μM to 2 mM, 300 μM and 3 mM and 12.5 nM and 1250 nM, respectively, for SAM, epinephrine and hSCOMT. Another major optimization was carried out concerning the inclusion of stabilizers in the protein solution. As a matter of fact, since the protein is unstable, especially at higher temperatures (as in the case of this test occurring at 37°C), it loses its biological activity thereby, leading to the misinterpretation of the results. Therefore, in order to prevent these losses and keep the protein stable in an active state during this assay, a stabilizer solution was added to the reaction chamber once this trial is somewhat delayed (about 2 hours).

As we can see in the figure 20, the optimization of the process was achieved resulting in a greater signal of the system being easier in the future the comparison between a normal enzymatic assay and one were the inhibitor is added to the reaction. The best results were obtained in the following experimental conditions: SAM and epinephrine at 2 mM to, and 1250 nM of protein with the addition of the stabilizing solution previous described.

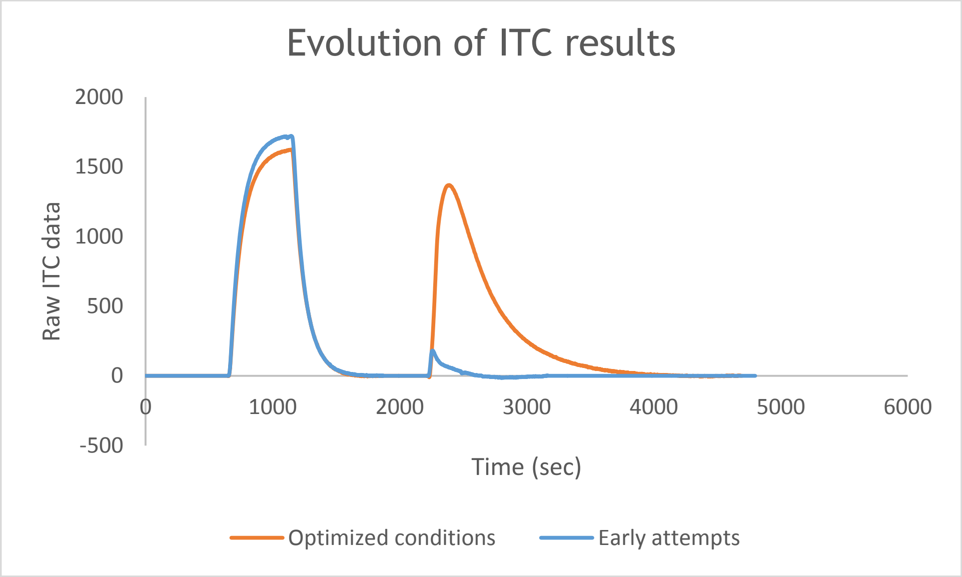


Figure 17 - Raw isothermal titration calorimetry data comparing the initial results and the results after the optimization of the reactional conditions (the first peak is due to the system calibration).

4.5.2.1. Thermodynamic characterization of catechol-*O*-methyltransferase using the single injection method

After the optimization of the signal obtained, the next step was the thermodynamic characterization of the COMT enzyme by ITC and the comparison the results given by others techniques described in the literature and reached by our research group [77].

With the values of rate and substrate concentration the Michaelis-Menten and Lineweaver-Burk plots are generated (Figure 21 and Figure 22).

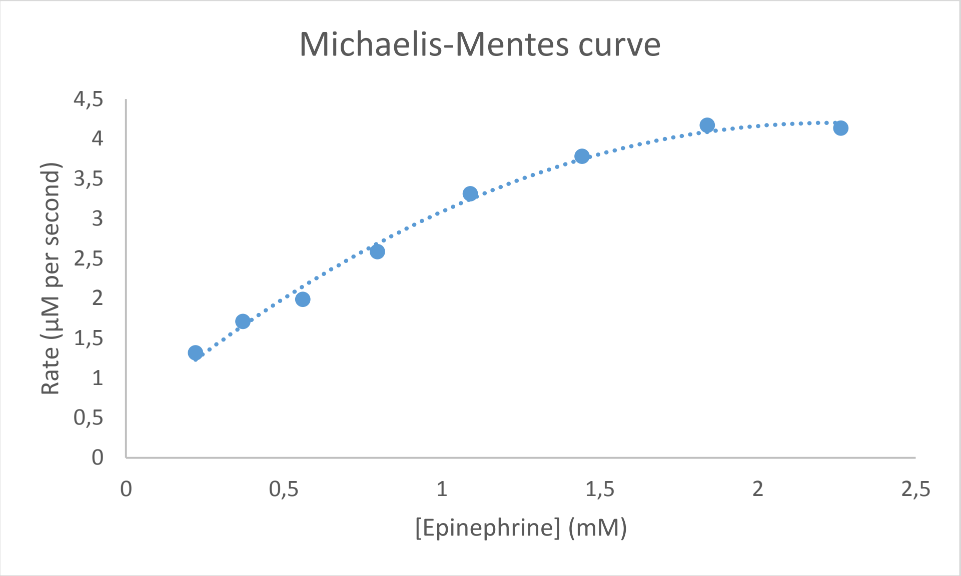


Figure 18 - Example of an experimental Michaelis-Menten curve.

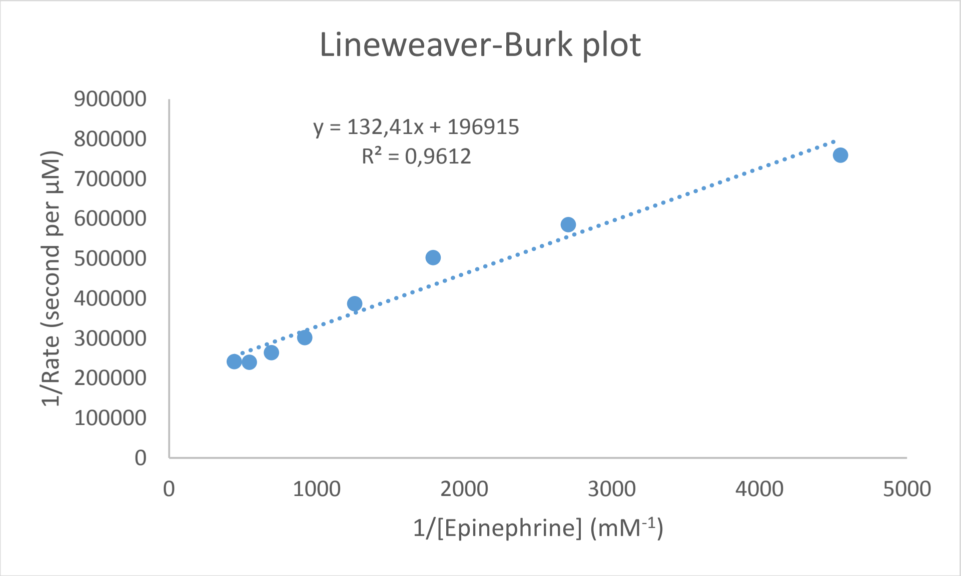


Figure 19 - Example of an experimental Lineweaver-Burk plot.

Then the values of V_{max} and K_m are determinate with the equation from the Lineweaver-Burk plot. From the ITC experiments the calculate K_m for epinephrine was 486.26 [444.97;510.77] µM. This value is not very close from that described in the literature, however this variance from different techniques is also mentioned in literature [119]. The V_{max} value achieved is 4.19 ± 1.75µM/s.

4.5.2.2. Interaction of catechol-O-methyltransferase with the inhibitor 3,5-dinitrocatechol

COMT is one of the most important enzymes in dopamine breakdown. As a therapeutic target, their inhibition could be a treatment for several neurodegenerative diseases, particularly in Alzheimer's and PD. Hence, the thermodynamic characterization between the COMT and the inhibitor is relevant. In this study was tested several concentrations of the second generation inhibitor 3,5-DNC.

First off all, were tested the highest concentration of the proposed range [0.316nM to 3162.3 nM] so as to promote the complete inhibition of protein. As we can see in figure 23, these total inhibition was achieved, being the curves obtain in the test with the protein almost overlapping with the blank run.

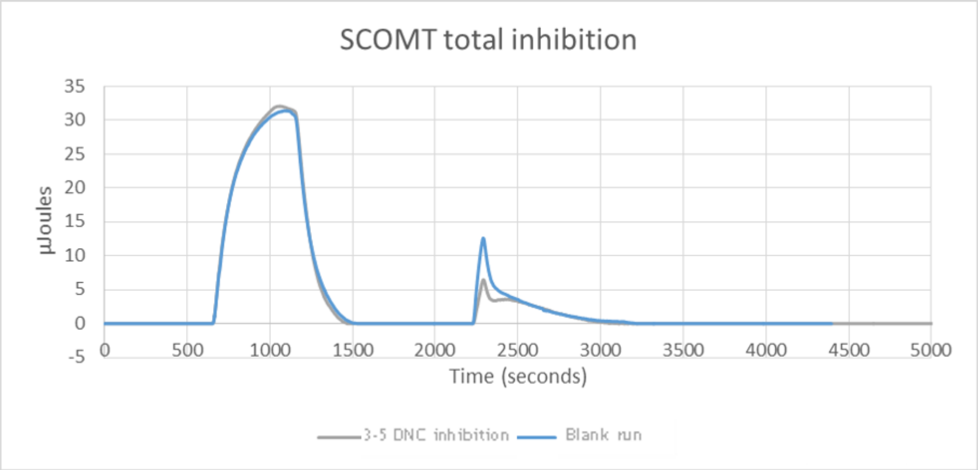


Figure 20 - Isothermal titration calorimetry data comparing a blank run with a run were 3162,3 mM of 3,5-dinitrocatechol was applied leading to a total inhibition of the catechol-O-methyltransferase.

The 3,5-DNC is an uncompetitive inhibitor. These kind of inhibition affects both kinetics parameters reducing so much V_{max} as the K_m.

As we can see in table 4, experimentally, these kind of conclusions was also achieved, principally in the K_m values, giving good directions to the analysis and thermodynamic characterization of this inhibitor with the protein.

Table 4 - Comparison of V_{max} and K_m values when the concentration of inhibitor is increased.

Test	*Inhibitor concentration (nM)*	*V_{max} (M/s)*	*K_m (μM)*
1	0.00	3.78E-06	500.73
2	1.00	1.74E-06	166.44
3	3.16	1.00E-06	94.79
4	316.20	1.77E-06	90.85
5	316.20	2.55E-06	83.15
6	1000.00	1.33E-06	40.93

Looking more closely to the table, we can see that the value of K_m decreases as the inhibitor concentration increases, as expected. In the case of the V_{max}, it is possible to see a tendency to decrease, but there is some fluctuation in results. Yet, the highest value for V_{max} is for the test without inhibitor.

Chapter V - Conclusion

Proteins are recognized as drug targets for a wide range of diseases. Actually, the SCOMT, which has received an increasing interest in the past years due its implication in several neurodegenerative diseases, like Parkinson's. Indeed, because COMT is one of the primordial enzymes involved in the dopamine breakdown, the use of COMT inhibitors are, undoubtedly, a remarkable therapeutic strategy in this kind of diseases. Actually, the inhibition of the COMT activity and, consequently, the inhibition of the dopamine breakdown, will result in an increase of its levels in the brain of the patients. The *P. pastoris* bioprocess employed in this thesis for biosynthesis of hSCOMT proved to be efficient as catalytically active hMBCOMT was obtained from mini-bioreator methanol-induced cultures. In addition, we also wanted to evaluate if there are any *P. pastoris* X33 endogenous proteins that interfere in the methylation of epinephrine to metanephrine. As expected, de conversion was at basal levels, don't interfering with the results when the COMT production is inducted.

The majority of biophysical techniques including ITC, requires samples with high homogeneity in a high purity degree. Therefore, after hSCOMT biosynthesis in *P. pastoris* and recuperation using an appropriated procedure involving glass beads, IMAC was applied as the main chromatographic purification step. Indeed, IMAC proved to be selective and accurate in purifying hSCOMT once fractions with high purity were obtained. Then, after establishing an appropriate bioprocess for hSCOMT biosynthesis and purification, it was evaluated the ability of lyophilization to storage hSCOMT in a catalytically active form and its kinetic properties were determined using ITC in the absence and presence of 3,5-DNC, a COMT inhibitor.

Initially, a lyophilization process for COMT was first described with the objective to keep the protein in a stable and active state further in time, and more robust to storage and transportation conditions like changes in temperature, a condition to which COMT is highly sensitive. One cryoprotective solution (mannose 20% (w/v) trehalose 4% (w/v) sucrose 6% (w/v)) was made in order to protect the protein from the freeze-drying process. After that, the stabilizing solution (trehalose [200mM] and cysteine [300mM]) was also added to the aliquot in order to retard the degradation of COMT. Unfortunately these solutions do not have the capability to fully protect the protein during the lyophilization process. The activity data, showed the more extended protein inactivation when compared with a sample that suffer the same processes except the lyophilization one. However, as far as we know, this is the first report concerning hSCOMT lyophilization and despite the target enzyme activity recovery was low after the lyophilization, we believe that these results may be improved once further optimizations on the cryo and lyoprotective solutions will be carried out. Nevertheless, we can conclude that no more than 24 hours is necessary to lyophilization the sample until a 1ml of volume

On the other hand, concerning ITC, two different methods were evaluated, namely the single and the multiple injection method. Although several optimizations were carried out, the weak heat signal obtained with the multi-injection approach didn't allow obtaining a mensurable heat signal. Therefore, the single injection method is preferred and the experimental conditions were concerning the heat signal were optimized using 2mM to SAM and epinephrine, and 1250 nM of protein, alongside the addition of stabilizing solution comprising 100 mM trehalose, 150 mM of cysteine, and 10% of glycerol.

As well, some parameters were calculated, like the K_m and V_{max}, for the epinephrine substrate. Although the values obtained (486.26 [444.97;510.77] µM and 4.19 ± 1.75µM/s for K_m and V_{max} respectively) are slightly different from those already described. they are in the same order of magnitude. Besides, is normal this divergence in the values when compared different techniques for calculation thermodynamic constants.

With the 3,5-DNC inhibitor some good indicators are given. We can see not only complete inhibition of the enzyme at the highest concentration of the inhibitor applied as the K_m and V_{max} values decrease with the increase in 3,5-DNC concentration, as it is characteristic for uncompetitive inhibition.

Chapter VII - Future perspectives

COMT, which has an important role in the metabolism of dopamine, and which is directly related with PD, emerged as a primordial therapeutic target for the treatment of PD. Actually, the improvement of the clinical efficacy of the currently available COMT inhibitors has depended of the structural and functional studies performed with recombinant forms of this enzyme.

Regarding to the lyophilization process, performed with the aim of improving the stability of COMT, some modifications could be applied in future experiments. Indeed, other lyo and cryoprotectant agents, like lactose and polyethylene glycol, respectively, could be tested. Moreover, different freezing methods could be evaluated. For example, the passage of the samples of -20°C to -80°C and then to liquid nitrogen is a process that could be applied. Moreover, the time spent between all these steps can be appropriately varied. Additionally, the conditions of pressure and temperature can also be change in order to improve the lyophilization process. Indeed, the optimal formulation including the optimal experimental conditions may be accomplished through the application of an experimental design (DOE) using the above mentioned factors.

Regarding the microcalorimetric assays, some improvements could be done through the use of COMT standards with a pre-defined concentration. In spite of this alterations, the quantification methodology could also be ameliorate. Actually, the use of mass spectrometry equipment, like a MALDI TOF-TOF, which is an ongoing work in our group, would allow to obtain more accurate results.

Finally, in addition to the COMT inhibitor 3,5-DNC that was used in the present work, other inhibitors, mainly of third generation like opicapone, could be studied and conveniently analyzed from the thermodynamic point of view.

Chapter VIII - Bibliography

[1] J. AXELROD, R. TOMCHICK, Enzymatic O-methylation of epinephrine and other catechols., J. Biol. Chem. 233 (1958) 702–705.

[2] M.J. Bonifácio, P.N. Palma, L. Almeida, P. Soares-Da-Silva, Catechol-O-methyltransferase and its inhibitors in Parkinson's disease, CNS Drug Rev. 13 (2007) 352–379.

[3] P.T. Mannisto, S. Kaakkola, Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors., Pharmacol. Rev. 51 (1999) 593–628.

[4] K. Rutherford, I. Le Trong, R.E. Stenkamp, W.W. Parson, Crystal Structures of Human 108V and 108M Catechol O-Methyltransferase, J. Mol. Biol. 380 (2008) 120–130.

[5] E.M. Tunbridge, P.J. Harrison, D.R. Weinberger, Catechol-o-Methyltransferase, Cognition, and Psychosis: Val158Met and Beyond, Biol. Psychiatry. 60 (2006) 141–151.

[6] L.M. Williams, J.M. Gatt, S.M. Grieve, C. Dobson-Stone, R.H. Paul, E. Gordon, et al., COMT Val108/158Met polymorphism effects on emotional brain function and negativity bias, Neuroimage. 53 (2010) 918–925.

[7] B. Borroni, M. Grassi, C. Agosti, C. Costanzi, S. Archetti, S. Franzoni, et al., Genetic correlates of behavioral endophenotypes in Alzheimer disease: Role of COMT, 5-HTTLPR and APOE polymorphisms, Neurobiol. Aging. 27 (2006) 1595–1603.

[8] M. Flirski, T. Sobow, I. Kloszewska, Behavioural genetics of Alzheimer's disease: A comprehensive review, Arch. Med. Sci. 7 (2011) 195–210.

[9] S.J. Glatt, S. V Faraone, M.T. Tsuang, Association between a functional catechol O-methyltransferase gene polymorphism and schizophrenia: Meta-analysis of case-control and family-based studies, Am. J. Psychiatry. 160 (2003) 469–476.

[10] R.D. Strous, N. Bark, S.S. Parsia, J. Volavka, H.M. Lachman, Analysis of a functional catechol-O-methyltransferase gene polymorphism in schizophrenia: Evidence for association with aggressive and antisocial behavior, Psychiatry Res. 69 (1997) 71–77.

[11] H.M. Lachman, K.A. Nolan, P. Mohr, T. Saito, J. Volavka, Association between catechol O-methyltransferase genotype and violence in schizophrenia and schizoaffective disorder, Am. J. Psychiatry. 155 (1998) 835–837.

[12] B. Borroni, M. Grassi, C. Costanzi, M. Zanetti, S. Archetti, S. Franzoni, et al., Haplotypes in cathechol-O-methyltransferase gene confer increased risk for psychosis in Alzheimer disease, Neurobiol. Aging. 28 (2007) 1231–1238.

[13] M. Karayiorgou, M. Altemus, B.L. Galke, D. Goldman, D.L. Murphy, J. Ott, et al., Genotype determining low catechol-O-methyltransferase activity as a risk factor for obsessive-compulsive disorder., Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 4572–4575.

[14] S. Wedrén, T.R. Rudqvist, F. Granath, E. Weiderpass, M. Ingelman-Sundberg, I. Persson, et al., Catechol-O-methyltransferase gene polymorphism and post-menopausal breast cancer risk, Carcinogenesis. 24 (2003) 681–687.

[15] L. Diatchenko, G.D. Slade, A.G. Nackley, K. Bhalang, A. Sigurdsson, I. Belfer, et al., Genetic basis for individual variations in pain perception and the development of a chronic pain condition, Hum. Mol. Genet. 14 (2005) 135–143.

[16] T.E. Goldberg, M.F. Egan, T. Gscheidle, R. Coppola, T. Weickert, B.S. Kolachana, et al., Executive subprocesses in working memory: relationship to catechol-O-methyltransferase Val158Met genotype and schizophrenia., Arch. Gen. Psychiatry. 60 (2003) 889–896.

[17] N.J. Bray, P.R. Buckland, N.M. Williams, H.J. Williams, N. Norton, M.J. Owen, et al., A haplotype implicated in schizophrenia susceptibility is associated with reduced COMT expression in human brain., Am. J. Hum. Genet. 73 (2003) 152–161.

[18] M. Akil, B.S. Kolachana, D.A. Rothmond, T.M. Hyde, D.R. Weinberger, J.E. Kleinman, Catechol-O-methyltransferase genotype and dopamine regulation in the human brain., J. Neurosci. 23 (2003) 2008–2013.

[19] S. Shifman, M. Bronstein, M. Sternfeld, A. Pisanté, A. Weizman, I. Reznik, et al., COMT: a common susceptibility gene in bipolar disorder and schizophrenia., Am. J. Med. Genet. B. Neuropsychiatr. Genet. 128B (2004) 61–64.

[20] S.J. Bishop, J.D. Cohen, J. Fossella, B.J. Casey, M.J. Farah, COMT genotype influences prefrontal response to emotional distraction., Cogn. Affect. Behav. Neurosci. 6 (2006) 62–70.

[21] M.-A. Enoch, M.A. Schuckit, B.A. Johnson, D. Goldman, Genetics of alcoholism using intermediate phenotypes., Alcohol. Clin. Exp. Res. 27 (2003) 169–176.

[22] B.S. Oken, D. Zajdel, S. Kishiyama, K. Flegal, C. Dehen, M. Haas, et al., Randomized, controlled, six-month trial of yoga in healthy seniors: Effects on cognition and quality of life, Altern. Ther. Health Med. 12 (2006) 40–47.

[23] Z. Ma, H. Liu, B. Wu, Structure-based drug design of catechol-O-methyltransferase inhibitors for CNS disorders, Br. J. Clin. Pharmacol. 77 (2014) 410–420.

[24] J.L. Martin, F.M. McMillan, SAM (dependent) I AM: The S-adenosylmethionine-dependent methyltransferase fold, Curr. Opin. Struct. Biol. 12 (2002) 783–793.

[25] J. Vidgren, L.A. Svensson, A. Liljas, Crystal structure of catechol O-methyltransferase., Nature. 368 (1994) 354–358.

[26] E. Tsuji, K. Okazaki, K. Takeda, Crystal structures of rat catechol-O-methyltransferase complexed with coumarine-based inhibitor, Biochem. Biophys. Res. Commun. 378 (2009) 494–497.

[27] E. Tsuji, K. Okazaki, M. Isaji, K. Takeda, Crystal structures of the Apo and Holo form of rat catechol-O-methyltransferase, J. Struct. Biol. 165 (2009) 133–139.

[28] M. Ellermann, C. Lerner, G. Burgy, A. Ehler, C. Bissantz, R. Jakob-Roetne, et al., Catechol-O-methyltransferase in complex with substituted 3́-deoxyribose bisubstrate inhibitors, Acta Crystallogr. Sect. D Biol. Crystallogr. 68 (2012) 253–260.

[29] M. Ellermann, R. Jakob-Roetne, C. Lerner, E. Borroni, D. Schlatter, D. Roth, et al., Molecular recognition at the active site of catechol-omethyltransferase: Energetically favorable replacement of a water molecule imported by a bisubstrate inhibitor, Angew. Chemie - Int. Ed. 48 (2009) 9092–9096.

[30] M. Ellermann, R. Paulini, R. Jakob-Roetne, C. Lerner, E. Borroni, D. Roth, et al., Molecular recognition at the active site of catechol-O-methyltransferase (COMT): Adenine replacements in bisubstrate inhibitors, Chem. - A Eur. J. 17 (2011) 6369–6381.

[31] C. Lerner, A. Ruf, V. Gramlich, B. Masjost, G. Zrcher, R. Jakob-Roetne, et al., X-ray crystal structure of a bisubstrate inhibitor bound to the enzyme catechol-O-methyltransferase: A dramatic effect of inhibitor preorganization on binding affinity, Angew. Chemie - Int. Ed. 40 (2001) 4040–4042.

[32] P.N. Palma, M.L. Rodrigues, M. Archer, M.J. Bonifácio, A.I. Loureiro, D.A. Learmonth, et al., Comparative study of ortho- and meta-nitrated inhibitors of catechol-O-methyltransferase: Interactions with the active site and regioselectivity of O-methylation, Mol. Pharmacol. 70 (2006) 143–153.

[33] M.J. Bonifácio, M. Archer, M.L. Rodrigues, P.M. Matias, D.A. Learmonth, M.A. Carrondo, et al., Kinetics and crystal structure of catechol-o-methyltransferase complex with co-substrate and a novel inhibitor with potential therapeutic application., Mol. Pharmacol. 62 (2002) 795–805.

[34] J. Volavka, R. Bilder, K. Nolan, Catecholamines and aggression: The role of COMT and MAO polymorphisms, in: Ann. N. Y. Acad. Sci., 2004: pp. 393–398.

[35] J.A. Gogos, M. Morgan, V. Luine, M. Santha, S. Ogawa, D. Pfaff, et al., Catechol-O-methyltransferase-deficient mice exhibit sexually dimorphic changes in catecholamine levels and behavior., Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 9991–9996.

[36] M. Huotari, J.A. Gogos, M. Karayiorgou, O. Koponen, M. Forsberg, A. Raasmaja, et al., Brain catecholamine metabolism in catechol-O-methyltransferase (COMT)-deficient mice, Eur. J. Neurosci. 15 (2002) 246–256.

[37] E.M. Tunbridge, D.M. Bannerman, T. Sharp, P.J. Harrison, Catechol-o-methyltransferase inhibition improves set-shifting performance and elevates stimulated dopamine release in the rat prefrontal cortex., J. Neurosci. 24 (2004) 5331–5335.

[38] J. Meiser, D. Weindl, K. Hiller, Complexity of dopamine metabolism., Cell Commun. Signal. 11 (2013) 34.

[39] L.M. Laatikainen, T. Sharp, P.J. Harrison, E.M. Tunbridge, Sexually Dimorphic Effects of Catechol-O-Methyltransferase (COMT) Inhibition on Dopamine Metabolism in Multiple Brain Regions, PLoS One. 8 (2013) e61839.

[40] R.C. Brown, A.H. Lockwood, B.R. Sonawane, Neurodegenerative diseases: An overview of environmental risk factors, Environ. Health Perspect. 113 (2005) 1250–1256.

[41] J.-F. Emard, J.-P. Thouez, D. Gauvreau, Neurodegenerative diseases and risk factors: A literature review, Soc. Sci. Med. 40 (1995) 847–858.

[42] V.K. Ramanan, A.J. Saykin, Pathways to neurodegeneration: mechanistic insights from GWAS in Alzheimer’s disease, Parkinson's disease, and related disorders., Am. J. Neurodegener. Dis. 2 (2013) 145–75.

[43] M. Chin-Chan, J. Navarro-Yepes, B. Quintanilla-Vega, Environmental pollutants as risk factors for neurodegenerative disorders: Alzheimer and Parkinson diseases, Front. Cell. Neurosci. 9 (2015) 124.

[44] C. Reitz, G. Jun, A. Naj, R. Rajbhandary, B.N. Vardarajan, L.-S. Wang, et al., Variants in the ATP-binding cassette transporter (ABCA7), apolipoprotein E ϵ4,and the risk of late-onset Alzheimer disease in African Americans., JAMA. 309 (2013) 1483–92.

[45] S.M. Hague, S. Klaffke, O. Bandmann, Neurodegenerative disorders: Parkinson's disease and Huntington's disease., J. Neurol. Neurosurg. Psychiatry. 76 (2005) 1058–1063.

[46] S. Nuber, E. Petrasch-Parwez, B. Winner, J. Winkler, S. von Hörsten, T. Schmidt, et al., Neurodegeneration and motor dysfunction in a conditional model of Parkinson's disease., J. Neurosci. 28 (2008) 2471–2484.

[47] C.M. Tolleson, J.Y. Fang, Advances in the mechanisms of Parkinson's disease., Discov. Med. 15 (2013) 61–6.

[48] C.G. Goetz, The history of Parkinson's disease: Early clinical descriptions and neurological therapies, Cold Spring Harb. Perspect. Med. 1 (2011) a008862–a008862.

[49] B.S. Connolly, A.E. Lang, Pharmacological treatment of Parkinson disease: a review., JAMA. 311 (2014) 1670–83.

[50] D. Salat, E. Tolosa, Levodopa in the treatment of Parkinson's disease: Current status and new developments, J. Parkinsons. Dis. 3 (2013) 255–269.

[51] M.M. Goldenberg, Medical management of Parkinson's disease., P T. 33 (2008) 590–606.

[52] A.J. Lees, R. Katzenschlager, J. Head, Y. Ben-Shlomo, Ten-year follow-up of three different initial treatments in de-novo PD: a randomized trial., 2001.

[53] R.A. Hauser, O. Rascol, A.D. Korczyn, A. Jon Stoessl, R.L. Watts, W. Poewe, et al., Ten-year follow-up of Parkinson's disease patients randomized to initial therapy with ropinirole or levodopa., Mov. Disord. 22 (2007) 2409–2417.

[54] C. Waters, Catechol-O-methyltransferase (COMT) inhibitors in Parkinson's disease., J. Am. Geriatr. Soc. 48 (2000) 692–698.

[55] K. Haasio, Toxicology and safety of comt inhibitors, Elsevier Inc., 2010.

[56] N. Jatana, A. Sharma, N. Latha, Pharmacophore modeling and virtual screening studies to design potential COMT inhibitors as new leads, J. Mol. Graph. Model. 39 (2013) 145–164.

[57] L.E. Kiss, P. Soares-Da-Silva, Medicinal Chemistry of Catechol O-Methyltransferase (COMT) Inhibitors and their Therapeutic Utility., J. Med. Chem. 57 (2014) 8692–717.

[58] T. Muller, Catechol-O-Methyltransferase Inhibitors in Parkinson's Disease, Drugs. 75 (2015) 157–174.

[59] C. dos Santos Passos, L.C. Klein-Júnior, J.M. de Mello Andrade, C. Matté, A.T. Henriques, The catechol-O-methyltransferase inhibitory potential of Z-vallesiachotamine by in silico and in vitro approaches, Rev. Bras. Farmacogn. (2015).

[60] L.E. Kiss, H.S. Ferreira, L. Torrão, M.J. Bonifácio, P.N. Palma, P. Soares-Da-Silva, et al., Discovery of a long-acting, peripherally selective inhibitor of catechol- O -methyltransferase, J. Med. Chem. 53 (2010) 3396–3411.

[61] F. Assal, L. Spahr, A. Hadengue, L. Rubbia-Brandt, P.R. Burkhard, Tolcapone and fulminant hepatitis., 1998.

[62] J.F. Rocha, L. Almeida, A. Falcão, P.N. Palma, A.I. Loureiro, R. Pinto, et al., Opicapone: A short lived and very long acting novel catechol-O-methyltransferase inhibitor following multiple dose administration in healthy subjects, Br. J. Clin. Pharmacol. 76 (2013) 763-775.

[63] I. Ulmanen, J. Peränen, J. Tenhunen, C. Tilgmann, T. Karhunen, P. Panula, et al., Expression and intracellular localization of catechol O-methyltransferase in transfected mammalian cells., Eur. J. Biochem. 243 (1997) 452–459.

[64] C. Tilgmann, K. Melen, K. Lundstrom, a. Jalanko, I. Julkunen, N. Kalkkinen, et al., Expression of recombinant soluble and membrane-bound catechol O-methyltransferase in eukaryotic cells and identification of the respective enzymes in rat brain, Eur. J. Biochem. 207 (1992) 813–821.

[65] J. Kelloniemi, K. Mäkinen, J.P.T. Valkonen, A potyvirus-based gene vector allows producing active human S-COMT and animal GFP, but not human sorcin, in vector-infected plants, Biochimie. 88 (2006) 505–513.

[66] F. Baneyx, Recombinant protein expression in Escherichia coli, Curr. Opin. Biotechnol. 10 (1999) 411–421.

[67] L.A. Passarinha, M.J. Bonifácio, J.A. Queiroz, Application of a fed-batch bioprocess for the heterologous production of hSCOMT in Escherichia coli, J. Microbiol. Biotechnol. 19 (2009) 972–981.

[68] G.M. Espírito Santo, a. Q. Pedro, D. Oppolzer, M.J. Bonifácio, J. a. Queiroz, F. Silva, et al., Development of fed-batch profiles for efficient biosynthesis of catechol-O-methyltransferase, Biotechnol. Reports. 3 (2014) 34–41.

[69] R. Silva, S. Ferreira, M.J. Bonifácio, J.M.L. Dias, J.A. Queiroz, L.A. Passarinha, Optimization of fermentation conditions for the production of human soluble catechol-O-methyltransferase by Escherichia coli using artificial neural network., J. Biotechnol. 160 (2012) 161–8.

[70] S.Y. Lee, High cell-density culture of Escherichia coli., Trends Biotechnol. 14 (1996) 98-105.

[71] K. Terpe, Overview of bacterial expression systems for heterologous protein production: From molecular and biochemical fundamentals to commercial systems, Appl. Microbiol. Biotechnol. 72 (2006) 211–222.

[72] A. Domînguez, E. Ferminân, M. Sânchez, F.J. Gonzalez, F.M. Pérez-Campo, S. Garcia, et al., Non-conventional yeasts as hosts for heterologous protein production, Int. Microbiol. 1 (1998) 131–142.

[73] A. Gonçalves, A. Pedro, C. Maia, S. Fani, J. Queiroz, L. Passarinha, Pichia pastoris: A recombinant microfactory for antibody and human membrane proteins, Trends Biotechnol. 23 (2012) 587–601.

[74] R. Daly, M.T.W. Hearn, Expression of heterologous proteins in Pichia pastoris: A useful experimental tool in protein engineenring and production, J. Mol. Recognit. 18 (2005) 119–138.

[75] P. Li, A. Anumanthan, X.-G. Gao, K. Ilangovan, V. V Suzara, N. Düzgüneş, et al., Expression of recombinant proteins in Pichia pastoris., Appl. Biochem. Biotechnol. 142 (2007) 105–124.

[76] L. Passarinha, F. Correia, A. Pedro, D. Oliveira, J. Queiroz, Biosynthesis, purification and biointeraction of human SCOMT with Parkinson's disease inhibitors, N. Biotechnol. 31 (2014) S193.

[77] L.A. Passarinha, M.J. Bonifácio, P. Soares-da-Silva, J.A. Queiroz, A new approach on the purification of recombinant human soluble catechol-O-methyltransferase from an Escherichia coli extract using hydrophobic interaction chromatography., J. Chromatogr. A. 1177 (2008) 287–96.

[78] F.M. Santos, A.Q. Pedro, R.F. Soares, R. Martins, M.J. Bonifácio, J.A. Queiroz, et al., Performance of hydrophobic interaction ligands for human membrane-bound catechol-O-methyltransferase purification., J. Sep. Sci. 36 (2013) 1693–702.

[79] F.F. Correia, F.M. Santos, a. Q. Pedro, M.J. Bonifácio, J. a. Queiroz, L.A.P. Passarinha, Recovery of biological active catechol-O-methyltransferase isoforms from Q-sepharose, J. Sep. Sci. 37 (2014) 20–29.

[80] S.R. Costa, M.J. Bonifácio, J.A. Queiroz, L.A. Passarinha, Analysis of hSCOMT adsorption in bioaffinity chromatography with immobilized amino acids: the influence of pH and ionic strength., J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 879 (2011) 1704–6.

[81] C. Tilgmann, I. Ulmanen, Purification methods of mammalian catechol-O-methyltransferases, J. Chromatogr. B Biomed. Sci. Appl. 684 (1996) 147–161.

[82] M. Urh, D. Simpson, K. Zhao, Affinity chromatography: general methods., Methods Enzymol. 463 (2009) 417–38.

[83] M.C. Heller, J.F. Carpenter, T.W. Randolph, Protein formulation and lyophilization cycle design: Prevention of damage due to freeze-concentration induced phase separation, Biotechnol. Bioeng. 63 (1999) 166–174.

[84] W. Abdelwahed, G. Degobert, S. Stainmesse, H. Fessi, Freeze-drying of nanoparticles: formulation, process and storage considerations., Adv. Drug Deliv. Rev. 58 (2006) 1688–713.

[85] F. Franks, Freeze-drying of bioproducts: putting principles into practice, Eur. J. Pharm. Biopharm. 45 (1998) 221–229.

[86] X. Tang, S.L. Nail, M.J. Pikal, Freeze-drying process design by manometric temperature measurement: Design of a smart freeze-dryer, Pharm. Res. 22 (2005) 685–700.

[87] E.A. Boss, R.M. Filho, E.C.V. de Toledo, Freeze drying process: real time model and optimization, Chem. Eng. Process. Process Intensif. 43 (2004) 1475–1485.

[88] A.A. Barresi, R. Pisano, D. Fissore, V. Rasetto, S.A. Velardi, A. Vallan, et al., Monitoring of the primary drying of a lyophilization process in vials, Chem. Eng. Process. Process Intensif. 48 (2009) 408–423.

[89] T. Serno, R. Geidobler, G. Winter, Protein stabilization by cyclodextrins in the liquid and dried state, Adv. Drug Deliv. Rev. 63 (2011) 1086–1106.

[90] N.A. Williams, G.P. Polli, The Lyophilization of Pharmaceuticals: A Literature Review, PDA J. Pharm. Sci. Technol. 38 (1984) 48–60.

[91] T.W. Patapoff, D.E. Overcashier, The Importance of Freezing on Lyophilization Cycle Development, BioPharm. (2002) 16–21,72.

[92] M. Pikal, S. Shah, M. Roy, R. Putman, The secondary drying stage of freeze drying: drying kinetics as a function of temperature and chamber pressure☆, Int. J. Pharm. 60 (1990) 203–207.

[93] F. Franks, ed., Water and Aqueous Solutions at Subzero Temperatures, Springer US, Boston, MA, 1982.

[94] K. Imamura, K. Murai, T. Korehisa, N. Shimizu, R. Yamahira, T. Matsuura, et al., Characteristics of sugar surfactants in stabilizing proteins during freeze-thawing and freeze-drying, J. Pharm. Sci. 103 (2014) 1628–1637.

[95] J.F. Carpenter, S.J. Prestrelski, T. Arakawa, Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. I. Enzyme activity and calorimetric studies., Arch. Biochem. Biophys. 303 (1993) 456–64.

[96] J.H. Crowe, J.F. Carpenter, L.M. Crowe, T.J. Anchordoguy, Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules, Cryobiology. 27 (1990) 219–231.

[97] T. Arakawa, S.J. Prestrelski, W.C. Kenney, J.F. Carpenter, Factors affecting short-term and long-term stabilities of proteins, Adv. Drug Deliv. Rev. 46 (2001) 307–326.

[98] S.J. Prestrelski, T. Arakawa, J.F. Carpenter, Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. II. Structural studies using infrared spectroscopy., Arch. Biochem. Biophys. 303 (1993) 465–73.

[99] L.M. Crowe, D.S. Reid, J.H. Crowe, Is trehalose special for preserving dry biomaterials?, Biophys. J. 71 (1996) 2087–2093..

[100] S.D. Allison, A. Dong, J.F. Carpenter, Counteracting effects of thiocyanate and sucrose on chymotrypsinogen secondary structure and aggregation during freezing, drying, and rehydration, Biophys. J. 71 (1996) 2022–2032.

[101] S.D. Allison, T.W. Randolph, M.C. Manning, K. Middleton, A. Davis, J.F. Carpenter, Effects of Drying Methods and Additives on Structure and Function of Actin: Mechanisms of Dehydration-Induced Damage and Its Inhibition, Arch. Biochem. Biophys. 358 (1998) 171–181.

[102] E. Frelre, O.L. Mayorgal, M. Straume, Isothermal Titration, Anal. Chem. 62 (1990) 950-959.

[103] M.W. Freyer, E.A. Lewis, Isothermal Titration Calorimetry: Experimental Design, Data Analysis, and Probing Macromolecule/Ligand Binding and Kinetic Interactions, Methods Cell Biol. 84 (2008) 79–113.

[104] R. Perozzo, G. Folkers, L. Scapozza, Thermodynamics of protein-ligand interactions: history, presence, and future aspects., J. Recept. Signal Transduct. Res. 24 (2004) 1–52.

[105] N.A. Demarse, M.C. Killian, L.D. Hansen, C.F. Quinn, Determining enzyme kinetics via isothermal titration calorimetry., Methods Mol. Biol. 978 (2013) 21–30.

[106] R. Ghai, R.J. Falconer, B.M. Collins, Applications of isothermal titration calorimetry in pure and applied research-survey of the literature from 2010, J. Mol. Recognit. 25 (2012) 32–52.

[107] Y. Liang, Applications of isothermal titration calorimetry in protein science, Acta Biochim. Biophys. Sin. (Shanghai). 40 (2008) 565–576.

[108] W.F. de Azevedo, R. Dias, Experimental approaches to evaluate the thermodynamics of protein-drug interactions., Curr. Drug Targets. 9 (2008) 1071–1076.

[109] W.H. Ward, G.A. Holdgate, Isothermal titration calorimetry in drug discovery., Prog. Med. Chem. 38 (2001) 309–376.

[110] S. Leavitt, E. Freire, Direct measurement of protein binding energetics by isothermal titration calorimetry, Curr. Opin. Struct. Biol. 11 (2001) 560–566.

[111] W.B. Peters, V. Frasca, R.K. Brown, Recent developments in isothermal titration calorimetry label free screening., Comb. Chem. High Throughput Screen. 12 (2009) 772–790.

[112] X. Zhou, R.M. Kini, J. Sivaraman, Application of isothermal titration calorimetry and column chromatography for identification of biomolecular targets., Nat. Protoc. 6 (2011) 158–165.

[113] X. Zhou, Q. Sun, R.M. Kini, J. Sivaraman, A universal method for fishing target proteins from mixtures of biomolecules using isothermal titration calorimetry., Protein Sci. 17 (2008) 1798–1804.

[114] R.W. Sarver, J. Peevers, W.L. Cody, F.L. Ciske, J. Dyer, S.D. Emerson, et al., Binding thermodynamics of substituted diaminopyrimidine renin inhibitors, Anal. Biochem. 360 (2007) 30–40.

[115] G.G. Ferenczy, G.M. Keserũ, Thermodynamics guided lead discovery and optimization, Drug Discov. Today. 15 (2010) 919–932.

[116] A. Velazquez-Campoy, I. Luque, E. Freire, The application of thermodynamic methods in drug design, Thermochim. Acta. 380 (2001) 217–227.

[117] J.E. Ladbury, G. Klebe, E. Freire, Adding calorimetric data to decision making in lead discovery: a hot tip., Nat. Rev. Drug Discov. 9 (2010) 23–27.

[118] J.B. Chaires, Calorimetry and thermodynamics in drug design., Annu. Rev. Biophys. 37 (2008) 135–151.

[119] M.J. Todd, J. Gomez, Enzyme kinetics determined using calorimetry: a general assay for enzyme activity?, Anal. Biochem. 296 (2001) 179–187.

[120] R. Noske, F. Cornelius, R.J. Clarke, Investigation of the enzymatic activity of the Na+,K+-ATPase via isothermal titration microcalorimetry, Biochim. Biophys. Acta - Bioenerg. 1797 (2010) 1540–1545.

[121] J.J. Christensen, R.M. Izatt, D. Eatough, Thermodynamics of Metal Cyanide Coordination. V. Log K, ΔH°, and ΔS° Values for the Hg 2+ -CN- System, Inorg. Chem. 4 (1965) 1278–1280.

[122] B.A. Williams, E.J. Toone, Calorimetric evaluation of enzyme kinetic parameters, J. Org. Chem. 58 (1993) 3507–3510.

[123] Energetics of Protein-Drug Binding Division of Bioengineering National University of Singapore, Division of Bioengineering Nation University of Singapore (2006).

[124] M. Kabiri, L.D. Unsworth, Application of isothermal titration calorimetry for characterizing thermodynamic parameters of biomolecular interactions: peptide self-assembly and protein adsorption case studies., Biomacromolecules. 15 (2014) 3463–73.

[125] S. Núñez, J. Venhorst, C.G. Kruse, Target-drug interactions: First principles and their application to drug discovery, Drug Discov. Today. 17 (2012) 10–22.

[126] G.A. Holdgate, W.H.J. Ward, Measurements of binding thermodynamics in drug discovery, Drug Discov. Today. 10 (2005) 1543–1550.

[127] E. Edink, C. Jansen, R. Leurs, I.J.P. de Esch, The heat is on: Thermodynamic analysis in fragment-based drug discovery, Drug Discov. Today Technol. 7 (2010) e147–202.

[128] M.L. Bianconi, Calorimetry of enzyme-catalyzed reactions., Biophys. Chem. 126 (2007) 59–64.

[129] L. Cai, A. Cao, L. Lai, An isothermal titration calorimetric method to determine the kinetic parameters of enzyme catalytic reaction by employing the product inhibition as probe., Anal. Biochem. 299 (2001) 19–23.

[130] S.N. Olsen, Applications of isothermal titration calorimetry to measure enzyme kinetics and activity in complex solutions, Thermochim. Acta. 448 (2006) 12–18.

[131] P.L. Privalov, A.I. Dragan, Microcalorimetry of biological macromolecules, Biophys. Chem. 126 (2007) 16–24.

[132] Use of Isothermal Titration Calorimetry to Measure Enzyme Kinetics Parameters ITC and Enzyme Kinetics, ITC Application Note, MicroCal, Europe. (2012).

[133] I. Wadsö, Isothermal microcalorimetry in applied biology, Thermochim. Acta. 394 (2002) 305–311.

[134] A.Q. Pedro, L.M. Martins, J.M.L. Dias, M.J. Bonifácio, J. a Queiroz, L. a Passarinha, An artificial neural network for membrane-bound catechol-O-methyltransferase biosynthesis with Pichia pastoris methanol-induced cultures, Microb. Cell Fact. 14 (2015) 113.

[135] M.J. Bonifácio, M.A. Vieira-Coelho, P. Soares-da-Silva, Kinetic inhibitory profile of BIA 3-202, a novel fast tight-binding, reversible and competitive catechol-O-methyltransferase inhibitor., Eur. J. Pharmacol. 460 (2003) 163–70.

[136] A. Pedro, R. Soares, D. Oppolzer, F. Santos, L. Rocha, A. Gonçalves, et al., An Improved HPLC Method for Quantification of Metanephrine with Coulometric Detection, J. Chromatogr. Sep. Tech. 05 (2014).

[137] L.A. Passarinha, M.J. Bonifácio, J.A. Queiroz, The effect of temperature on the analysis of metanephrine for catechol-O-methyltransferase activity assay by HPLC with electrochemical detection., Biomed. Chromatogr. 20 (2006) 937–44.

[138] N. Borges, M.A. Vieira-Coelho, A. Parada, P. Soares-da-Silva, Rat liver catechol-O-methyltransferase kinetics and assay methodology., J. Enzyme Inhib. 13 (1998) 473–83.

[139] T. Vogl, F.S. Hartner, A. Glieder, New opportunities by synthetic biology for biopharmaceutical production in Pichia pastoris., Curr. Opin. Biotechnol. 24 (2013) 1094–101.

[140] E.S. Hemdan, Y.J. Zhao, E. Sulkowski, J. Porath, Surface topography of histidine residues: a facile probe by immobilized metal ion affinity chromatography., Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 1811–1815.

[141] W. Wang, Instability, stabilization, and formulation of liquid protein pharmaceuticals, Int. J. Pharm. 185 (1999) 129–188.

[142] L. a. Passarinha, M.J. Bonifćio, J. a. Queiroz, The effect of temperature on the analysis of metanephrine for catechol-O-methyltransferase activity assay by HPLC with electrochemical detection, Biomed. Chromatogr. 20 (2006) 937–944.