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Quantification of Oxidative Stress Biomarkers:
Development of a Method by Ultra Performance
Liquid Chromatography
Liliana da Silva Rodrigues

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UNIVERSIDADE da MADEIRA

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MASTER DISSERTATION

Liliana da Silva Rodrigues
MASTER IN APPLIED BIOCHEMISTRY

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Quantification of oxidative stress biomarkers: Development of a Method by Ultra Performance Liquid Chromatography

Dissertation submitted at the University of Madeira in order to obtain the degree of Master in Applied Biochemistry

Liliana da Silva Rodrigues

Work developed under the orientation of:
Supervisor Prof. Doctor Helena Cardeira Araújo
Co-supervisor Prof. Doctor José de Sousa Câmara

Funchal, Portugal
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To my Family

“Sem eles nada disto seria possível”

*****

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Abstract

Thiol and purine compounds are widely distributed in nature. They are involved in physiological processes, such as, homeostasis and redox signalling, and disturbances of these functions are the basis of many human diseases. Therefore, there has been a great effort to better understand the metabolomics of these compounds.

The goal of the present work was to optimize an Ultra-High Performance Liquid Chromatography method for simultaneous detection and quantification of some thiol, and purine compounds in cells lysates and biological fluids.

For this purpose, an UPLC® system, equipped with an HSS T3 column, with fluorescence and photodiode array detection was used. Preliminary experiences to optimize chromatographic separation of standard compounds were accomplished and involved testing of conditions for sample pre-analytical treatments, mobile phase compositions and detection conditions.

A good resolution and separation was obtained for the standards tested. The thiols Glutathione, Cysteine and Homocysteine eluted at 0.655 ± 0.005, 2.189 ± 0.000 and 3.752 ± 0.001 minutes. Intra-day precision of the method was 12.05, 9.87 and 9.06% for areas. Farther, inter-day precision for the retention times were 29.49, 1.63 and 2.24% and for areas were 41.55, 28.28 and 29.46%, respectively. Purines Adenosine and Inosine eluted at 2.249 ± 0.001 and 2.584 ± 0.005 minutes. Inter-day precision for the retention times were 0.06 and 0.01% and for areas were 6.99 and 6.63%, respectively. Linearity was tested in a range of concentrations from 5 to 100 μM for thiols and 25 to 500 μM for purines, with good results. Additionally, the detection and quantification limits were too high. Unfortunately, our analyses do not shown intra or inter-day precision. Therefore, the validation was not completed because reproducibility was inconsistent due to the mechanical failure of the chromatographic equipment used. However, we concluded that the identification of these analytes is possible with the methods established.

Keywords: Thiols; Purines; Ultra-High Performance Liquid Chromatography; Oxidative stress.

Resumo

Os compostos tiólicos e as purinas encontram-se amplamente distribuídos na natureza. Estão envolvidos em vários processos fisiológicos, como a homeostasia e a sinalização redox. Perturbações destas funções estão na base de muitas patologias humanas. Por essa razão, existe um grande esforço da comunidade científica para entender estes compostos, particularmente ao nível da metabolómica.

O objetivo deste trabalho foi otimizar um método de cromatografia líquida de ultra precisão para a deteção e quantificação destes compostos, em lisados celulares e fluidos biológicos.

Com este propósito, foi usado o sistema cromatográfico UPLC®, equipado com uma coluna HSS T3 acoplado a detetores de fluorescência e fotodiodos em série para os tióis e purinas, respetivamente. Foram realizadas diversas experiências preliminares de otimização da separação dos padrões, onde foram testadas várias condições de pré-tratamento das amostras, diversas composições das fases móveis e condições de deteção.

Foi possível obter uma boa resolução com os compostos tiólicos e com as purinas. O Glutatião, a Cisteína e a Homocisteína eluíram aos 0.655 $\pm$ 0.005, 2.189 $\pm$ 0.000 e 3.752 $\pm$ 0.001 minutos. A precisão inter-diária dos tempos de retenção foi de 29.49, 1.63 e 2.24% e das áreas foi de 41.55, 28.28 e 29.46%, respetivamente. A linearidade do método foi testada numa gama de concentrações entre 5 e 100 μM, com bons coeficientes de correlação. Os compostos purínicos Adenosina e Inosina eluíram aos 2.249 $\pm$ 0.001 e 2.584 $\pm$ 0.005 minutos. A precisão inter-diária dos tempos de retenção foi 0.06 e 0.01% e das áreas 6.99 e 6.63%, respetivamente. A linearidade do método foi testada numa gama de concentrações entre 25 e 500 μM, com bons coeficientes de correlação. Infelizmente os resultados foram inconsistentes e a validação não foi alcançada, devido a falhas mecânicas do equipamento cromatográfico utilizado. Conclui-se que a identificação dos analitos é possível com os métodos estabelecidos.

Palavras-chave: Tióis; Purinas; Cromatografia em fase líquida de ultra performance; *Stress* oxidativo

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Scientific Notation	
%	Percentage
°C	Degrees Celsius
µL	Microlitres (1 x 10^{-6} litres)
µM or µmol/L	Micromole per litres (1 x 10^{-6} moles)
µV	MicroVolt units
µV°sec	MicroVolt units per second
AU	Absorbance units
D-amino acid	Chirality (-)*levorotatory* (R-enantiomer)
EU	Emission units
L-amino acid	Chirality (+)*dextrorotatory* (S-enantiomer)
mg	Milligrams (1 x 10^{-3} grams)
min	Minute
mL	Millilitres (1 x 10^{-9} litres)
mM or mmol/L	Millimole per liter (1 x 10^{-3} moles)
nm	Nanometer (1 x 10^{-9} metres)
β	Greek letter beta
γ	Geek letter gamma
λ	Greek letter lambda which expresses the wavelength
A	
A	Alanine transporter system
AC	Adenylyl cyclase
ACN	Acetonitrile
ADA	Adenosine deaminase
ADHD	Dimethylarginine dimethylamino hydrolase
ADK	Adenosine kinase
ADMA	Asymmetric dimethylargenine
Ado	Adenosine
ADP	Adenosine 5'diphosphate
ADSL	Adenylosuccinate lyase
ADSS	Adenylosuccinate synthetase
AICAR	Aminoimidazolecarboxamide ribonucleoside
AICARFT	Aminoimidazole carboxamide ribonucleotide formyltransferase
AIRC	Aminoimidazole ribonucleotide carboxylase
AIRS	Aminoimidazole ribonucleotide synthetase
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine 5'monophosphate
APOBEC	Apolipoprotein-β mRNA aditing anzyme, catalytic polypeptide-like
APRT	Adenosine phosphoribosyl transferase
APS	Antiphospholipid antibody syndrome
Arg	Arginine
ASC	Alanine-serine-cysteine transporter system
Asp	Aspartate
ATIC	Aminoimidazole carboxamide ribonucleotide transformylase/inosine monophosphate cyclohydrolase
ATP	Adenosine 5'triphosphate

B	
BHMT	Betaine-homocysteine methyltransferase
B_2 | B_6 | B_{12}	Cofactors derived from B vitamin complex
C	
2Cys	Cystine
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CBS	Cystathionine β-synthase
CEC	Circulating endothelial cells
CEPC	Circulating endothelial progenitor cells
cGMP	Cyclic guanosine monophosphate
CG-X	Conjugate cysteinylglycine
CH_2	Methylene group
Cl^-	Chlorine ion
CPK	Creatinine phosphokinase
CSE	Cystathionase γ-liase
CSIF	Human cytokine synthesis inhibitory factor
CVD	Cardiovascular diseases
C-X	Cysteine conjugates
Cys	Cysteine
CysGly	Cysteinylglycine
Cys-SS-Hcy	Cysteinylhomocysteine
D	
DBP	Diastolic blood pressure
DHFR	Dihydrofolate reductase
DMG	Dimethylglycine
DNA	Deoxyribonucleic acid
E	
e^-	Free electron
EC	Endothelial cells
eNOS	Endothelial nitric oxide synthase
ENT1 and 2	Equilibrative nucleoside transporter 1 and 2
$ERK_{½}$	Extracellular signal-regulated kinase ½
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
F	
FA	Formic acid
FAD	Flavine adenine dinucleotide oxidized (active form of vitamin B_2)
$FADH_2$	Flavine adenine dinucleotide reduced
FGAMS	Formylglycinamidine ribonucleotide synthase
G	
G6PD	Glucose-6-phosphate dehydrogenase enzyme
GARS	Glycinamide ribonucleotide synthetase
GART	Glycinamide ribonucleotide transformylase
GCL	Gamma-glutamylcysteine ligase
GGT	Gamma-glutamyl transpeptidase
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine

GNMT	Glycine N-methyltransferase
GPx	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxine
GS	Glutathione synthetase
GS(O)SG	Glutathione disulphide S-monoxide
$GS^{\bullet}$	Glutathione radical
GSH	Reduced glutathione
GSNO	S-Nitrosoglutathione
GSOH	Glutathione sulphenic acid
GS-R or GS-X	Glutathione conjugates
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
Gua	Guanosine
γ-GGCT	Gamma-glutamyl cycle transferase
γ-Glu-aa	Gamma-glutamylaminoacid
γ-GluCys	Gamma-glutamylcysteine
H	
2Hcy	Homocystine
H^+	Hydrogen cation
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
HCO_2^-	hydrogen carbonate anion
Hcy	Homocysteine
HDL	High-density lipoprotein
HGPRT	Hypoxanthine-guanine phosphoribosyltranferase
HHcy	Hyperhomocysteine
HMGCR	3-Hydroxy-3-methylglutaryl-CoA reductase
HNE	4-Hydroxynonenal
HNO_2	Nitrous acid
$HO^{\bullet}$	Hydroxyl radical
HOCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
HUVEC	Human Umbilical Vein Endothelial Cells
I	
IL-10	Interleukin
IMP	Inosine 5'monophosphate
IMPCH	Inosine monophosphate cyclohydrolase
L	
L	Large branched-chain neutral amino acids transporter system
L/T-type Ca^{2+}	L and T-type calcium channels
LDL	Low-density lipoprotein
LOH	Lipid hydroxide
$LOO^{\bullet}$	Lipid
LOOH	Lipid hydroperoxides
LVCa	L-type calcium channels
M	

3MST	3-Mercaptopyruvate sulphur transferase
MAPK	Mitogen-activated protein kinase
MAT	Methionine S-adesonyltranferase
Met	Methionine
meTHF	5,10-Methylenetetrahydrofolate
Met-R-O	Methionine sulfoxide residues
MetRS	Methyonyl-tRNA synthetase
miRNA	Micro RNA
MPx	Methionine peroxidase
MRP	Protein complex of transporter
MS	Methionine synthase
MSR	Methionine sulfoxide reductase
mTHF	5-Methyltetrahydrofolate
MTHFR	Methylene tetrahydrofolate reductase
N	
N_2O_3	Dinitrogen trioxide
Na^+	Sodium ion
NAC-X	Mercapturic acid
NAD^+	Nicotinamide adenine dinucleotide oxidized
NADH	Nicotinamide adenine dinucleotide reduced
$NADP^+$	Nicotinamide adenine dinucleotide phosphate oxidized
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NF-kβ / NrF-2	Nuclear factor
$-NH_2$	Amino group
$-NH_4^+$	Ammonia group
NO	Nitric oxide
NO^-	Nitroxyl ion
$NO^\bullet$	Nitric oxide radical
NO_2^-	Nitrite ion
$NO_2^\bullet$	Nitrogen dioxide radical
NO_2Cl	Nitryl chloride
NOH	Nitroxide
NOOH	Nitro hydroperoxides
NOOR	Nitroperoxide
O	
$^\bullet OH$	Hydroxyl radical
5-Oxo	5-Oxoprolinase
O_2	Oxygen molecule
$O_2^{\bullet-}$	Superoxide radical anion
OH^-	Hydroxyl anion
$ONOO^-$	Peroxynitrite
$ONOO^\bullet$	Peroxynitrite radical
ONOOH	Peroxynitrous acid
P	
Pept	Peptidase
PGI2	Prostacyclin
Pi	Inorganic phosphate
Pii	Inorganic pyrophosphate

PKA	Protein kinase A
PKC	Protein kinase C
PLAT	Plasminogen tissue activator
PLC	Phospholipase C
PLP	Pyridoxal-5'-phosphate (active form of vitamin B_6)
PNP	Purine-nucleoside phosphorylase
PPAT	Phosphoribosyl pyrophosphate amidotransferase
PPP	Pentose phosphate pathway
Protein-Met	Methionine residue in the protein complex
Protein-Met-O	Protein complex with methionine sulfoxide residue
PRPP	Phosphoribosyl pyrophosphate
PRPPS	Phosphoribosyl pyrophosphate synthetase
Prx	Peroxiredoxins
R	
$R^{\bullet}$	Free radicals
$R\text{-}CH_3$	Acceptor methylated
RMVEC	Rat Microvascular endothelial cells
RNA	Ribonucleic acid
RNAi	Interference RNA
RNH	Nitroxyl
RNS	Reactive nitrogen species
ROH	Alcohol
R-OH	Alcohol group
ROOH	Hydroperoxide
ROS	Reactive oxygen species
RS	Reactive species
$R\text{-}S^-$	Thiol thiolate anion
R-SH	Thiol protein compound
$R\text{-}SO_2H$	Sulfinic acid
$R\text{-}SO_3H$	Sulfonic acid
R-SOH	Sulfenic acid
S	
S	Sulfur
SAH (or AdoHcy)	S-adenosylhomocysteine
SAHH	S-adenosyl-homocysteine hydrolase
SAICARS	Aminoribosyl-aminoimidazole succinocarboxamide ribonucleotide synthetase
SAM (or AdoMet)	S-adenosylmethionine
Sar	Sarcosine
SBP	Systolic blood pressure
Ser	Serine
SHMT	Serine hydroxymethyltransferase
SOD	Peroxide dismutase
T	
tHcy	Total homocysteine
THF	Tetrahydrofolate
TM	Thrombomodulin
TMG	Trimethylglycine

tRNA	Transfer ribonucleic acid
TRPV	Transient receptor potential cation channel
TrxOxi	Thioredoxin oxidazed form
TrxR	Thioredoxin reductase
TrxRed	Thioredoxin reduced form
TX	Diffusion or active transport
U	
UA	Uric acid
UMP	Uridine monophosphate
UHPLC	Ultra-High Performance Liquid Chromatography
V	
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
X	
X	Xenobiotic molecule or Toxic compound
X_{AG}	Aspartate and glutamate system transporter
XMP	Xanthosine monophosphate
XO	Xanthine oxidase

Aims

The project presented in this thesis had as the main purpose to obtain a master's degree in Applied Biochemistry. This work was developed in the *Centro de Química* (CQM) of the University of Madeira.

Specific objectives

In this dissertation, an extensive bibliographic search and laboratory research were performed. The theoretical introduction highlights all relevant information for the development, analysis and discussion of the experimental work.

The experimental part consisted of two approaches, developed with the following purpose:

First, developed and optimized a pre-analytical procedures for standard treatment of some biomarkers of oxidative stress, for analysis by Ultra-High Performance Liquid Chromatography (UHPLC). The optimization procedure was held for the detection of the standard glutathione in reduced form (GSH), homocysteine (Hcy), cysteine (Cys) and cysteamine (Cyst) with fluorescence (FLR) detection. Additionally, a method of photodiode array (PDA) detection for purine compounds analysis, like adenosine (Ado) and inosine (Ino) was developed.

In the second approach, two analytical methods by UHPLC with FLR and PDA detection for a high sensitivity quantification of thiols and purines was established and optimized. Finally, application in biological samples.

Chapter 1

Introduction

In this chapter, the most relevant aspects of thiol compounds and their derivatives will be reviewed, as biomarkers of oxidative stress. Also, some purine compounds will be mentioned, referring and emphasizing cardiovascular pathologies and some of their factors, like the unbalance of the physiological and homeostatic state of endothelial cells.

Additionally, analytical techniques for their assessment will be focused, including pre-analitical considerations.

1. Oxidative Stress and Redox Systems

The term “stress”, expresses the tension exerted in an organism mediated by a physical or chemical injury, which consequently triggers several mechanisms of defense and adaptation [1]. Cellular stress is a cause of pathology in certain human diseases, such as, hypoxia, immune reactions and infections [2].

The cell is composed of organelles, small structural units bounded by the membrane, which are the basis of their operation and integrity. As such, it can be said that the redox state is also divided into compartments, highlighting the mitochondria, the endoplasmic reticulum, the nucleus and the cytoplasm, as the main areas of redox signaling. Comparatively, it is in the mitochondria, where there is a greater redox activity, since its membrane has five multiprotein complexes, which are responsible for the oxidative phosphorylation. These are the NADH-quinone oxidoreductase, succinate dehydrogenase, coenzyme Q cystochrome reductase, cytochrome oxidase and ATP synthase [3].

In cell metabolism, it is possible to find different redox systems, such as, the pairs flavine adenine dinucleotides ($FADH_2$/FAD), nicotinamide adenine dinucleotides (NADH/NAD^+ and NADPH/$NADP^+$), thioredoxins (TrxRed/TrxOxi) and glutathiones (GSH/GSSG) [4, 5]. They promote a dynamic regulation that maintains the cells in good oxy-reductive conditions, by balancing the concentrations of each coupled form [6].

Under normal conditions, the formation and neutralization of the reactive oxygen (ROS) and nitrogen (RNS) species and all the species listed in table 1, are counterbalanced by the above antioxidant systems [7].

Table 1 - Different reactive species that are found in organisms, under normal or pathological conditions. Adapted from [8 - 10]:

Reactive Oxygen Species (ROS)	Reactive Nitrogem Speies (RNS)
Superoxide radical ($O_2^{\bullet -}$)	Nitrogen dioxide radical ($NO_2^{\bullet}$)
Hydrogen peroxide (H_2O_2)	Nitrite ion (NO_2^-)
Hydroxyl radical ($HO^{\bullet}$)	Nitric oxide radical ($NO^{\bullet}$)
Hydroxyl ion (HO^-)	Nitroxyl ion (NO^-)
Hydroxyl (ROH)	Nitroxyl (RNH)
Hydroperoxide (ROOH)	Nitroperoxide (NOOR)
Lipid peroxyl radical ($LOO^{\bullet}$)	Dinitrogen trioxide (N_2O_3)
Lipid hydroxide (LOH)	Nitroxide (NOH)
Lipid hydroperoxide (LOOH)	Nitrous acid (HNO_2)
Hydrochloric acid (HOCl)	Nitryl chloride (NO_2Cl)
Peroxynitrite radical ($ONOO^{\bullet}$)	
Peroxynitrite ion ($ONOO^-$)	
Peroxynitrous acid (ONOOH)	

Antioxidants are molecules that prevent oxidative reactions caused by ROS and RNS occurring with damage effects [7]. The regulation of antioxidant systems is crucial for a proper cell function, which is achieved by lowering levels of reactive species. Several studies have shown that these systems are important protective agents, because they avoid abrupt and irreversible changes on metabolic molecules and irreparable damage of deoxyribonucleic acid (DNA), which in turn could induce cell death [4].

In some physiological conditions, the regulatory mechanisms may not be effective, for example, when the levels of antioxidants are very low or nonexistent, or when there is absence or inactivation of some of the enzymes involved in those mechanisms [11]. In these cases, excess of reactive species compromises the metabolic balance or even the cell viability. However, the consequences of these possible situations depend on where it happens, the availability of energy and the plasticity of the cells [2]. Therefore, oxidative stress may arise and translate into an imbalance between oxidants and neutralizing compounds, leading to disruption of the redox signalling, loss of homeostatic control and can consequently, cause genetic damage [12]. Therefore, a condition that results from the accumulation of oxidative compounds, in detriment of the reducing ones, reduction of the antioxidant agents, or even, by synergy of these two situations [13].

In aerobic metabolism, the inevitable oxygenated status can be a hostile environment, what is a contradiction of life. The oxygen molecule (O_2) is essential in the respiration process and energy metabolism but, at the same time, is the pathogenic basis of many diseases or degenerative conditions of living beings. The theoretical principle, states that a single inspiration contains O_2 capable of producing billions of reactive species that may have signaling or destructive action [14]. However, the cells have antioxidant compounds and enzymes that develop protection strategies, which are effective pathways adapted to survival, in this

oxygenated and stress conditioned environment [12]. Still, there is always a chance of something going wrong, at metabolic level, and oxidative damage may arise.

Accordingly, the main consequences of oxidative stress are the molecular changes in nucleic acids, lipids and proteins that compromise cell stability or even, viability [15]. Thus, in extreme cases, the cell promotes secondary responses to induce death by necrosis or apoptosis. Moreover, in most fortunate cases, the cell is able to restore the redox homeostatic state and the metabolism back to be in equilibrium without negative consequences [11].

1.1. Oxidative Stress and Endothelial Cells

The endothelial cells (EC) line on the entire surface of the vascular system, forming a barrier between the blood and the other organs. It is a versatile and multifunctional monolayer that is essential in the structural support and integrity of the vascular wall throughout all circulation system. These are cells with high metabolic activity, with endocrine, paracrine and autocrine functions. They form a dynamic and semi-permeable barrier, which controls the transport of a large number and variety of metabolites between the different tissues [16]. In its metabolic properties, regulation of the vascular tone that is characterized by the normal state of firmness and elasticity of an organ or tissue, through the production of vasodilator and vasoconstrictor molecules, stands out. At this level, the control of the flow and blood clotting happens by the action of platelet factors and regulation of inflammatory responses [17].

Under physiological conditions, EC express and secrete a number of specific metabolic compounds in response to different stimulations. Among the activating molecules, we present some of those that stand out in the anticoagulant reactions, such as, prostacyclin (PGI2), nitric oxide (NO), thrombomodulin (TM) and plasminogen tissue activator (PLAT); the latter, also presents pro-coagulant action; the vasodilators NO, PGI2 and endothelin, and the vasoconstrictors endothelin, thromboxane and prostaglandins [18, 19]. Furthermore, EC mediate vascular proliferation (angiogenesis) in synergy with platelets, smooth muscle cells and the matrix constituents (fibronectin, laminin, collagen and proteoglycans) [20, 21].

A relevant fact, is that the growth and survival of EC depend on the intracellular production of reactive species, such as, superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), as they are responsible for the activation of certain essential metabolic proteins, such as, mitogenic kinases (ERK1/2 MAPK) and vascular endothelial growth factors (VEGF) [22]. In addition, their circulation integrity results from the action of thrombin and adenosine diphosphate (ADP) in synergy with the changes of pressure and blood flow, which in turn induce the secretion of prostacyclin, NO and vasoactive substances that inhibit platelet aggregation and cause vasodilation [20]. Futhermore, an inflammatory response outcome from damage on the vascular endothelium, leads to multiple hemodynamic changes, such as, dilation, increased blood pressure and segregation of specific mediators, in order to inhibit and repair the injury [23]. Thus, it is understandable that cell barrier disruptions are the source of certain pathological states in the cardiovascular system.

The syndrome of endothelial dysfunction is a disorder that expresses vascular abnormalities, which are the consequence of damage in the thrombogenic and angiogenic properties. This may result from loss of tone by the vascular smooth muscle or, from blocking of the normal immunologic response [24]. This disturbance is characterized essentially by reduced NO bioavailability and production. Also, by increased adhesion of monocytes and polymorphonucleate cells, cholesterol and oxidized low-density lipoprotein (LDL) accumulation, signaling failure of endothelium derivatives, expression of pro-fibrotic genes and premature cell senescence or apoptosis [24, 25].

Risk factors of oxidative stress and endothelial dysfunction are classified as i) behavioral, such as, smoking, malnutrition, physical inactivity and obesity, and ii) biological determinants, like gender, aging, dyslipidemia, hypertension, diabetes, family history and genetic defects [26, 27]. These factos affect functional integrity of the endothelium, and should always be taken into consideration at the clinical level.

The endothelial health is balanced (figure 1) by damage factors and regenerative capacity [28]. In the cardiovascular system, the regeneration is promoted by a decrease in the inflammatory response, which is achieved by reducing cell interactions, such as, decreasing macrophage phagocytosis of LDL, secreting platelets and inhibiting foam cells formation [29]. That balance is also promoted by the circulating endothelial cells (CEC), which derive from the endothelial layer of vessels, and by the circulating endothelial progenitor cells (CEPC) from bone marrow, in the bloodstream [28]. These cell types are major promoters of the integrity, are responsible for angiogenesis and regeneration of the endothelium. Therefore, they prevent, attenuate or delay the development of atherosclerosis and improve the blood flow [29].

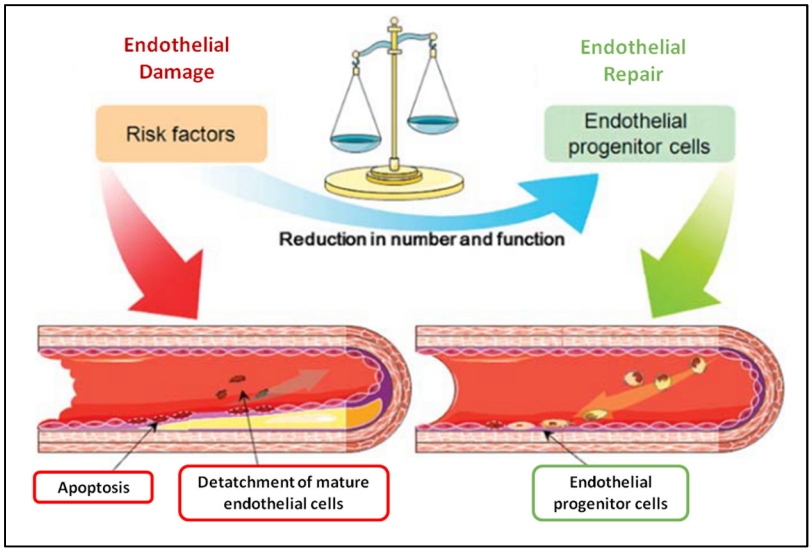

Figure 1 - Counterbalancing of endothelial damage with the regenerative capacity. Image obtained from Fadini *et al.* [28].

1.2. Biomarkers of Oxidative Stress

The term biomarker features a measurable compound as an indicator of normal biological metabolism, pathological conditions or pharmacological responses to therapeutic processes [30]. Therefore, a biomarker should help in symptomatic or pre-symptomatic disease diagnosis and provide data results showing the clinical effectiveness of treatments [15]. In theory, to be validated as a biomarker of oxidative stress, a compound must have the following characteristics: i) be stable, not susceptible to oxidative induction or loss due to handling, processing, analysis or storage; ii) be the main product that is directly involved in the initiation or progression of the pathological condition; iii) be accessible on the target tissue and quantitatively translate the metabolic changes [30]; iv) be a significant product present in high concentrations and free of confounding factors, such as from diet; v) be identified and quantified by noninvasive and painless methods; vi) its analysis must be specific, sensitive and reproducible [30]; vii) be easy to detect and quantify between populations; viii) have relatively stable concentrations, which do not vary widely in the same person, in different times and conditions; ix) be measurable with relatively small variation in intra and inter sample tests [30].

Validation requires multiple steps that are often difficult to achieve, because of the complexity of the metabolic pathways.

Measurement of oxidative stress and antioxidant defense biomarkers is a major challenge in research studies. Next, the most relevant topics for each thiol and purine compound analyzed in this dissertation are presented, including properties, functional characteristics, metabolic and regulatory pathways, as well as, some pathological associations. Finally, the relationship and metabolic link between them is showed.

1.2.2. Thiol Compounds

Thiol compounds and their derivatives are widely distributed in nature, either in prokaryotic or eukaryotic cells, in plant and animal tissue [31]. These compounds are synthesized and then sent to the required locations, where they are essential for the proper functioning of organisms. Thiols are involved in crucial physiological processes that include elimination of toxins, redox signalling, transport, metabolic storage and protein functionality, as well as, gene expression, proliferation, differentiation and even in cell death [32]. Several studies have shown that the maladjustment of these roles is the basis of many human diseases, which is why there is a great effort of the scientific community to better understand their metabolic implications.

Thiol compounds and their derivatives are important scientific targets, because they are ubiquitous and critical in many physiological processes. Several studies have been carried out in different biological samples trying to demonstrate that they are the basis of many human pathologies and their importance as biomarkers at clinical and pharmacological levels has been shown [33]. They are low molecular wietgh compounds, considered below 900 Da [34]. In their structure there is one (or more) sulfhydryl groups (R-SH), sulphur and hydrogen

linked to a carbon atom, also known as thiol or mercaptan group. The R-SH group is analogue to alcohol group (R-OH) but form weaker hydrogen bonds. Therefore, these compounds are volatile [35], highly polar, soluble in water and with low boiling points [36]. The sulfur (S) is an abundant non-metal atom, which can be incorporated into molecules, giving rise to sulfate or be reduced to sulfite, the main substrate in cysteine synthesis [37]. Thiol group is considered to be the most reactive group present in cells. Actually, it is involved in many metabolic reactions that depend on the oxidative capacity, a specific biochemical property that distinguishes them from other molecules with metabolic functions [37]. In figure 2 the most relevant organic sulfur compounds in cell metabolism, are highlighted.

The thiolate anions ($R\text{-}S^-$) are more reactive than the sulfhydryl group, becouse they are easily oxidized to different products, such as, sulfenic acid (R-SOH), sulfinic acid ($R\text{-}SO_2H$) and sulfonic acid ($R\text{-}SO_3H$) [7]. The sulfite compound is less reactive than the thiol that gives rise to it, but it is extremely important in the stability of protein complexes, in polypeptide chains with tertiary and quaternary three-dimensional structure, as well as, in the functionality of the active site of some metabolic enzymes. The biosyntheses of organosulfur compounds occur mainly in bacteria and plants, while the oxidation commonly occurs in animals [37].

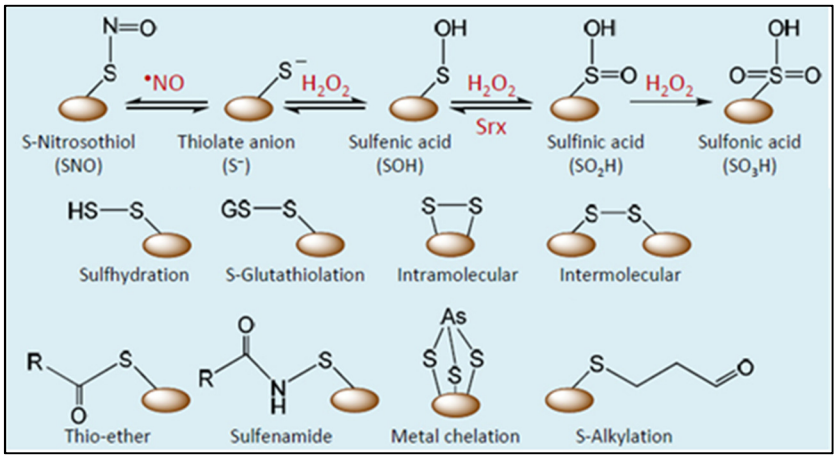

Figure 2 - Different thiol compounds that are present in biological systems. Abbreviations: H_2O_2, hydrogen peroxide; •NO, nitric oxide; Srx, sulfiredoxin. Image obtained from Ma [7].

Endogenous hydrogen sulfide (H_2S) is considered an important signaling molecule for EC and cardiovascular system (Figure 3) [38]. It is produced by enzymatic reactions of cystathionase γ-liase (CGL), cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulphur transferase (3MST). Subsequently, it suffers different conversions. It may be oxidized to hydrosulfide ion (HS^-) or thiolsulfate forms, may produce sulfhemoglobin reacting with methemoglobin, and can be methylated to methanethiol or dimethylsulfide. It has effects on endothelial, but also in smooth muscle and inflammatory cells, at mitochondria and over nuclear transcription factors [39]. It is considered a toxic molecule, but it also has beneficial effects in blood pressure control and regulation of inflammation.

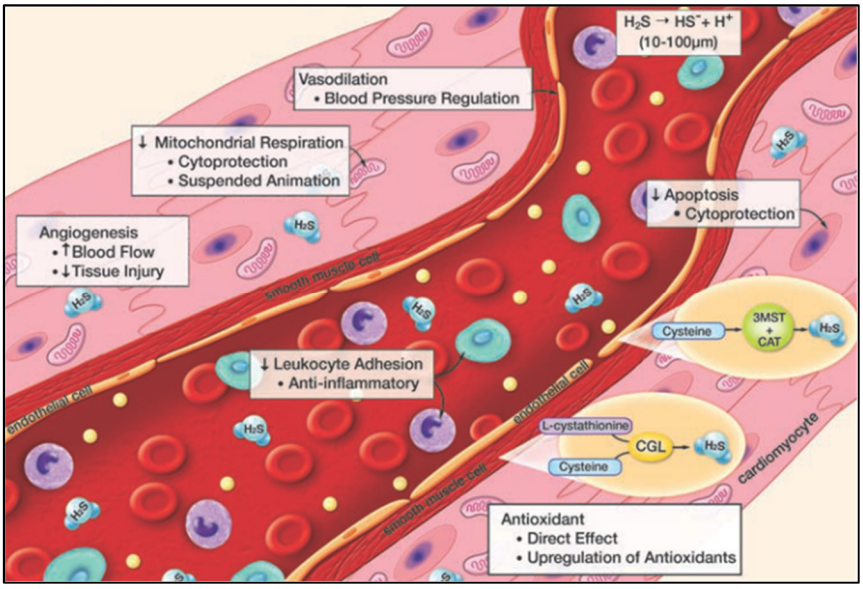

Figure 3 - Cardiovascular functions of hydrogen sulfide (H_2S). Abbreviations: H_2O_2, hydrogen peroxide; 3MST, 3-mercaptopyruvate sulphur transferase; SH^-, hydrosulfide ion; H^+, free hydrogen cation; CAT, cysteine amino transferase; CGL, cystathionase γ-liase. Image obtained from Calvert *et al.* [38].

According to its concentration, H_2S (ranging from 10 to 100 μM [38]) can produce cytoprotection actions, through i) protein modifications (S-sulfhydration); ii) modulation of ion channels, such as, vascular K_{ATP} channels, transient receptor potential cation channel (TRPV) and calcium channel (L/T-type Ca^{2+}) iii) stimulation of the production of antioxidant molecules, like nuclear factors (NrF-2) and glutathione [39]; and iv) decrease of mitochondrial functions [40]. The purpose is to prevent oxidative stress, apoptoctic and necrotic mechanisms. In some conditions, like in heart failure, administration of H_2S has shown to be a promoter of remodelling processes [41]. Similarly, in atherosclerosis, this molecule modulates vasodilatation or vasoconstriction reactions, inhibits the expression of leukocyte adhesion molecules and formation of foam cells. In addition, it activates pro-angiogenic mechanisms, prevents edema formation and it is an anti-apoptotic mediator [39]. This is a compound related to the thiol homocysteine, and its metabolic importance will be specified further on.

Also important in this group of thiol compounds, are some amino acids, which have been implicated in the pathophysiology of a wide range of chronic conditions [42]. As a matter of fact, sulphur amino acids like methionine (Met), homocysteine (Hcy) and cysteine (Cys) are being investigated as potential indicators of health status and disease risk. Glutathione, the most abundant mammalian antioxidant, a tripeptide that is linked to Hcy metabolism via the transsulphuration pathway, which will be specified later on.

Some human disorders are associated with increase or decrease of the concentrations of thiol compounds and their derivatives, which in synergy with the reactive species cause oxidative damage, dysfunction and activation of the vascular endothelium. There are numerous disorders related to these conditions, such as, hypertension, heart attack or failure, angina, pulmonary hypertension, stroke, peripheral artery disease, macular degeneration,

Alzheimer disease, dementia, sleep apnea, renal failure, diabetes, erectile dysfunction, among others [43].

1.2.2.1. Glutathione

In 1888, Rey-Pailhade identified a compound that he called *Philothione*, a Greek expression, which means "sulfur friend", in yeasts. Later in 1927, he changed the name to glutathione (GSH), when Hopkins identified it as a dipeptide of glutamic acid (Glu) and Cys [44]. However, in the same year, Hunter and Eagles conducted more studies and realized that there was an error [45]. As such, in 1929 [46] the compound was reanalyzed in yeast and erythrocytes, and GSH was recharacterized as a tripeptide also composed by glycine (Gly) (figure 4). Hopkins [45, 46] noted that, biochemically, this molecule exhibited an exceptional behavior, that it was a very unstable compound due to the sulfur atom in the Cys residue. He even stated that this instability resided in all molecules formed by Cys or its derivatives, which could lead to GSH misidentification [45, 46].

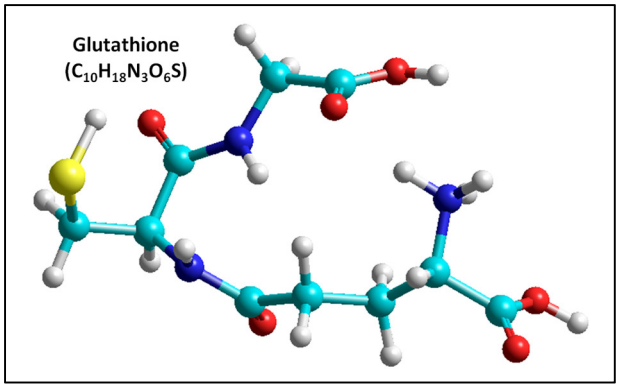

Figure 4 - Representation of the molecular structure of glutathione.

I. Glutathione syntesis

The thiol compound GSH results from enzymatic reactions**,** which occur in the cytoplasm of cells and extracellular space of all organic tissues (Figure 5). The formation, from the constituent amino acids starts with the activation of two ATP-dependent enzymes [47]. Initially, the γ-glutamylcysteine ligase (GCL) catalyzes the peptide bond between Glu and Cys, creating the dipeptide γ-GluCys. Subsequently, glutathione synthetase (GS) adds Gly amino acid, forming GSH [48].

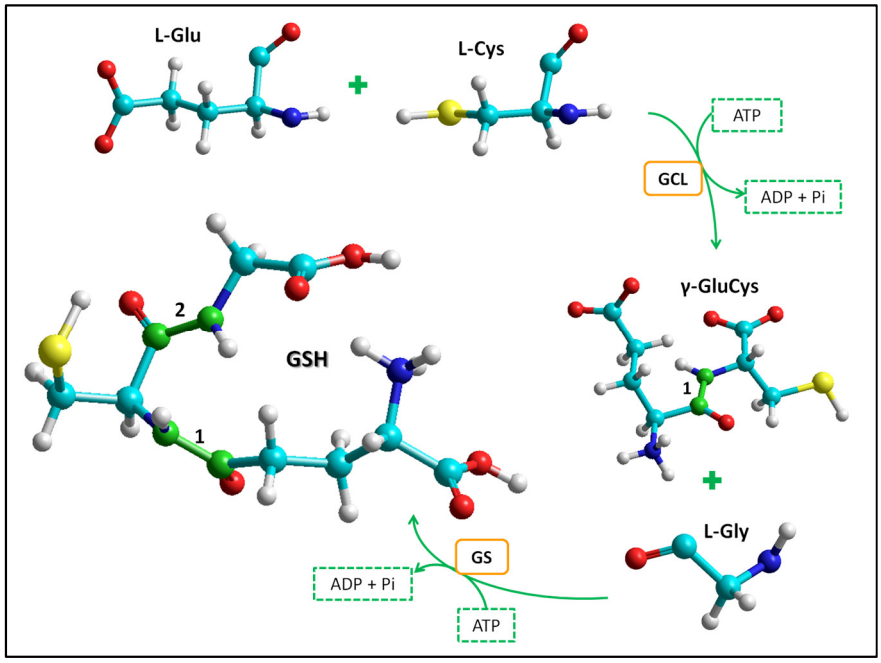

Figure 5 – Enzymatic synthesis of glutathione. Abbreviations: ADP, adenosine 5'diphosphate; ATP, adenosine 5'triphosphate; GCL, γ-glutamylcysteine ligase; GS, glutathione synthetase; L-Cys, cysteine; L-Glu, glutamic acid; L-Gly, glycine; Pi, inorganic phosphate; γ-GluCys, gamma-glutamylcysteine; **1.** Chemical bond between the γ-carbonyl group of glutamic acid and the amine group of cysteine; **2.** Chemical bond between the α-carbonyl group of cysteine and the amine group of glycine. Adapted from Lu [47].

The biosynthesis of GSH is initially regulated by gene expression and metabolism is further controlled by feedback signaling mechanisms, following homeostatic conditions [49]. GSH synthesis is limited by ATP bioavailability and by the catalytic action of GCL and MS enzymes. Besides, GCL is inhibited by negative feedback when GSH concentrations reach high levels [4].

In GSH molecule, each amino acid has unique features that are critical in its general skills and properties. GSH presents a unique structure due to the binding between Glu and Cys, which uses the γ-carbonyl group rather than the typical α-carboxyl bond. This rare union is resistant to the endogenous action of peptidases and is only degraded by the gamma-glutamyl transpeptidase (GGT) [50]. GGT is an ecto-enzyme, it has an active centre facing the outside and a higher affinity for Met, Glu, Cys and arginine (Arg) [51]. Orlowski and Meister [51] studied the activity of this enzyme in mice kidney and found that the reactions of GSH synthesis and degradation are coupled to major transport ways.

II. Glutathione and the gamma-glutamyl cycle

Described by Meister in 1970 [51] the gamma-glutamyl cycle represented in figure 6, is a key route in the bioavailability of GSH and Cys in the living organism. Meister investigated GSH metabolism in mice kidneys and characterized the catalytic reactions of this compound, which are associated with the transport of amino acids. He observed that GSH is clived by GGT

and, at the same time, the latest mediates the uptake of amino acids by other specific carriers, from the extracellular space up to the cytoplasm [51, 52]. So, GGT converts GSH into γ-glutamylaminoacid (γ-Glu-aa) releasing cysteinylglycine (CysGly), in the extracellular space. This dipeptide, suffers degradation by some exopeptidases and their amino acids are provided for new metabolic reactions. The γ-Glu-aa is then imported and, in the intracellular space, it is catalysed by γ-glutamyl cycle transferase (γ-GGCT), which releases the transported amino acid and 5-oxoproline complex. It is then converted to Glu through the action of 5-oxoprolinase (5-Oxo) [47, 51]. Following the cycle, GSH is regenerated by the subsequent reactions, which have already been described and schematized in figure 5.

GGT is a canalicular enzyme [1], which has been identified through histological studies, in the apical surfaces of several epithelial tissues, such as, the cells covering the villi in the jejunum, external portion of pancreas, liver, bile, seminal vesicles, epididymis, fallopian tubes, ovaries, endometrium, prostatic ducts, submaxillary and mammary glands, ependymal cells of the brain and in the endothelium of blood capillaries [51], among others.

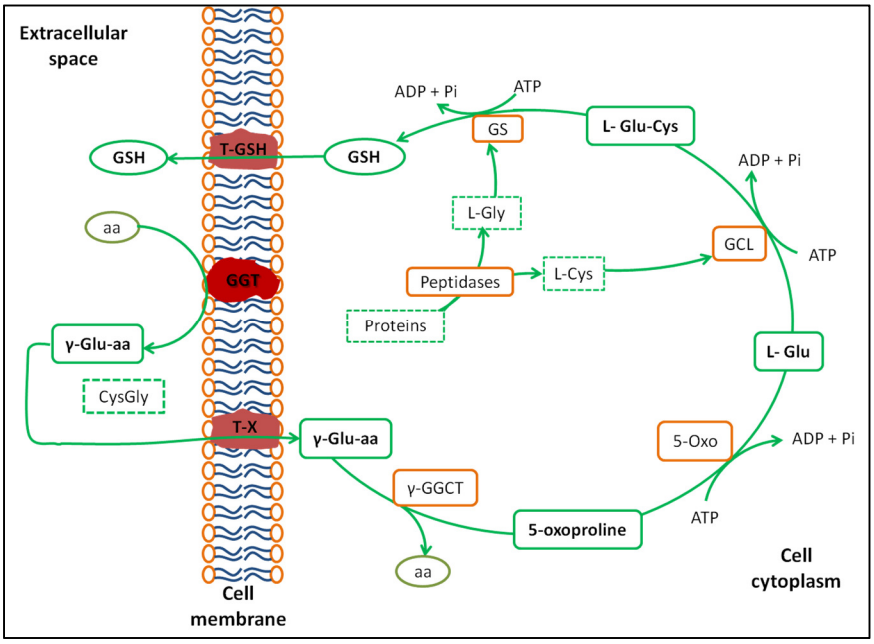

Figure 6 - Gamma-glutamyl cycle expressing glutathione metabolism associated with the transport of amino acids. Abbreviatons: 5-Oxo, 5-oxoprolinase; aa, amino acid; ADP, adenosine 5'diphosphate; ATP, adenosine 5'triphosphate; GCL, gamma-glutamylcysteine ligase; GGT, gamma-glutamyl transpeptidase; GS, glutathione synthetase; L-Cys, cysteine; L-Glu, glutamic acid; L-Glu-Cys, glutamylcysteine; L-Gly, glycine; Pi, inorganic phosphate; T-GSH, glutathione transporter protein complex; T-X, transporter X; γ-GGCT, gamma-glutamyl cyclotransferase; γ-Glu-aa, gamma-glutamyl amino acid. Adapted from [47, 51].

Therefore, GSH synthesis and catabolism have a number of enzymatic steps regulated and controled by specific transporters. GSH degradation occurs exclusively on the surface of the cell membrane that has GGT, as mentioned above. GGT is the only ecto-enzyme capable of degrading GSH complex (conjugated and oxidized glutathione) [47].

In 1970, Meister [51] speculated that the gamma-glutamyl cycle could be crucial for metabolic transport and/or secretion and, that the presence of GSH was indispensable. Fifteen years later [52], he confirmed that the transport of γ-Glu-aa is one step of the cycle, which mediates the uptake of amino acids. He also found that this transport system is inhibited by high GSH concentrations in the extracellular space, as well as, low GSH concentrations inside the cells [52, 53].

A limiting factor of GSH synthesis is the bioavailability of Cys [53]. This amino acid is in higher concentration in the cytoplasm and, externally, it is more abundant in the oxidized form, as cystine (2Cys). Therefore, it seems that cells perform the gamma-glutamyl cycle also as a Cys source [54]. However, numerous studies have tried to clarify the uptake of Cys and other amino acids by cells, but until now, there is no consensus, because in the several reports, different cells were used (for example: astrocytes [55], red blood cells, endothelium or epithelium [56]). The uptake of Cys and 2Cys is surely mediated by different carriers, which can be coupled or in antiport. It also depends on sodium (Na^+) or chlorine (Cl^-) ions. However, all studies confirm that the transport of amino acids is an important feature in antioxidant defence mechanisms, as induced by GSH [57, 58].

III. Glutathione oxidation pathway

GSH is a molecule with a high redox potential [4]. In certain physiological conditions, it may be in the reduced (GSH) or in the oxidized (GSSG) tripeptide form (Figure 7). The latter, results from the intermolecular binding of two reduced forms by a bisulfite bond (GH-S-S-GH), a reaction that is mediated by direct oxidation of ROS or by enzymatic action of glutathione peroxidase (GPx) a key selenoprotein [4, 59].

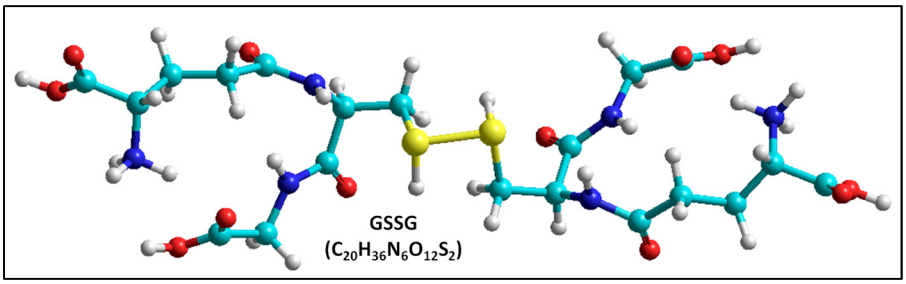

Figure 7 - Representation of the molecular structure of oxidized glutathione.

The reduced form GSH is mainly responsible for maintaining the redox state in optimal levels, contributing to proper cell function, since 90% of total GSH is in this form. The GSH:GSSG ratio is maintained by synergy of all metabolic pathways: synthesis, enzymatic reduction of GSSG and external degradation of GSH forms [60]. Glutathione reductase (GR) is the enzyme responsible for maintaining a constant balance of the pair GSH:GSSG, a critical feature in living beings [4]. While GR uses NADPH in the conversion of GSSG to GSH, oxygen free radicals convert GSH back into GSSG [61], as shown in figure 8. In addition, GSH can form conjugates (GS-R) by association to proteins. Therefore, overall GSH levels are balanced between production and consumption [53].

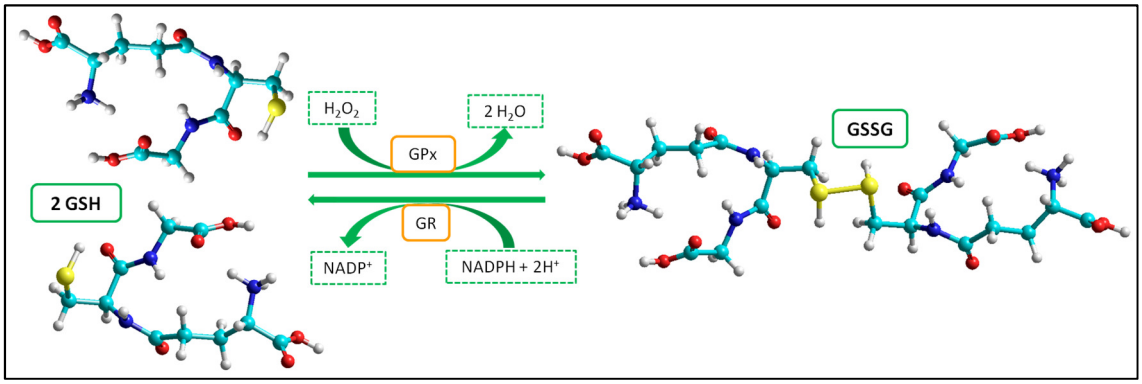

Figure 8 - Oxidation and reduction reactions of glutathione. Abbreviations: H^+, free hydrogen cation; H_2O, water; H_2O_2, hydrogen peroxide; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; NADPH, nicotinamide adenine dinucleotide phosphate reduced; $NADP^+$, nicotinamide adenine dinucleotide phosphate oxidized. Adapted from Lushcak [4].

IV. Physiological levels of glutathione

GSH is present in all types of cells. GSH total concentration, quantified on plasma samples, ranges from 2 to 20 µM [62] and inside cells varies between 0.1 and 10 mM [63]. These values depend on the type of tissue cell and the overall homeostatic conditions displayed. For instance, in neurons it is approximately 2.5 mM and in astrocytes about 3.8 mM [53]. Intracellular GSH is in the millimolar (mM or mmol/L) range and extracellularly it is in the micromolar (µM or µmol/L) range. GSH levels decrease with increasing transpeptidation [64].

Quantification is not accurate so far. However, it is estimated that cells have three major GSH reservoirs, which are 80 to 85% in the cytoplasm, about 10% in the mitochondria and a small percentage in the endoplasmic reticulum [47, 48]. It is considered that 99.5% of the circulating GSH is linked to the red blood cells [65] and, in the extracellular space, the concentration is very low, because is rapidly metabolized. In 1980, Wendel and Cikryt [66] performed an *in vivo* experiment and determined that, in human plasma, the halt-life time of GSH is around 1.6 minutes. It is important to note that GSH concentrations are different between tissues due to its functions. In accordance, there is a higher concentration in the liver. However, it also depends on the prevalence of some pathological conditions.

V. Specific functions of glutathione

GSH is a multifunctional molecule. Hence, it interferes with metabolic pathways that are vital to cells [67]. Until now, it has been associated with numerous functions and others are still being evaluated. GSH chemical structure is the main determinant of the biological roles that it plays. This compound has two structural features, the gamma-glutamyl bond and the sulfhydryl group, which are closely linked to the performed functions [49]. We can say that GSH functions are linked to the antioxidant defense and regulation of some metabolic pathways. It is involved in a variety of reactions that emphasize its protective action: i)

elimination of ROS and ERN; ii) detoxification of endogenous and exogenous toxins [68]; iii) maintenance of normal levels of essential thiols, for proteins synthesis and other molecules, such as vitamin C and E; iv) control of Cys storage; v) mediation of copper and iron ions transfer [69]; vi) intervention in hormone metabolism, like prostaglandins, estrogen and leukotriene [4]; vii) mediation of ribonucleotides reduction both in DNA synthesis and signal transduction, by enzymatic regulation; viii) regulation of mitochondrial function and integrity [70, 71]; ix) control of cell proliferation and apoptosis [4].

Some of the above functions are here specified:

Detoxification - The detoxifying action mediated by GSH is translated in the mercapturic acid pathway [72], shown in figure 9. It consists of a sequence of reactions that neutralize and eliminate toxic compounds and their metabolic derivatives, harmful to cells. This protection mechanism begins with the conjugation of GSH with xenobiotic (X) molecules, by a spontaneous reaction or, it may be mediated by glutathione S-transferase (GST) enzyme, which produces glutathione conjugates (GS-X) [47]. Subsequently, the conjugate compounds are transported across the membrane by the human multidrug resistant-associated protein (MRP), an ATP-dependent complex. Outside the cell, they are degraded by the GGT enzyme followed by the action of peptidases, giving rise to a conjugate of cysteine (C-X). This comes later into the cell, where it is acetylated to mercapturic acid by N-acetyl transferase enzyme, or is transformed into a very reactive thiol compound, by the action of β-liase enzyme [47, 72]. Therefore, the resulting compounds are polar and highly hydrophilic, which facilitates their cell transportation and elimination. In this way, GGT is an enzyme that protects cells from potentially mutagenic and carcinogenic compounds.

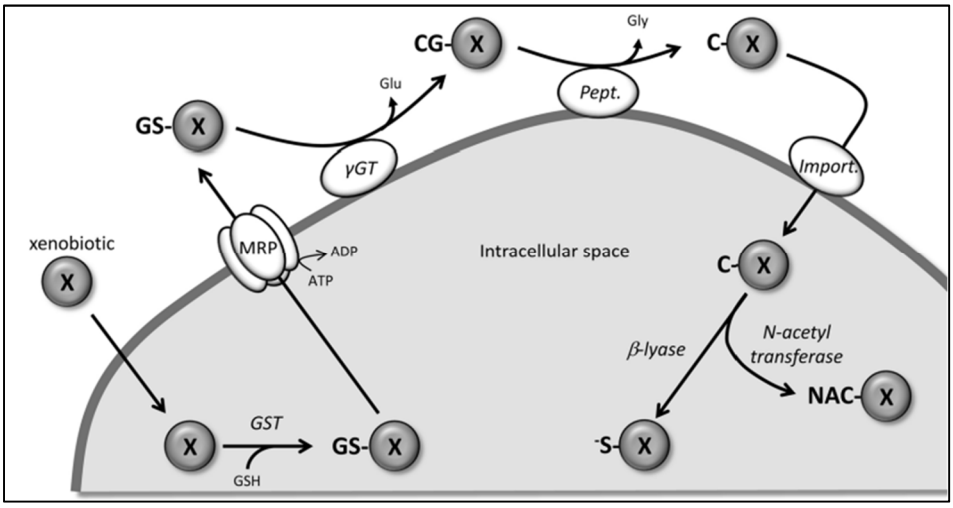

Figure 9 - Detoxification pathway mediated by glutathione. Abbreviatons: ADP, adenosine 5'diphosphate; ATP, adenosine 5'triphosphate; CG-X, conjugate cysteinylglycine; C-X, cysteine conjugate; Glu, glutamic acid; Gly, glycine; GSH, reduced glutathione; GST, glutathione S-transferase; GS-X, conjugate glutathione; MRP, protein complex of transporter; NAC-X, mercapturic acid; Pept, peptidase; $^{-}$S-X, conjugated thiol; X, toxic compound; γGT (or GGT), gamma-glutamyl transferase. Image obtained from Ramsay *et al.* [72].

Elimination of reactive species - In living organisms, over 90% of the O_2 consumed is directly reduced to H_2O by the electron transport chain, at the mitochondrial membrane of cells, with the purpose of producing energy in ATP form [3]. The remaining O_2 is reduced, as shown in figure 10, by elimination of an electron, giving rise to $O_2^{\bullet -}$. After this, an electron is removed and two hydrogen cations (H^+) are accepted, forming H_2O_2. The latter, accepts an electron, dividing in a hydroxyl radical ($HO^{\bullet}$) and a hydroxyl anion (HO^-), which subsequently, produce water [11, 49]. Therefore, this is one of the means responsible for the emergence of ROS and free radical ($R^{\bullet}$) compounds.

The amount and types of species that are generated depends on the physiological state of the organism [3]. It is important to note, that ROS are important signaling molecules, whose action can have negative or positive consequences. It depends on the time and intensity of the products, which can mediate an adaptive or a pathological condition [73]. Therefore, low levels of ROS favour cell proliferation, intermediate levels can lead to senescence and high concentrations may induce cell death by apoptosis or necrosis.

The mechanisms that regulate ROS levels arise not only by direct elimination, but also by the anti-oxidation systems, where low molecular weight compounds like GSH, intervene. Proteins with enzymatic activity, such as, glutathione peroxidase (GPx), glutaredoxin (Grx), peroxiredoxins (Prx) and thioredoxin reductase (TrxR) are associated to those systems [3, 5]. For example, GSH and Trx are involved in the electron transport chain, in an antioxidant pathway of mitochondria [74]. Moreover, the GSH:GSSG pair relies on the availability of each molecule, since glutathione is synthesized in the cytoplasm and is then targeted to the organelles. So, transport is a crucial process [47]. In figure 10 the reaction mediated by peroxide dismutase (SOD), which catalyses the reduction of $O_2^{\bullet -}$ originating H_2O_2 and catalase, is represented. The scheme shows the reduction of H_2O_2 by GPx coupled to GSH, originating H_2O and GSSG [4, 11]. In addition, the oxidation products from fatty acids and phospholipids, like lipid hydroperoxides (LOOH) and nitro hydroperoxides (NOOH), which are neutralized by GSH associated to GPx or GST, are also shown. Similar protective actions can be mediated by GSH over the RNS compounds, like the peroxynitrites ($ONOO^-$) [11].

In order to maintain GSH levels available for the reduction of ROS, the oxidative form GSSG is constantly regenerated by GR through the reaction shown in figure 5.

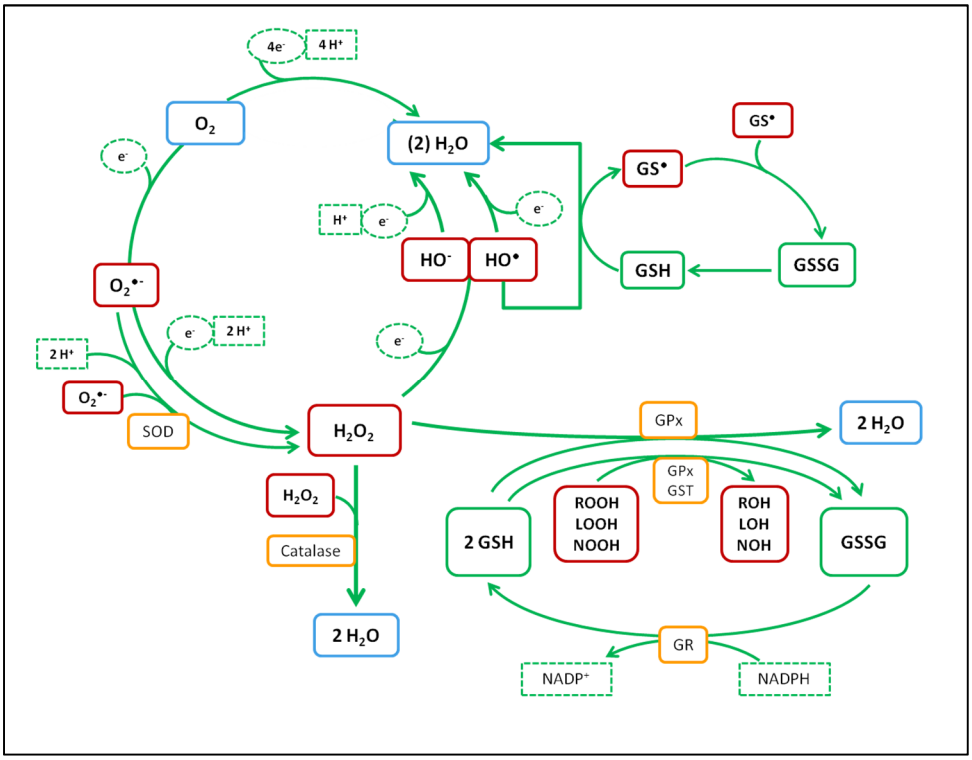

Figure 10 - Synthesis and neutralization of reactive species. Abbreviatons: e^-, free electron; GPx, glutathione peroxidase; GR, glutathione reductase; $GS^\bullet$, glutathione radical; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; H^+, hydrogen cation; H_2, free hydrogen; H_2O, water; H_2O_2, hydrogen peroxide; HNE, 4-hydroxynonenal; $HO^\bullet$, hydroxyl radical; LOH, lipid hydroxide; LOOH, lipid hydroperoxide; $NADP^+$, nicotinamide adenine dinucleotide phosphate oxidazed; NADPH, nicotinamide adenine dinucleotide phosphate reduced; NOH, nitroxide; NOOR, nitroperoxide; O_2, oxygen; $O_2^{\bullet-}$, superoxide radical; ROH, alcohol; ROOH, hydroperoxide; SOD, dismutase peroxide. Adapted from [4, 11].

VI. Oxidative stress and glutathionylation

GSH is seen as the main cellular redox buffer. Therefore, the detection and quantification of the GSH:GSSG ratio has been considered an important indicator of the redox state of cells [75]. In case of oxidative stress, protein modifications mediated by GSH molecules emerge, being called glutathionylation. These are considered important functional changes, which are responsible for metabolic, transcriptional and signalling processes [76]. That reaction is also a way to prevent the loss of GSH, by cellular exportation of the GSSG form (in case of oxidative stress, where GSSG is accumulated).

The Cys residues from protein complexes suffer oxidative modifications, mediated by the presence of reactive species and GSH, which result in the formation of the different organic sulfur compounds, previously mentioned. In figure 11A, it is possible to verify that in the presence of ROS, thiol protein compounds (highlighted in red) can be oxidized to sulfenic, sulfinic or sulfonic, and even induce intra or intermolecular modifications. In addition, when the oxidation involves RNS, S-nitrosylated proteins emerge [77]. Each of these changes can

have positive (activation) or negative (inhibition) consequences in signaling, regulation and protein functionality of cells, particularly in proteins with enzymatic action [76]. At this level, the presence of GSH and the reaction of glutathionylation (Figure 11B) are decisive in redox regulation [77], and we emphasize the reversibility and specificity of this reaction.

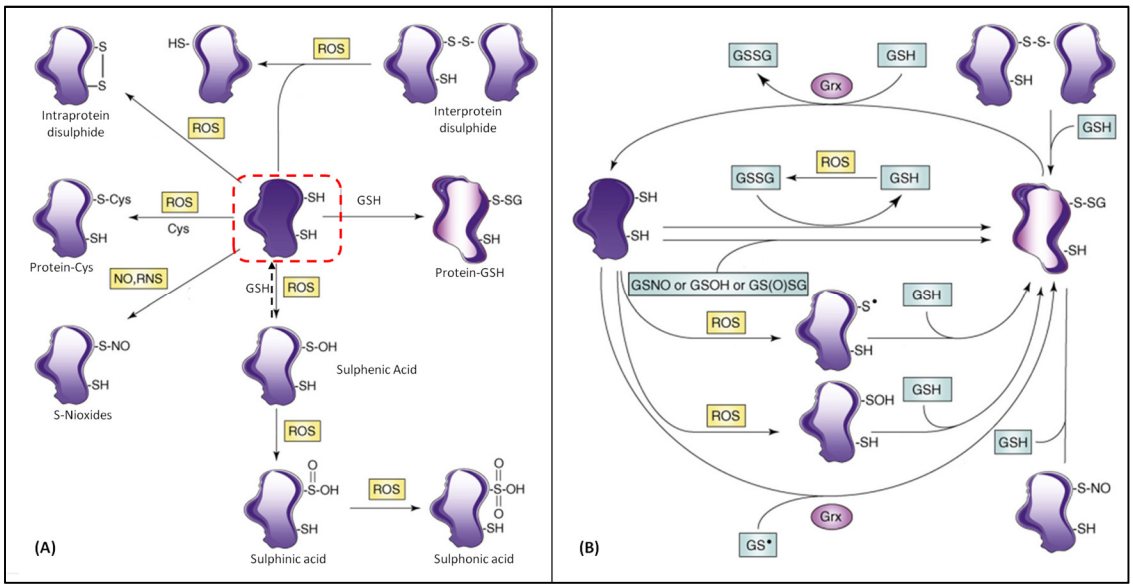

Figure 11 - Oxidative modifications of the thiol containing in proteins (A) and the glutathionylation reaction (B). Abbreviatons: GPx, glutathione peroxidase; GS(O)SG, glutathione disulphide S-monoxide; GS•, glutathione radical; GSH, reduced glutathione; GSNO, S-nitrosoglutathione; GSOH, glutathione sulphenic acid; GSSG, oxidized glutathione; NRS, reactive nitrogen species; ROS, reactive oxygen species. Adapted from Dalle-Donne *et al.* [77].

These post-transcriptional modifications, which occur in protein complexes and rely on redox reactions are important in the cell-based mechanisms, such as, gene expression, protein signaling, energy metabolism, ionic transport, protein folding, degradation and even in cell death and survival [78, 79]. On the one hand, it appears that proteins with thiol groups should be in a reduced state, because it facilitates their transport and mobilization. On the other hand, changes that occur in sulfur residues are crucial in cell signalling or protein activation. Hence, dysregulation of redox balance or protein glutathionylation may give rise to human pathology.

VII. Glutathione and pathological relationship

GSH deficiency manifests itself mainly through an increase in susceptibility to oxidative stress. Moreover, high levels of GSH increase the antioxidant capacity and the resistance to damage.

Some pathological conditions are associated with genetic abnormalities affecting enzymes involved in GSH metabolism [53]. There are several mutations and inborn deficiencies like those related to GCS, GS, GR, GST, 5-oxoprolinase and GPx enzymes, but the pathological pictures are complex and remain unclear [80]. Thus, genetic modifications usually result into

enzyme deficiency, inhibition or inactivation (truncated/non-functional or destabilized/short-lived enzymes) that leads to metabolic dysregulation [81].

As mentioned previously, on the one hand the presence of ROS is one of the determinant factors in endothelial activation and that leads to the emergence of atherosclerosis. On the other hand, GSH is a crucial antioxidant compound, and disturbance of its metabolism may also promote this disease. Some studies have shown this relationship, as held by Ashfaq *et al.* [82], where they proved that the GSH:GSSG ratio is a novel biomarker that helps prevent the onset of atherosclerosis, through early detection. Also, Lapenna *et al.* [83] analyzed mammary arteries in a normal state and with atherosclerotic plaques. They observed that in the plaque condition there was no activity of GPx enzyme and that in both cases, GST enzyme had a similar level of function. The first enzyme, promotes the elimination of reactive species and the second acts on toxins. Additionaly, Prasad *et al.* [84], conducted a study, which showed that administering GSH to patients with atherosclerosis reduced the risk factors and the endothelial injury and, consequently, increased NO concentration and antioxidant action.

Hypertension is a chronic increase of blood pressure. It may result from genetic trait or acquired condition and presents different degrees of intensity, such as, mild, moderate and severe [85]. Some studies, conducted in animals and humans, suggest that an increased concentration of reactive species and oxidative stress are the cause and the consequence, respectively, of that condition. The vascular system has antioxidant defense mechanisms, such as, SOD, GSH, catalase, Trx and Prx mentioned above, but the failure of these systems in synergy with increase of ROS, causes hypertension [86, 87]. It has been observed that the GSH:GSSG ratio is superior in whole blood and in EC of hypertensive individuals, and that inhibition of GSH pathways induces an increase of blood pressure [87].

Diabetes is characterized by elevated glucose levels in the blood, a condition that affects physiological metabolism and causes cellular dysfunction. This status represents a group of other conditions, such as, hyperglycemia and hyperlipidemia that express the excess of free fatty acids and insulin resistance [88]. It induces a set of events, such as, elevated oxidative stress, inflammation and platelet hyperactivity, in the vessel wall, causing severe dysfunction [19]. Hyperglycemia promotes the production of ROS and RNS by NADPH oxidase activation. Additionally, it inhibits the pentose phosphate pathway (PPP) by down-regulation of glucose-6-phosphate dehydrogenase enzyme (G6PD). This last route is an important source of NADPH, which is also essential in the conversion of oxidized GSSG to the reduced GSH form. Therefore, by reducing the PPP flow, levels of glucose are elevated and elimination of NADPH contributes to ROS accumulation [89]. Thus, it seems that diabetes is a condition that increases the oxidative stress of cells.

1.2.2.2. Homocysteine

Hcy is a non-protein amino acid, discovered in 1931 by DuVigneaud [90] when he tried to get 2Cys by making Met react with sulphuric acid. Hcy is an endogenous thiol that, in physiological conditions, is not incorporated into functional or structural proteins [91]. It derives from Met and, when it is further catabolized, results in Cys [92]. So, these three amino acids are structurally similar. Met has one more methylene group ($–CH_2$) than Hcy and Cys has one less $–CH_2$ (Figure 12). These thiols present a sulphur atom in their molecular structure, which determines a large reactive capacity. Met has another biochemical feature that stands out, the methyl group ($-CH_3$). It provides a high hydrophobic character and is important for proteins at structural and fuctional levels [93] (for example in interaction with lipid barriers [94]).

The conversion of Met to Cys is an irreversible reaction, the bioavailability of the latter is directly related to dietary habits and the metabolism of Hcy, which will be discussed ahead [95]. These three amino acids are important molecules for the normal functioning of living organisms, and nutritionally, only Met is considered essential, since it comes from food.

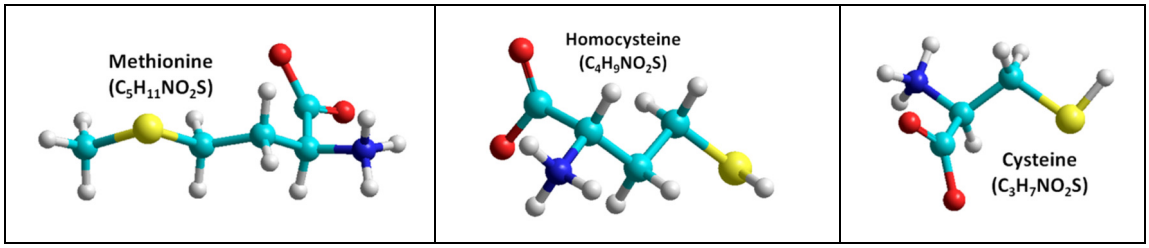

Figure 12 - Representation of the molecular structure of homocysteine, methionine and cysteine.

I. Relevance of cysteine and methionine

The Cys residue of Hcy is responsible for dimer formation, through disulfide bonds between homocysteines, known as homocystine (2Hcy), or with other Cys, called cystinylhomocysteine (Cys-SS-Hcy). These oxidation and reduction reactions occur spontaneously (Figure 13).

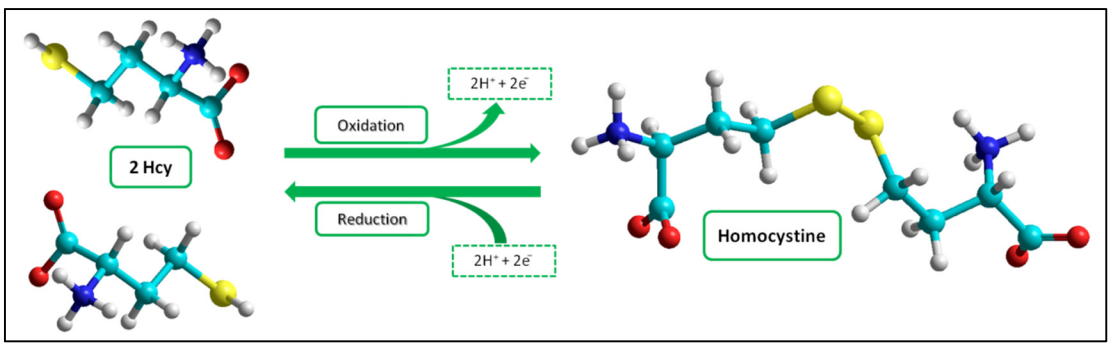

Figure 13 - Redox reaction of homocysteine. Abbreviations: H^+ - free hydrogen cation; e^- - free electron.

At protein level, Cys residues are responsible for forming intra (P1-SS-P1) and inter (P1-SS-P2) disulphide bonds [96] that ensure structural stability and even protein functionality [94]. These chemical bonds also play an important role in the post-transcriptional processing and balance between synthesis and degradation of intracellular proteins [97].

The Cys proteinases are important precursors of other molecules, like endorphin and insulin. They have been related to some pathologic processes, like those that originate arthritis, muscular dystrophy and tumor metastasis [98].

Like Cys, Met residues are critical in protein complexes, as their oxidation leads to enzyme activation or metabolic signalling [93]. The reaction is represented in figure 14, where the Met residue of a protein is oxidized by methionine peroxidase (MPx), through the interaction with H_2O_2. In addition, the reduction reaction, mediated by methionine sulfoxide reductase (MSR) is also shown [94]. This is an important path for eliminating some of the reactive oxygen species, which are harmful to cell metabolism. However, some studies have shown that the increase of methionine sulfoxide residues (Met-R-O) is responsible for vascular cell dysfunction, thrombosis, neurodegenerative diseases and a decrease in average life expectation [94, 99].

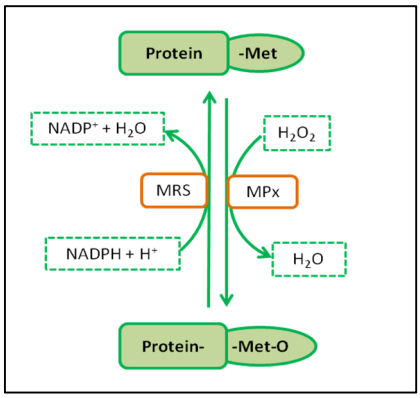

Figure 14 - Redox reaction of the methionine residue in proteins. Abbreviatons: H^+, hydrogen cation; H_2O, water; H_2O_2, hydrogen peroxide; MPx, methionine peroxidase; MSR, methionine synthase reductase; $NADP^+$, nicotinamide adenine dinucleotide phosphate oxidized; NADPH, nicotinamide adenine dinucleotide phosphate reduced; Protein-Met, methionine residue in the protein complex; Protein-Met-O, protein complex with methionine sulfoxide residue. Adapted from Brosnan *et al.* [93].

II. Homocysteine metabolism

Hcy results from the demethylation reaction (Figure 15), consisting in the removal of one $–CH_3$ group from Met, which is acquired from diet [100]. This pathway is also called methionine metabolism [93]. It involves the catalytic action of methionine S-adesonyltranferase (MAT), an ATP-dependent enzyme that induces the transfer of the adenosyl moiety of ATP to Met. From this reaction, S-adenosylmethionine (SAM or AdoMet) a high-energy compound results, due to the sulfuric bond established between the carbon atom from the ribose and the sulfur atom of the amino acid. Then, SAM is further converted to S-adenosylhomocysteine (SAH or AdoHcy) by removal of a $–CH_3$ group [93, 100]. The conversion of SAM to SAH is also performed by glycine N-methyltransferase (GNMT) to give sarcosine

(Sar), which is subsequently regenerated to Gly in the mitochondria [96]. Following the reaction, SAH is hydrolyzed by S-adenosylhomocysteine hydrolase (SAHH) to Hcy and adenosine. This hydrolysis is generally irreversible, but in certain physiological conditions, the equilibrium may favor SAH synthesis as Ado and Hcy are rapidly removed [100, 101].

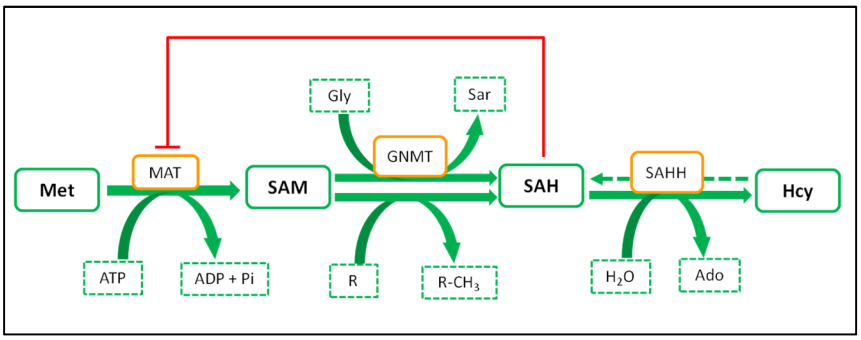

Figure 15 - Homocysteine synthesis, also called demethylation of methionine. Abbreviatons: Ado, adenosine; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Gly, glycine; GNMT, glycine N-methyltransferase; H_2O, water; Hcy, homocysteine; MAT, methionine S-adenosyltransferase; Met, methionine; Pi, inorganic phosphate; R, methyl group acceptor; R-CH_3, methylated acceptor; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine; Sar, sarcosine. Adapted from [93, 100].

Regulation of this pathway occurs by a negative feedback mechanism of SAH on MAT activity, if there is an increase of SAH concentration. However, SAM levels are controlled by allosteric interaction when Met levels are high, inactivated MAT returns to the active form by interaction with Met [93].

SAM is a powerful methyl donor in many reactions, involving DNA, RNA, proteins and lipids, such that the SAM:SAH ratio allows to estimate the cellular methylation capacity [100].

In humans, Hcy metabolism is ubiquitous and the fate of Hcy depends on the tissue where it is produced and the cellular metabolic state. After its synthesis, this thiol compound may intervene in two pathways, the remethylation or the transsulfuration, with the main role of transfering methyl groups [102].

The remethylation pathway (figure 16) consists in capturing a methyl group from the 5-methyltetrahydrofolate (mTHF or 5-MethylTHF), which is converted into tetrahydrofolate (THF) and regenerating the amino acid Met [101]. This reaction is catalysed by methionine synthase (MS), an enzyme whose activation depends on the cofactor cobalamin, also called vitamin B_{12} [103]. It takes place in nearly all cells, with the exception of erythrocytes and hepatocytes where the methyl group comes from the reduction of betaine, also named trimethylglycine (TMG), catalysed by betaine-homocysteine methyltransferase (BHMT), an enzyme that does not require cofactors [100]. The remethylation pathway consists of a step in the folate cycle and its relevance is specified ahead.

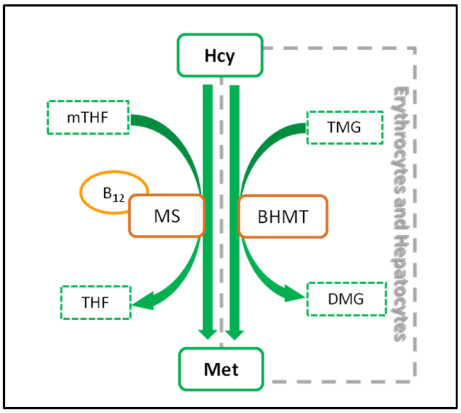

Figure 16 - Remethylation reaction of homocysteine. Abbreviatons: B_{12}, vitamin of complex B; BHMT, betaine-homocysteine methyltransferase; DMG, dimethylglycine; Hcy, homocysteine; Met, methionine; MS, methionine synthase; mTHF, 5-methyltetrahydrofolate; THF, tetrahydrofolate; TMG, trimethylglycine. Adapted from House *et al.* [101].

The transsulfuration pathway (figure 17) is an irreversible reaction of cystathionine β-synthase (CBS), an enzyme dependent on pyridoxal-5'-phosphate (PLP - active form of vitamin B_6), which binds Hcy to serine (Ser) and forms cystathionine [100]. Thereafter, the latest is hydrolyzed by cystathionase γ-liase (CSE), also PLP dependent, cleaving the molecule into Cys, α-ketobutyrate and ammonia (-NH_4^+) [103]. This pathway occurs mainly in the liver, kidney, pancreas and small intestine, where the excess of Hcy is adjusted, and is an alternative route for Cys synthesis [93, 94]. Moreover, the concentration of Cys is controlled by oxidation to taurine, which is eliminated by urinary excretion in the inorganic sulfate form [100], or is metabolically directed to gluthatione metabolism, that was discussed above. The regulation of this reaction is mediated by SAH, which inhibits CBS enzyme and, by synergic action, of SAM that blocks the methylene tetrahydrofolate reductase (MTHFR) from the folate cycle, promoting the transsulfuration and secondarily limiting the Hcy remethylation [100, 103].

It should be emphasized that the reaction of CSE enzyme produces H_2S, a gaseous molecule mentioned above, which modulates blood pressure and is crucial in cardiovascular homeostasis [104, 105].

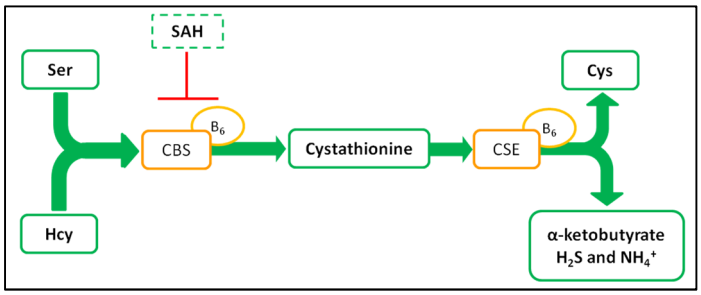

Figure 17 - Transsulfuration reaction of homocysteine metabolism. Abbreviatons: B_6, vitamin from B complex; CBS, cystathionine β-synthase; Cys, cysteine; Hcy, homocysteine; H_2S, hydrogen sulfide, SAH, S-adenosylhomocysteine; Ser, serine. Adapted from [100, 103].

The reactions of demethylation of Met, the remethylation and the transsulfuration of Hcy are all interconnected and form the Hcy metabolism, as shown in figure 18. The pathway is

mainly controlled by feedback mechanisms over key enzymes and by regulating the concentration of each intermediate [106]. In addition, the diet also has a major contribution, since it is the main souce of methionine, an essential amino acid [94]. As mentioned above, Hcy metabolism is directly related to bioavailability of methyl groups. Therefore, when the organism has deficiencies of these metabolites, cells promote methylation reactions instead of catabolism. But, in the opposite situation, there is a decrease of the remethylation pathway [93].

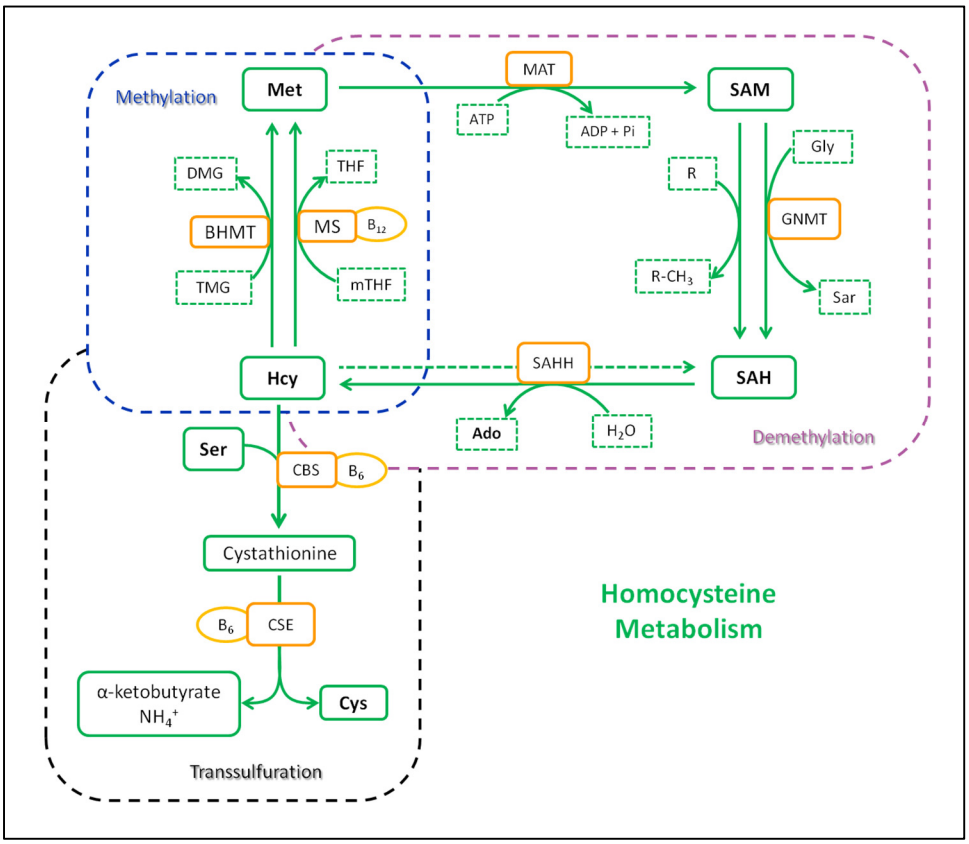

Figure 18 - Homocysteine metabolism. Abbreviations: Ado, adenosine; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; B_6 and B_{12}, cofactors derived from B vitamin complex; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; Cys, cysteine; DMG, dimethylglycine; Gly, glycine; GNMT, glycine N-methyltransferase; H_2O, water; Hcy, homocysteine; MAT, methionine S-adenosyltransferase; Met, methionine; MS, methionine synthase; mTHF, 5-methyltetrahydrofolate; Pi, inorganic phosphate; R, methyl group acceptor; R-CH_3, acceptor methylated; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; SAM, S-adenosilmethionine; Sar, sarcosine; Ser, serine; THF, tetrahydrofolate; TMG, trimethylglycine. Adapted from [100, 106].

III. Homocysteine and the folate cycle

As seen above, Hcy is involved in another metabolic pathway, critical for all types of cells, the folate cycle, which is illustrated in figure 19. This cycle consists on the catalytic transfer of carbon units through derivatives of folic acid, also called folate or vitamin B_9 [100]. The cycle begins when this compound is available in the cells, where it is recognized by

dihydrofolate reductase (DHFR), the enzyme that converts it into THF, by transfering one carbon atom. Subsequently, THF accepts one carbon from Ser amino acid, being transformed into 5,10-methylenetetrahydrofolate (meTHF), mediated by serine hydroxymethyltransferase (SHMT) a vitamin B_6-dependent enzyme [91]. Then, meTHF suffers reduction by MTHFR, which requires FAD, the active form of vitamin B_2, and becomes mTHF [93]. At this point, Hcy the intermediate from the remethylation pathway that receives a methyl group from mTHF, arises. This reaction is catalysed by MS coupled to vitamin B_{12} [94]. Thus, THF and Met are regenerated, available to initiate a new cycle or metabolically directed. SAM regulates this cycle by inhibition of MTHFR. Therefore, when SAM concentration is low, mTHF synthesis occurs and the transsulfuration pathway is inhibited, which enhances the conversion of Hcy to Met. Oppositely, when SAM levels are elevated, Hcy is converted into Cys and the folate cycle is suppressed [100].

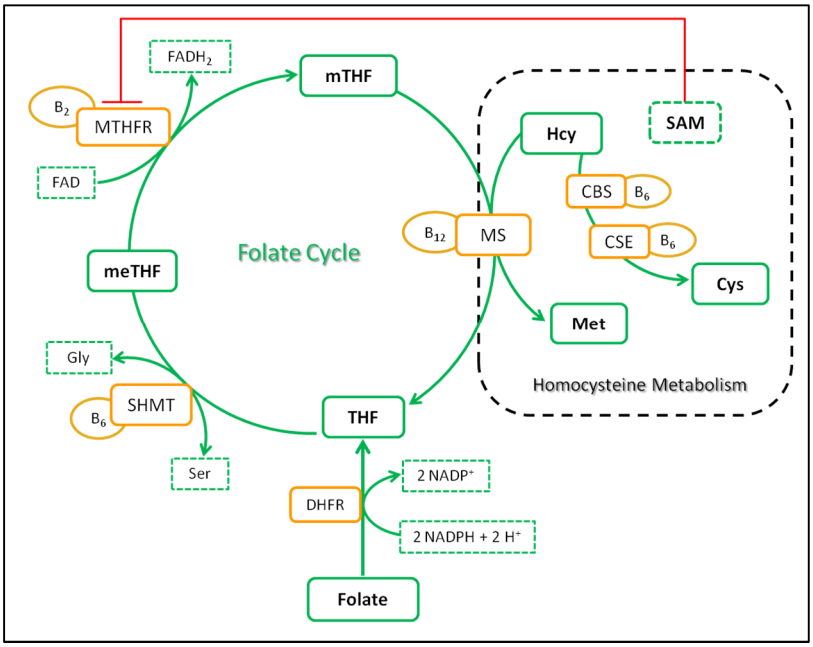

Figure 19 - Schematic representation of the folate cycle. Abbreviatons: B_2, B_6 and B_{12}, cofactors derived from B vitamin complex; DHFR, dihydrofolate reductase; FAD, flavine adenine dinucleotide oxidized; $FADH_2$, flavin adenine dinucleotide reduced; Gly, glycine; H^+, hydrogen cation; Hcy, homocysteine; Met, methionine; meTHF, 5,10-methylenetetrahydrofolate; MS, methionine sintetase; mTHF, 5-methyltetrahydrofolate; MTHFR, methyltetrahydrofolate reductase; $NADP^+$, nicotinamide adenine dinucleotide phosphate oxidized; NADPH, nicotinamide adenine dinucleotide phosphate reduced; Ser, serine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate. Adapted from [89, 100].

IV. Relevance of vitamins

Vitamins are essential in Hcy metabolism, because they mediate the activation of almost all the intervening enzymes. Among them, there are several cofactors derived from B complex vitamins. PLP, the active form of vitamin B_6, interacts with CBS, γ-cystathionine and SHMT; FAD, derived from B_2, is connected to MTHFR and MS; Methylated B_{12} activates MS; and B_9 is one of the main cofactors in Hcy metabolism [100]. B vitamin deficiencies are associated

with an increase in Hcy plasma levels and with the emergence of pathological conditions caused by hyperhomocysteinemia (HHcy**)**. On the other hand, vitamin bioavailability is directly related to diet and lack of these molecules is related to the development of some metabolic abnormalities [94].

HHcy is an established independent risk factor for vascular disease [107]. Therefore, the scientific community has been investigating the possibility of prevention and treatment of cardiovascular diseases (CVD) with oral administration of B vitamins. However, the studies of therapy supplementation with these molecules, in patients with CVD, have been a matter of great controversy, since results were not as expected. A couple of studies demonstrated that taking a supplement mixture of B vitamins (B_9, B_6 and B_{12}), successfully reduced plasma Hcy concentration. However, also showed that the risk for development vascular disease was not reduced [108, 109]. In another study, subjects who had previously suffered from myocardial infarction were followed and B vitamins did not decrease the prevalence or the possibility of new disease events. Although the concentration of Hcy was lower, the condition prevailed and the risk of health complications or even death, due to vascular abnormalities, was a reality [110]. Further studies have been inconclusive. They found no effective or significant relationship of cause and effect between vitamin supplementation and prevention of CVD [111, 112]. Anyway, these scientific studies have been important because they have demonstrated that CVD are multifactorial and that it is necessary to know more about them to find ways of prevention and treatment, which can be specific and effective.

V. Homocysteine levels

Hcy levels in humans are determined by physiological and genetic factors, as well as, nutritional elements and lifestyle. Sometimes the term “total homocysteine” is used in the wrong sense, since studies did not include a number of related molecules. Usually, total Hcy (tHcy) includes the free or reduced form, the oxidized forms and the ones that are linked to protein complexes. Apart from these, there are Hcy-thiolactones, SAH, homocysteic acid, homocysteine sulfinic acid, cystathionine, among others, which are not accounted as total Hcy [113]. In humans, it is considered that approximately 80% of this compound is coupled to proteins, such as, albumin and hemoglobin [96]. Nearly 19% is in the disulphide form and only 1% is free [113]. Either way, the normal concentration in human blood is considered to be in the range of 5 to 15 μM [101]. In cell cultures there is no specific range, because the concentration may vary more sharply, as it depends on the cell type and the method used.

Moreover, under pathological conditions plasma and urinary levels become higher, a disorder called HHcy and homocysteinuria, respectively [114]. In the most severe cases of homocysteinuria, the abnormal accumulation of Hcy can reach up to 500 μM [115]. HHcy cases are classified in tree cathegories, according to Hcy concentrations [114]: i) weak or mild (15 to 24 μM); ii) intermediate or moderate (25 to 100 μM); and iii) severe (above 100 μM).

VI. Homocysteine and pathological relationship

Hcy, as a risk factor for CVD, causes endothelial injury, vascular smooth muscle proliferation, high-density lipoprotein (HDL) cholesterol inhibition, thrombogenesis and autoimmune responses [114]. The increase of concentration alters the hemodynamic properties of the cardiovascular system, emphasizing a decreased NO bioavailability, by an up-regulation of endogenous asymmetric dimethylargenine (ADMA). The latest is an endogenous inhibitor of the endothelial nitric oxide synthase (eNOS) [116]. All these conduct to an induction of endothelial dysfunction. Also, as noted above, elevated levels of Hcy increase the concentration of reactive species and these, in turn, increase the production of oxidant molecules, such as, thiolactones that will be specified later, which damage the endothelium. Therefore, Hcy mediates metabolic disregulation by expression of pro-coagulant, pro-thrombotic and pro-inflammatory responses.

Several mechanisms have been proposed to try to explain Hcy associated pathologies. Metabolic disorders mediated by Hcy arise mainly from genetic abnormalities and vitamin deficiencies, which affect metabolic proteins, such as, CBS, MTHFR and MS. For example, a mutation in the *MTHFR* gene is associated with vascular disease, colon cancer, leukemia and impairment of neural tube formation [117]. Sultan *et al*. [118], reported a study where it was demonstrated that different *MTHFR* gene mutations resulted in different HHcy categories. Hence, plasma Hcy concentration is also dependent on the *MTHFR* genotype. The same may occur with any type of mutation in genes encoding enzymes of Hcy metabolic pathway.

In HHcy, the most important issues are the regulation of Hcy production and transport, within cells and between different tissues. In 2007, Jiang *et al.* [119] studied Hcy transport mechanisms in EC and vascular smooth muscle cells (VSMC), which occured through four systems called X_{AG}, ASC, A (Na^+ dependent) and L (Na^+ independent). They found that EC hold Hcy intracellular levels in high levels and against plasma concentration gradient. It was proved that Hcy transport is inhibited in basic medium and that it is highly dependent on an intact lysosomal system, since it has inhibitory molecules [119]. They also demonstrated that the excess of Hcy uptake by cells is not the cause of endothelial damage. They claim that the injury induced by Hcy cannot be attributed to an increase of absorption, it must be other biochemical mechanism, independent of Hcy absorption, which is responsible for the cytotoxic effects [119].

HHcy causes an increase of ROS production directly or through auto-oxidation pathways, such as, decreased action of antioxidant systems (GSH and superoxide dismutase) or increase of NADPH oxidase, respectively [73]. There is some evidence that subjects with sclerosis or amyotrophic lateral sclerosis (ALS), conditions that are characterized by decreased nerve and muscle functions or degeneration, have high plasma levels of Hcy. Some studies have shown that HHcy causes muscle cell metabolic abnormalities, due to increased production of creatinine phosphokinase (CPK), the enzyme that controls the energy potential [120]. All the above-mentioned conditions induce endothelium oxidative stress and injury, which affects the cardiovascular system, kidneys, brain or muscles and, consequently, can lead to multiple organ failure [121].

HHcy and hypercholesterolemia are considered independent risk factors in the emergence of CVD. Some studies have shown a positive correlation between Hcy and cholesterol, such as, the increase of one directly influences the increase of the other [122]. Therefore, the degree of endothelial dysfunction in HHcy is considered to be similar to that of hypercholesterolemia and hypertension conditions [123].

Some metabolic abnomalies result from Hcy thiolactones formation, due to errors of aminoacyl-tRNA synthetase, the enzyme that carries out esterification of amino acids in RNA transfer (tRNA) to form aminoacyl-tRNA, in protein transcription processes [124]. When Hcy concentration is high, some metabolic abnormalities emerge and affect the methylation process of Hcy-tRNA to Met-tRNA, due to the reduction of transmethylation or transsulfuration pathways [91]. As a result, formation of a cyclic thioester occurs, by the action of methyonyl-tRNA synthetase (MetRS), an ATP-dependent enzyme, that condenses the thiol group with the carboxylic acid, as shown in figure 20. This reaction was first described in *Escherichia Coli* bacteria [125] and was later identified in several eukaryotic cells, such as, fibroblasts, endothelial, hepatic and in breast cancer cells [91].

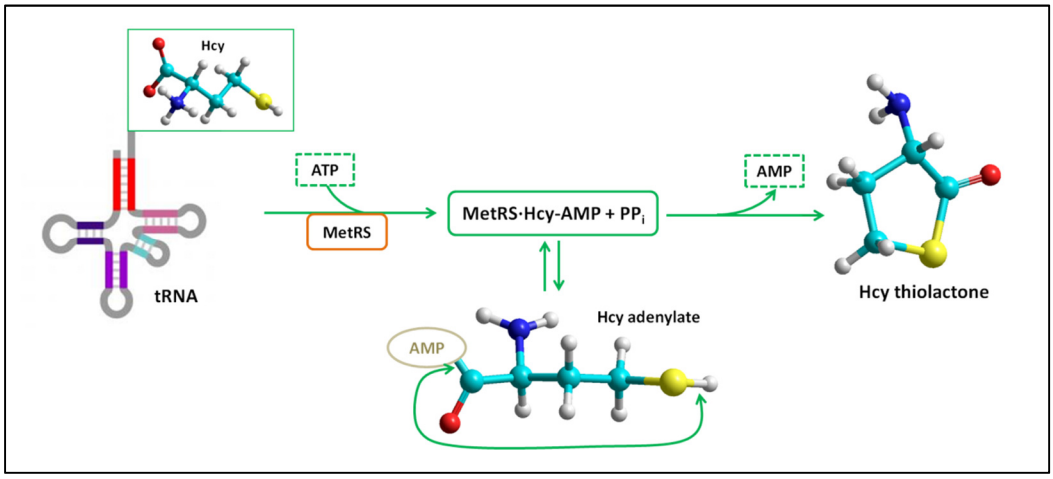

Figure 20 - Reaction of homocysteine thiolactones formation. Abbreviatons: AMP, adenosine 5'-monophosphate; ATP, adenosine 5'-triphosphate; Hcy, homocysteine; MetRS, methionyl-tRNA synthetase; MetRS·Hcy-AMP, methyonyl-tRNA bound to homocysteine adenylate; PP_i, inorganic pyrophosphate; tRNA, transfer ribonucleic acid. Adapted from Medina *et al* [91].

Thiolactones induce protein homocysteinylation by reacting with the amino group (-NH_2) of lysine residues, causing changes in the physical and chemical properties of the modified protein. The injury caused by this reaction is one of the conditions that lead to vascular endothelium damage and development of atherosclerosis [125]. Cells have a protection mechanism, which eliminates these reactive compounds and prevents homocysteinylation. It is mediated by an Hcy-thiolactonase hepatic enzyme also called paraoxonase, which is calcium and HDL dependent. Paraoxonase hydrolyzes thiolactones back to Hcy [124].

This way, Hcy is also considered a risk factor of hypertension, systolic and diastolic blood pressure (SBP/DBP) greater than 140/90 mmHg [85]. However, there is no consensus, or

an exact explanation for this relationship because the results of several studies were ambiguous [126]. Wang *et al* [127], investigated the relationship between plasma Hcy concentration and the incidence of hypertension. Like in other studies [128], they showed that men with high levels of Hcy had an increasead risk, but the data were not statistically significant in the incidence of hypertension. Furthermore, the results suggested that in an early stage of hypertension, lower Hcy levels might indicate higher risk. Thus, it is considered that HHcy cannot simply be related to the increased risk of incidence. Moreover, the levels of Hcy in women are lower and compared with these values, a significant risk factor was not observed [127, 128]. Therefore, we can say that the causal relationship of Hcy concentration and hypertension is not simple or absolute.

HHcy has been related to the antiphospholipid antibody syndrome (APS), also known as Hughes syndrome, which is characterized by the formation of blood clots due to autoimmune reactions [129]. However, the studies presented did not find a significant relationship between clinical manifestations and this pathological condition [130].

To summarize, Hcy plasma levels greater than 15 μM are considered to be pathologic. The increase may arise from disruptions of metabolic pathways due to nutritional deficiencies, enzymatic or genetic abnormalities, which further cause oxidative stress and damage in the vascular endothelium.

Since the late 60´s, Hcy plasma levels have been associated with CVD, especially in the emergence of atherosclerosis [131]. In 1962, McCully [132] reported the first cases of severe homocysteinuria in children with mental retardation, accelerated growth, osteoporosis, arterial thrombosis and atherosclerotic plaque. One child showed CBS deficiency so, did not synthesize cystathionine and evidenced high levels of Hcy and Met in plasma. Another child, who lacked MS enzyme, presented high plasma cystathionine and Hcy concentrations and low levels of Met [92]. Since then, a high concentration of Hcy was directly associated to the induction of atherosclerosis

Atherosclerosis describes a slowly progressive disease (Figure 21), which results from continuous inflammatory damage that increases the vascular permeability, induces fat accumulation and formation of atherosclerotic plaque in arterial walls [133]. Furthermore, this process can occur in veins subjected to arterial pressure and in the pulmonary system, in hypertension conditions [134]. Morphologically, arteries have a trilaminate structure composed of tunica adventitia of connective tissue, tunica media of smooth muscle, elastic tissue and collagen, and the intima tunic of endothelial tissue [135]. This pathological condition begins at the endothelial layer, with a series of hemodynamic changes, which subsequently, transmit molecular signals that activate several reactions throughout the vascular system, such as, cells recruitment, production of inflammatory molecules and lipid accumulation [17, 135]. In the EC, the chemical damage caused by Hcy is considered to be one of the earliest reactions that occur in the development of atherosclerosis [136].

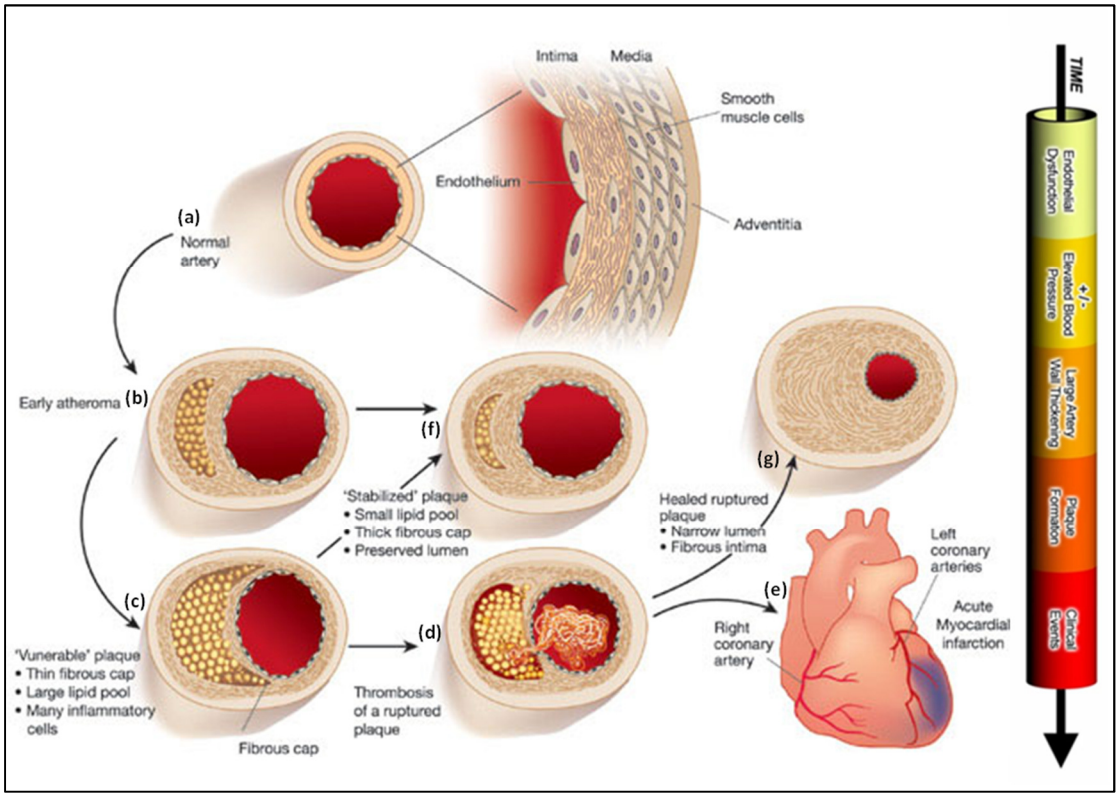

Figure 21 - Development and progression of atherosclerosis. Legend: (a) normal artery, a trilaminate structure; (b) early atheroma emergence due to endothelial dysfunction and inflammatory reaction; (c) growth of lipid plaque through a pro-inflamatory cascade; (d) rupture of the atherosclerotic plaque; (e) thrombus formation; (f) lipid-lowering, reduction of lipid content and control of the inflammatory response; (g) healing response may stimulate tissue proliferation and constrict the lumen. Image and information adapted from [136, 138].

Endothelial activation in atherosclerosis, as shown in figure 22, mediates the production of cytokines and the expression of molecular markers at the surface of EC, which cause adhesion and migration of monocytes. Increase of the endothelial permeability allows LDL-cholesterol to cross the vessel wall and, consequently, monocytes differentiate into macrophages that capture oxidized LDL-cholesterol and become foam cells (Macrophages loaded with fat) [139]. The pro-inflammatory cytokines promote more cell recruitment, smooth muscle proliferation and vascularization that lead to localized thickening of the vessel becoming an atherosclerotic plaque. Thus, as the plate increases, narrowing of the lumen and blood deprivation occur [140]. In certain cases, the weak vessel can leak blood, causing intra-plaque hemorrhage, which may accelerate the injury. Hypoxia and oxidative stress leads to apoptosis of the foam cells and the formation of a necrotic lipid cluster [141]. The inflammatory condition prevails when the risk factors are elevated, such as, in case of dyslipidemia and HHcy. Moreover, hypertension promotes this condition through multiple mechanisms and interaction with other risk factors, such as, poor diet and smoking [142].

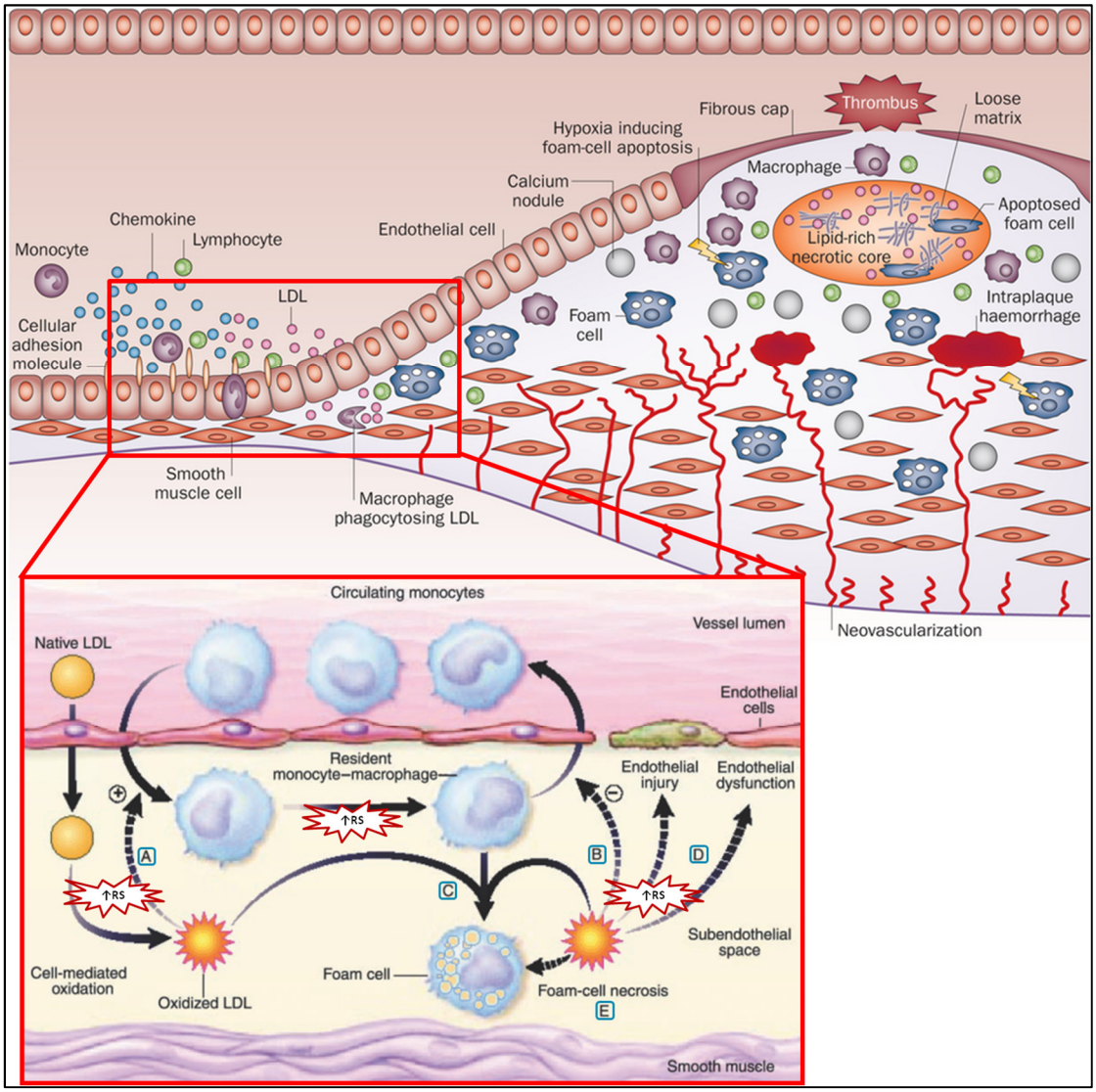

Figure 22 - Development of atherosclerotic plaque, highlighting the endothelial activation pathway and oxidative modifications that precede endothelial injury. Abbreviatons: LDL, low-density lipoprotein; RS, reactive species. Legend: Oxidized LDL-cholesterol stimulates chemotaxis (A), prevents the exit of monocytes from the subendothelial space (B) and mediates the formation of foam cells (C), which subsequently, become necrotic due to accumulation of oxidized LDL-cholesterol (E). As a final result, endothelial dysfunction emerges (D). Adapted from [139, 140].

Hypertension and atherosclerosis are pathologically linked. The NO molecule is an endothelial mediator of the inflammatory signal, which produces vascular relaxation and expression of specific factors that control oxidative stress, leukocytes adhesion and pro-thrombotic reactions [17]. In the case of chronic hypertension, it alters the homeostatic mechanisms expressing an early inflammatory response and formation of atherosclerotic plaque. Thus, hypertension, over the matrix vessel wall is associated with an increase of proteoglycans and a production of ROS of pro-inflammatory action, like O_2^- and HOCl [140]. The interaction of these particles increases the oxidation susceptibility of LDL-cholesterol and subsequent uptake by macrophages. In addition, the friction exerted on the vessel wall due to blood circulation, also stimulates the increased production of NO [142]. In atherosclerosis

condition, the presence of homocystine thiolactones promotes the formation of foam cells and accelerates the pathological condition state. Effectively, homocysteinilated LDL-cholesterol is more susceptible to oxidation than native LDL-cholesterol because it is more easily recognized and captured by macrophages [125]. Tsen *et al*. [143], found that in humam umbilical vein endothelial cells (HUVEC), Hcy inhibits ROS production and NO accumulation within the first hours of exposure (0.5 h), in a concentration from 0.25 to 1 mM. However, when the treatment was extended for longer than 22 to 24 hours, ROS synthesis was stimulated and NO was eliminated. They considered that endothelial dysfunction resulted from increased ROS over NO concentrations. They also showed that 5 mM of Hcy has cytotoxic effects in cells. On the contrary, Cys, which is a GSH precursor, has a protective effect and reduces ROS production in the initial state or after chronic exposure of Hcy, even exceeding 1 mM. Studies with Met were also performed but no significant effects were found [143]. Thereby, this work confirmed that Hcy limited NO bioavailability in HUVEC cells, directing metabolism to the formation of reactive compounds that cause oxidative stress and induce endothelial dysfunction.

In another study, an electrochemical detection of Hcy concentration was carried out in rat microvascular endothelial cells (RMVEC) [144]. Hcy was found to have an indirect action over NO production. Hcy caused suppression of NO production in cell culture *in vivo*, stimulating several eNOS dependent and independent receptors. They proved that Hcy oxidized eNOS sulfhydryl groups, inhibiting its activity [144].

Additionally, Li *et al*. [145] assessed gene expression in HHcy conditions, using cDNA microarrays, in HUVEC cells and found that there is an increase of the messenger RNA transcript, which expresses 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) intervening in cholesterol biosynthesis. They proved that Hcy increases cholesterol production and accumulation in EC. Control cultures showed that free cholesterol is accumulated in the endoplasmic reticulum and plasma membrane. However, HUVEC cell cultures in HHcy conditions evidenced lipid droplets throughout the cytoplasm, a condition observed in the formation of foam cells [145].

The relationship between Hcy, ADMA and NO, in HUVEC cell cultures, has also been studied and Hcy was found to induce synthesis and accumulation of NO and ADMA [146]. Other studies showed that Hcy blocks the enzymatic activity of dimethylarginine dimethylamine hydrolase (ADHD) with accumulation of ADMA and ROS, which are inhibitors of NO sysnthesis [116]. Tan *et al.* [147], demonstrated that HHcy stimulates the proliferation of VSMC, altering the structure of the vascular matrix through the production of collagen fibers. Consequently, it diminishes the elasticity of the vessel and leads to arterial remodeling and increased vascular resistance [147].

Although, many scientific research studies have been conducted, the mechanisms of pathologic action of thiol compounds in vascular homeostasis remain unknown.

1.2.3. Purines

Purines constitute a group of molecules, which are closely related to the previously mentioned thiol compounds. They are nitrogenous bases that bind to a pentose and one or more phosphate groups, forming nucleotides (Figure 23) [148]. Nucleotides are the monomeric units of nucleic acids, but they also play important functions in the cells, particularly as energy storage, cellular messengers and also act as enzyme cofactors. All nucleotides contain one type of sugar, the pentose ribose (RNA) or a deoxyribose (DNA). There are also two types of bases (heterocyclic amine) the purines with two ring in its structure and the pyrimidines with only one ring, the latest will not be discussed. Thus, nucleotides having purine bases are mainly, adenine, guanine and their derivatives [149].

Purines are precursors of DNA, RNA, coenzymes NAD^+, $NADP^+$, FAD and CoA, and of several metabolic derivatives as SAM and SAH referred to, above. They are substrates of some enzymes, such as, DNA and RNA polymerases and act as signal second messengers, like cyclic nucleotides adenosine monophosphate (cAMP) and guanosine monophosphate (cGMP) [150].

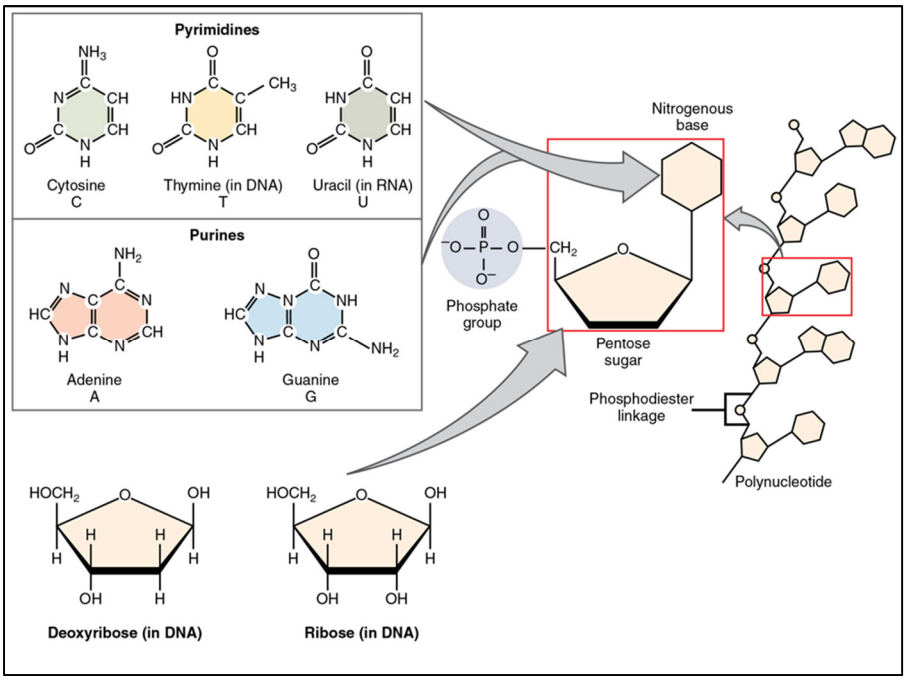

Figure 23 - Representation of the nucleoside and nucleotide structure. Image obtained from [151].

1.2.3.1. Purines metabolism

The purines and pyrimidines are ubiquitous in organisms. The purine metabolism can occur by two distinct pathways: “de novo”, a synthetic route for nucleotides, where energy is required and the “salvage” pathway, an energy saving mechanism of degradation and molecular recycling [150].

Purine “de novo” synthesis, also known as IMP pathway, is a set of reactions with the final goal of obtaining inosine 5'monophosphate (IMP), through a series of phosphoribosyl

pyrophosphate (PRPP) conversions [152]. PRPP is a precursor, which is common to the synthesis of purines and pyrimidines, and ribose-5-phosphate is another one. The latest, is the first compound in purine biosynthesis, which derives from the PPP pathway. It is the intermediate of PRPP and is activated by the enzyme phosphoribosyl pyrophosphate synthetase (PRPPS), through ATP consumption with AMP release (Figure 24). In the cells, when the concentration of PRPP is low, there is an increase of the "salvage" route, but when the concentration is high, there is activation of the "de novo" pathway [150].

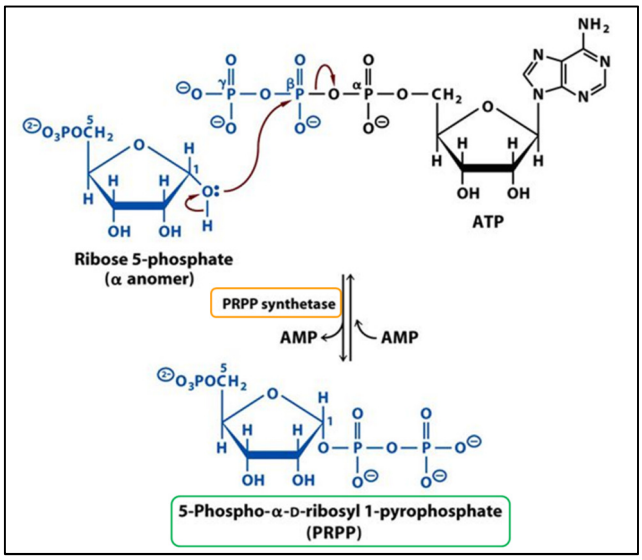

Figure 24 - Synthesis of phosphoribosyl pyrophosphate (PRPP). Abbreviations: AMP, adenosine 5'monophosphate; ATP, adenosine 5'triphosphate. Adapted from Moran *et al.* [153].

The complete pathway of purine "de novo" synthesis is represented in figure 25 [152] The first reaction is mediated by phosphoribosyl pyrophosphate amidotransferase (PPAT①) that displaces the pyrophosphoryl group of PRPP by the amine nitrogen of glutamine (Gln) [150]. The product phosphoribosylamine is then acylated by Gly to form glycinamide ribonucleotide by action of glycinamide ribonucleotide synthetase (GARS②), an ATP-dependent enzyme. Then, glycinamide ribonucleotide transformylase (GART③) performs the transfer of a formyl group from 10-formyltetrahydrofolate to the N-7 amino group of IMP [152]. Subsequently, formylglycinamidine ribonucleotide synthase (FGAMS④), another ATP-dependent enzyme, converts formylglycinamide-ribotide into formylglycine-amidinoribotide and Gln is the nitrogen donor [153]. The next reaction is a ring-closure step where aminoimidazole ribonucleotide synthetase (AIRS⑤), which also requires energy in ATP form, produces aminoimidazole-ribotide. Then, a carboxylation reaction (CO_2 incorporation) is performed by aminoimidazole ribonucleotide carboxylase (AIRC⑥) enzyme, requesting ATP and HCO_2^- anion as donor [152]. Following, aminoribosyl-aminoimidazole succinocarboxamide ribonucleotide synthetase (SAICARS⑦) incorporates an amine group from the amino acid aspartate (Asp) into the precursor and later, the enzyme adenylosuccinate lyase (ADSL⑧) reacts and releases fumarate. The following reaction is similar to GART③ action, but it is carried out by the aminoimidazole carboxamide ribonucleotide formyltransferase (AICARFT⑨). Finally, IMP cyclohydrolase (IMPCH⑩) completes this sequence of metabolic reactions and IMP

is formed [152, 153]. In this metabolic pathway, there is the intervention of other enzyme, the aminoimidazole carboxamide ribonucleotide transformylase/inosine monophosphate cyclohydrolase (ATIC) that also catalyzes the reactions mediated by AICARFT⑨ and IMPCH⑩ [154]. IMP is the main precursor of the purines adenine and guanine.

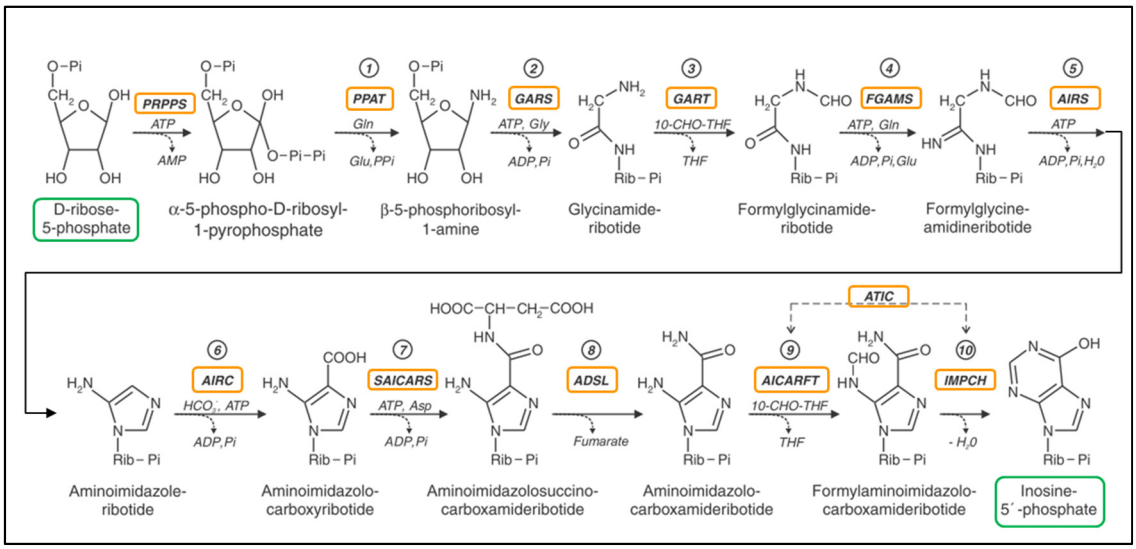

Figure 25 - Schematic representation of purine "de novo" synthesis pathway. Abbreviations: 10-CHO-THF, 10-formyl-tetrahydrofolate; ADP, adenosine 5'diphosphate; AMP, adenosine 5'monophosphate; Asp, aspartate; ATIC, aminoimidazole carboxamide ribonucleotide transformylase/inosine monophosphate cyclohydrolase; ATP, adenosine 5'triphosphate; Gln, glutamine; Glu, glutamic acid; Gly, glycine; H_2O, water; HCO_2^-, hydrogen carbonate anion; Pi, inorganic phosphate; PPi, inorganic pyrophosphate; THF, tetrahydrofolate; Legend: PPAT①, phosphoribosyl pyrophosphate amidotransferase; GARS②, glycinamide ribonucleotide synthetase; GART③, glycinamide ribonucleotide transformylase; FGAMS④, formylglycinamidine ribonucleotide synthase; AIRS⑤, aminoimidazole ribonucleotide synthetase; AIRC⑥, aminoimidazole ribonucleotide carboxylase; SAICARS⑦, aminoribosyl-aminoimidazole succinocarboxamide ribonucleotide synthetase; ADSL⑧, adenylosuccinate lyase; AICARFT⑨, aminoimidazole carboxamide ribonucleotide formyltransferase; IMPCH⑩, IMP cyclohydrolase. Adapted from Adam [152].

When sinthesyzed, IMP may subsequently lead to AMP or GMP, as presented in figure 26 [153]. IMP has a hypoxanthine base (or hydroxyl group) that in AMP synthesis suffers substitution by an amino group through the action of adenylosuccinate synthetase (ADSS) and ADLS, consuming aspartate and releasing fumarate. Afterwards, GMP results from oxidation of one IMP carbon atom, with reduction of NAD^+ by the enzyme IMP hydrogenase, forming xanthosine monophosphate (XMP). The latest suffers amination by a GMP syntethase with Gln intervention and is converted into glutamate [153].

It is important to note that, in AMP synthesis there is consumption of energy in GTP form, and in GMP synthesis there is an intake of ATP form [148]. That is, this is a way of energetic adjustment in cells.

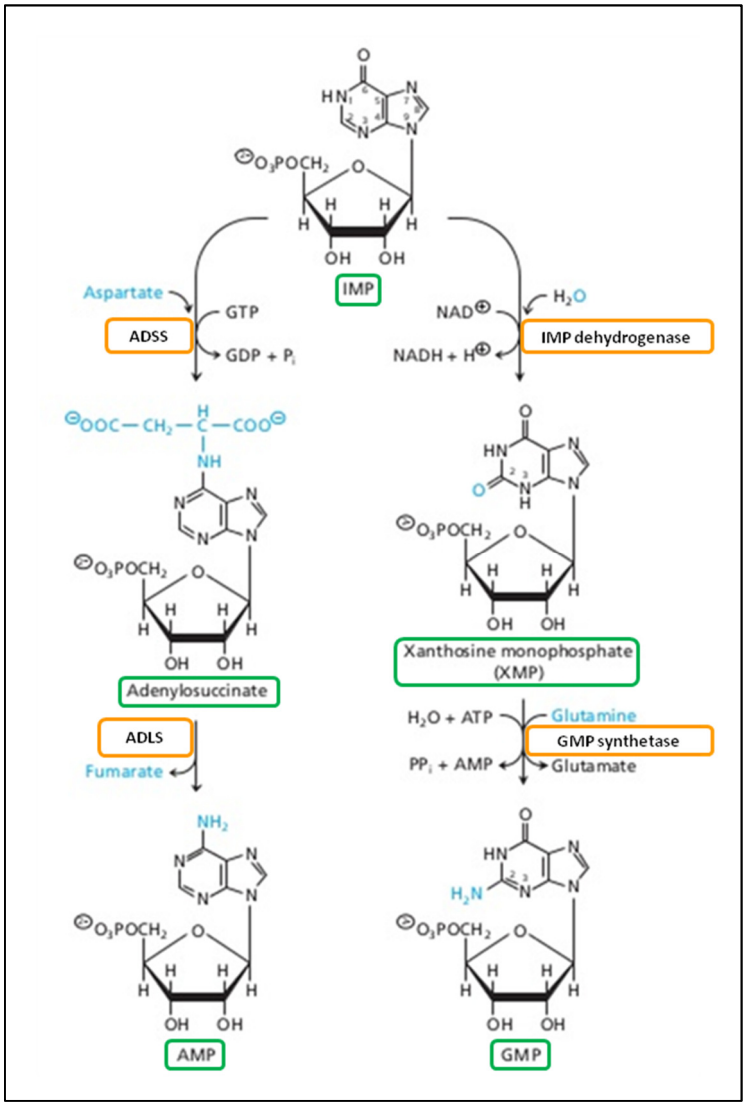

Figure 26 - Inosine monophosphate conversion. Abbreviations: AMP, adenosine 5'monophosphate; ATP, adenosine 5'triphosphate; GDP, guanosine diphosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; H^+, hydrogen cation; H_2O, water; IMP, inosine 5'monophosphate; NAD^+, nicotinamide adenine dinucleotide oxidized; NADH, nicotinamide adenine dinucleotide reduced; Pi, inorganic phosphate; PP_i, inorganic pyrophosphate. Adapted from Moran *et al.* [153].

Purine synthesis is regulated by complex mechanisms of feedback inhibition as shown in figure 27. PPAT enzyme is allosterically inhibited by high concentrations of IMP, AMP or GMP products. ADSL enzyme also catalyzes the conversion of IMP to AMP [153]. In transport mechanisms, aminoimidazolecarboxamide dephosphorylated ribonucleoside (AICAR) competes with Ado, which results in Ado accumulation in the extracellular space due to inhibition of A_1 receptor [152], receptor specified later on. Following, AICA riboside acts on AMP-activated protein kinase (AMP kinase), which is activated by glycogen phosphorylase [153]. The AICA ribose, also accelerates the replication of purine nucleotide pools in the heart, which consequently increase glucose uptake, gluconeogenesis, and inhibit fatty acids and sterol synthesis in hepatocytes [152].

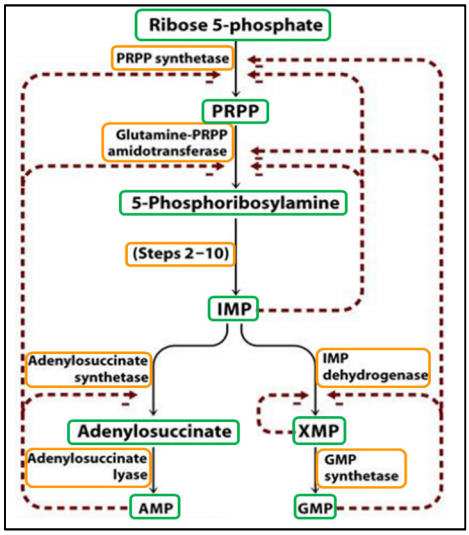

Figure 27 - Regulatory mechanisms in purine metabolism. Abbreviations: AMP, adenosine 5'monophosphate; GMP, guanosine 5'monophosphate; IMP, inosine 5'monophosphate; PRPP, phosphoribosyl pyrophosphate; XMP, xanthosine monophosphate. Adapted from Moran *et al.* [153].

The "salvage" pathways are a form of biochemical recycling. Nucleic acids are degraded by nonspecific nucleases, giving rise to polynucleotides, which are subsequently converted to nucleotide compounds by the action of phosphodiesterases or exonucleases. Nucleotide compounds are hydrolyzed by nucleosidases or nucleoside phosphorylases becoming nucleosides and inorganic phosphorus. In purine degradation, two functionally related key enzymes stand out: adenosine phosphoribosyl transferase (APRT), which catalyzes the reaction between adenine and PRPP to form AMP, and hypoxanthine-guanine phosphoribosyltranferase (HGPRT), which acts primarily in the recycling of DNA and RNA [153]. In addition, HGPRT catalyzes the reaction between guanine and PRPP to form GMP.

Figure 28 illustrates the systhesis of purines focusing on the relevant compounds in this study: adenosine and inosine.

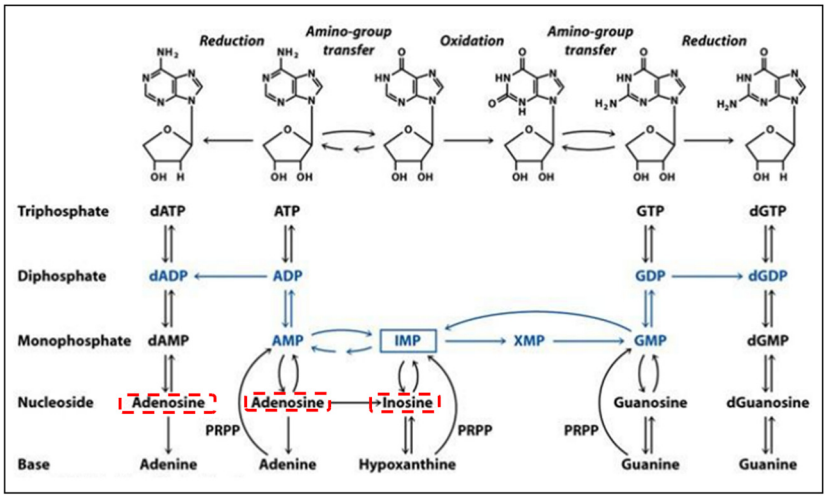

Figure 28 - Schematic representation of the metabolic conversions of purine compounds, emphasizing those who were determined in this study. Adapted from Moran *et al.* [153].

1.2.3.2. Adenosine and Inosine

Ado is a nucleoside belonging to the purine family first described by Drury and Szent-Györgyi in 1929 [155], as an important signaling molecule that is present in the extracellular space. It is widely distributed throughout the body, in the nervous, immune, cardiac, gastrointestinal, renal and in muscle systems. Under normal conditions, Ado cytoplasm concentration is around 0.01 to 0.10 µM and in the interstitial fluid it varies between 0.02 and 0.2 µM [156]. This nucleoside is a hydrophilic compound, so, its transport through the membrane is mediated by proteins [157]. It has protective and neuromodulating properties, which trigger physiological responses by interacting with specific receptors. It is a molecule of great importance at the scientific level, because of its high therapeutic potential [158].

Ino is another purine nucleoside, an Ado related metabolic compound in the pathways of synthesis and degradation. The deamination of Ado forms Ino through a reaction catalyzed by adenosine deaminase (ADA) [159]. As presented in figure 29, structurally, Ino has a nitrogen less and one more oxygen atom, than Ado. Functionally, Ino is also a relevant immunomodulator and neuroprotective compound [160]. Just as Ado in human plasma, Ino derives from dietary and endogenous purine metabolism.

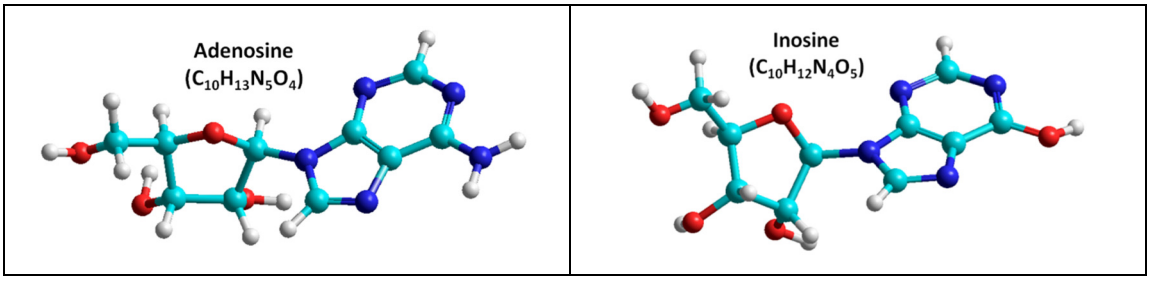

Figure 29 - Representation of the molecular structure of adenosine and inosine.

I. Adenosine specific pathway

As shown in figure 30, Ado results from the catabolism of ATP to ADP, and then to AMP, which subsequently regenerates Ado molecule by the action of endo and ecto-5-nucleotidase. In the cytoplasm, demethylation of Met in Hcy remethylation pathway is another source of Ado [161]. Reviewing the latest pathway, it involves the catalytic action of MAT with formation of SAM, which is then hydrolyzed to SAH that thereafter, originates Hcy and Ado. Subsequently, the nucleotide can be rephosphorilated to AMP by adenosine kinase (ADK), or catalyzed by ADA, giving Ino [157].

cAMP is a second messenger produced from AMP, by adenylyl cyclase (AC) activation, and it is dependent upon the action of other proteins that affect its synthesis and degradation [162]. Its fundamental role in cells is related to signal response of some hormones and neurotransmitters, for example in the activation of protein kinase A (PKA), a phosphorylating enzyme [163]. ATP, ADP, Ado and cAMP, when needed, are exported from cells, through diffusion or active transport (TX). Thus, anabolism and catabolism rate of ATP mediated by Ado is a pathway responsible for maintaining the normal energetic state of the cells. The

intracellular signaling cascade, mediated by Ado is a complex process, regulated at transcriptional level [164]. Ado transport to the intracellular space is made through specific transporters, named equilibrative nucleoside transporter 1 and 2 (ENT1 and ENT2), where it is transformed to Ino or AMP by the action of ADA or ADK, respectively [158].

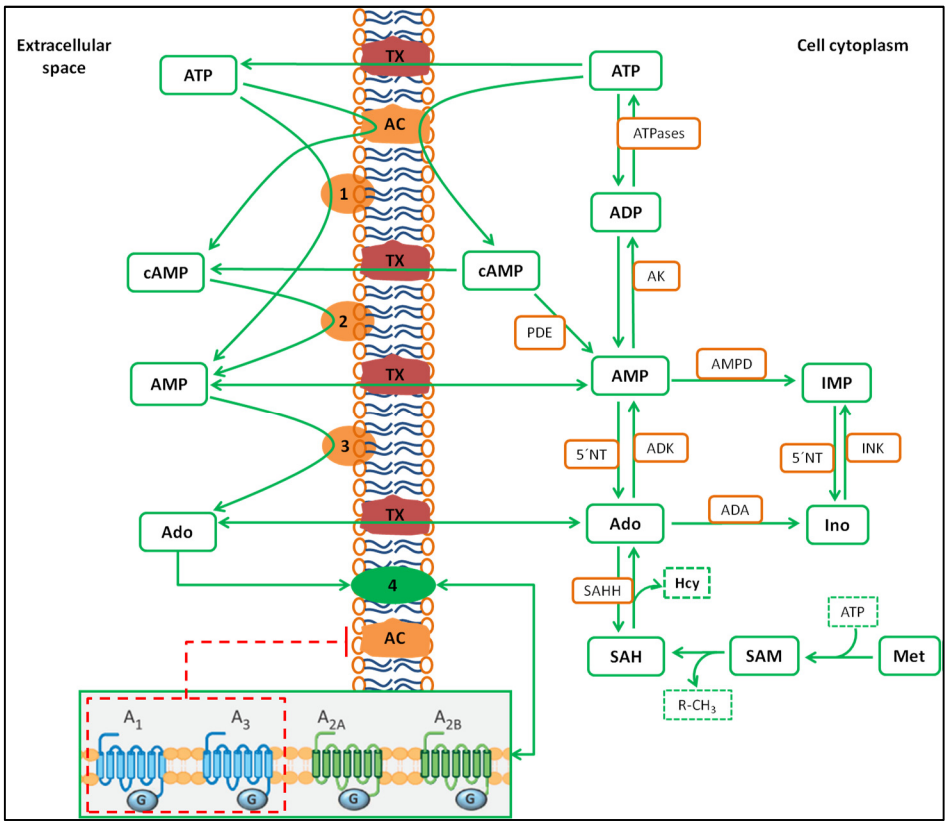

Figure 30 - Adenosine metabolism. Abbreviatons: 5'NT, 5'-nucleotidase; AC, adenylyl cyclase; ADA, adenosine deaminase; ADK, adenosine kinase; Ado, adenosine; ADP, adenosine diphosphate; AK, adenylate kinase; AMPD, adenosine monophosphate deaminase; ATP, adenosine triphosphate; cAMP, cyclic adenosine 3'5'-monophosphate; Hcy, homocysteine; IMP, inosine monophosphate; INK, inosine kinase; Ino, inosine; Met, methionine; PDE, phosphodiesterase; R-CH_3, methylated acceptor; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine; TX, protein carrier. Legend: 1, ecto-ATPases; 2, ecto-phosphodiesterase (Ecto-PDE); 3, ecto-5'-nucleotidase (Ecto-5'NT); 4, adenosine receptors A_1, A_{2A}, A_{2B} or A_3. Adapted from [165, 166].

As mentioned above, Ino is an important metabolic intermediate in Ado synthesis, but also, it has a crucial intervention in RNA editing. This mechanism is responsible for RNA and protein diversity, independently from the genetic code [167]. Ino is a modified nucleotide base, which derives from the reaction of ADA enzyme over RNA chain converting Ado into Ino. It is considered a rare base that usually, is not a part of RNA. However, it is common in tRNA [167], interference RNA (RNAi) [168] and micro RNA (miRNA) [169]. These modifications are responsible for variations in the genetic sequences encoding proteins, which may compromise their functional properties [167], as, for example, in cardiovascular system [170].

II. Specific functions of adenosine and pathological relationship

The functions mediated by Ado begin with interactions with the **adenosine receptors**, which in turn, modulate cell signaling. There are four receptor subtypes, A_1, A_{2A}, A_{2B} and A_3 (or A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R [175] as represented in figure 31), which are highly specific, belong to the heteromeric G proteins signaling family and are composed by seven transmembrane domains (Heptahelical receptor) with an extracellular amine terminus and a cytoplasmic carboxy terminus [171]. Extracellular Ado interacts with the G-protein complex and produces a response that can be activation (A_{2A} and A_{2B}) or inhibition (A_1 and A_3) regarding the AC enzyme activity, as illustrated in figures 30 [166]. This specificity is important in therapeutic applications, such as, at anti-inflammatory (A_{2A} and A_3), cardiac (A_1, A_{2B} and A_3) [172] neural (A_1 and A_3) levels, and control of pain/stress transmission (A_1) [164]. Some of the important actions mediated by each specific receptor are:

- A_1 stimulates the action of AC and phospholipase C (PLC) enzymes [156]. It has a negative chronotropic (heart rate) and dromotropic effect (stimulus conduction) on cardiac tissue and causes vasoconstriction [161]. Some studies, suggest the use of an antagonist of these receptors as a protective agent in the treatment of arrhythmias, atrial fibrillation [156] and supraventricular tachycardia [158];
- A_{2A} and A_{2B} stimulate AC, which increases the levels of cAMP [156];
- The activation of A_{2A} in EC, coronary smooth muscle, monocytes and macrophages induces vasodilatation, neo-angiogenesis and inhibit the production of pro-inflammatory cytokines, by acting on neutrophils and lymphocytes in inflammatory responses [173]. Binding to Ado, activates the pathway that increases cAMP production. It is associated with the activation of PKA, which in turn is able to inhibit nuclear factor (NF-kβ) thereby promoting anti-inflammatory activity [174];
- Receptor A_{2B} controls the degree of contraction of the vascular system through vascular tone [161], vasodilatation, decrease in heart ischemia and reperfusion [158]. Also, prevents cardiac remodeling after myocardial infarction and has a protective effect on ischemic post-conditioning [156]. As to anti-inflammatory response, it stimulates the secretion of cytokines or interleukin, a family of compounds also called human cytokine synthesis inhibitory factor (CSIF), such as, IL-10. The latest is produced by macrophages, and has anti-inflammatory properties [175];
- A_3 stimulates PLC enzyme, which increases calcium levels, modulates the activity of protein kinase C (PKC) [166] and chemotactic mechanisms [173]. It inhibits AC, decreasing available cAMP, also inhibiting the activity of PKA. Additionally, A_3 induces cardioprotection mechanisms in ischemia heart disease, through activation of PKC and regulation of mitochondrial ATP channels [164].

The immune capacity is one of the most important functions in our body and it is mediated and regulated by Ado release and respective binding to its specific receptors. This purine nucleoside regulates innate immunological reactions caused by microorganism invasions or tissue damage and reduces the inflammatory response that is triggered [175]. The extracellular Ado is a modulator of immunity, by linking to its receptors at the surface of some

immune cells, such as, macrophages, lymphocytes, neutrophils [173], dendritic and mast cells. Subsequently, as shown in figure 31 [175], each cell type is activated and sets off a particular mechanism and specific responses. For example, neutrophils decrease superoxide anion production and EC adhesion [175]. Another important reaction is increased secretion of VEGF by macrophages and EC, which is crucial in the process of tissue repair [173]. The protective role of Ado is also evidenced by the decrease of energy consumption in damaged tissue, which is mediated by a direct mechanism of inhibition. An example of this is the negative inotropic effect of Ado on heart muscle with ischemia, hypoxia or inflammation [172]. Furthermore, Ado indirectly protects cardiac tissue by increasing the availability of nutrients through vasodilatation [175]. Thus, Ado controls progression, intensity and time of an inflammatory process, by biochemical interactions with the immune system.

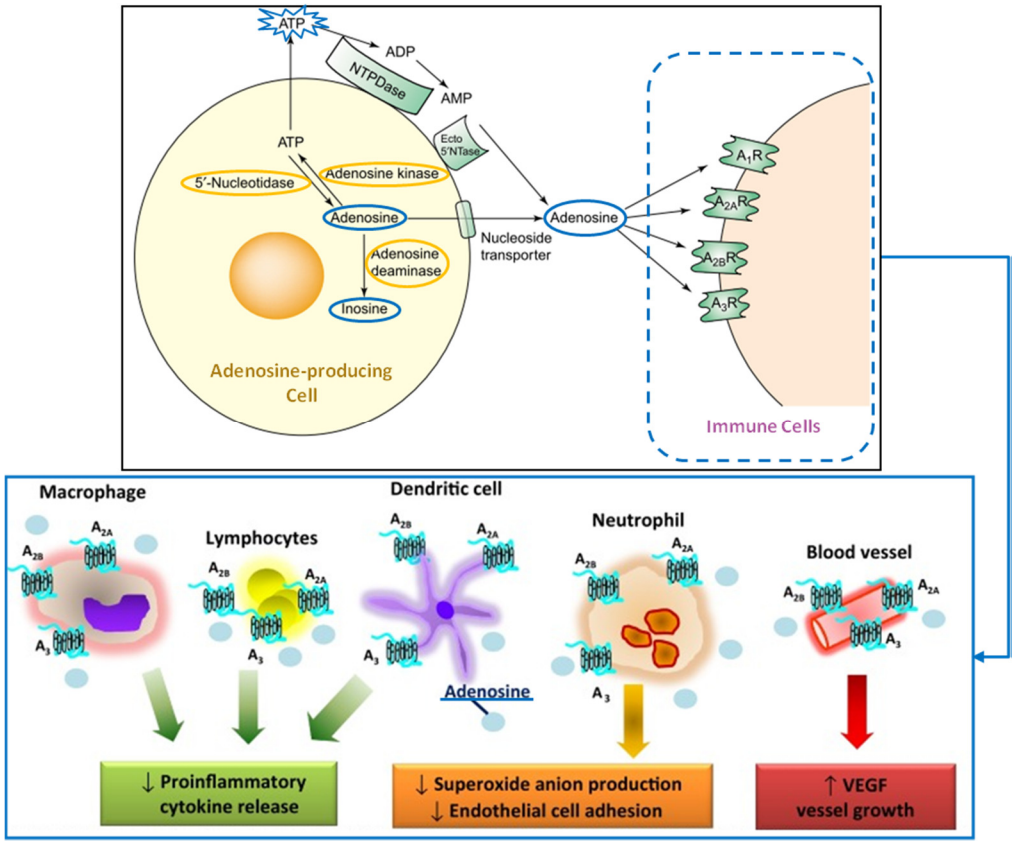

Figure 31 - Interaction of adenosine with immune cells leading to inflammation control response. Adapted from [175, 176].

Cells capture Ado from the external environment, which is needed for intracellular synthesis of ATP. Bodin and Burnstock in 1996 [177], studied the influence of extracellular ATP in HUVEC cells, and they showed that it induces the production and release of this energetic molecule, an important status in physiological and pathological conditions, as for example, in oxidative stress and hypoxia states. The same scientists [178] showed in 1991, that ATP release occurs exclusively in EC and not in muscle cells, and that the endothelial are the ones to respond in oxidative stress situations.

Ado and their derivatives, also display an important role in regulation of gastrointestinal function. Purinergic neurotransmitters mediate relaxation of gastrointestinal tract and bladder smooth muscle and, simultaneously, control secretions of intestinal mucosa, bowel fluid and electrolyte transport [179]. These mechanisms are intimately related to the down-regulation of activated immune response, mentioned above. Therefore, disregulation of these processes has pathophysiologycal consequences, such as, dysmotility, diarrhea and visceral pain [179].

Several disorders result from inborn errors affecting purine pathways and they are, usually, associated to enzymatic failures. Deficiency of several enzymes, such as, APRT (salvage of adenine to AMP), HGPRT (salvage of hypoxanthine and Gua into IMP and GMP, also called Lesch-Nyhan disease), ADA (conversion of Ado to Ino), purine-nucleoside phosphorylase (PNP; reversible phosphorylation of Ino and Gua), xanthine oxidase (XO; conversion of hypoxanthine into xanthine amino acid and then to UA) and ADSL (convertion of adenylosuccinate into AMP). Additionally, molybdenum cofactor deficiency (inability to synthesize a cofactor of XDH) leads to xanthinuria, hypouricemia and increased urinary sulfite [180].

Guanosine (Gua) is an important precursor of several energetic compounds, such as, GTP, GDP, GMT and cGMP [181], and it is considered as another protective agent due to the signaling modulating effect on cell growth, differentiation and survival [182, 183]. Jackson *et al*. [181], conducted several studies to observe the action of Gua in cultured cells of aortic and coronary artery vascular smooth muscle, coronary artery endothelial cells and cardiac fibroblasts. They reported that Gua, in the extracellular space, inhibits the availability of Ado and that this effect was neither attenuated by inhibition of certain enzymes, such as, ADK, ADA, SAH, or by Ado transport mechanism [181]. However, when the regulation of cell proliferation mechanisms was required, extracellular Gua increased Ado capacity through specific Ado receptors [184]. They also proved that certain metabolic toxins like iodoacetate and 2,4-dinitrophenol inhibit glycolysis and oxidative phosphorylation reactions, which eventually increase extracellular concentration of Gua and Ado [181, 185]. Therefore, it is considered that guanosine has an indirect signaling and regulatory function on Ado.

1.3. Metabolic relationship between all de above-mentioned compounds

Endothelial function and its homeostatic state are indicators of the risk of developing CVD. All molecules like Hcy, GSH, Ado, Ino or NO, which in high concentrations may induce activation of vascular endothelium or, shifts in metabolic pathways that, directly or indirectly, change physiological functions, are considered as risk factors. As previously mentioned, reactive species are essential secondary messengers on EC. However, an excessive increase of those compounds can cause changes with negative effects on the cardiovascular system, which lead to toxicity and development of pathological conditions.

In conclusion, we emphasize some significant aspects and relationship between the compounds analyzed, expressed through the figure 32 and 33.

Some studies have reported the relationship between Hcy transsulfuration pathway and GSH metabolism, which indicate that there are two sources of Cys, the transsulfuration pathway and import from external environment. It is also pointed out that about half of total GSH results from the metabolic pathway of Hcy, and the other half comes from external transportation and protein processing [186]. Weiss *et al*. [187], demonstrated that increased expression of GPx increases NO bioavailability and decreases adverse effects mediated by HHcy.

Ado is considered to have protective characteristics at cardiovascular level. However, when there is an increase of plasma Hcy concentration, like in HHcy cases, Ado concentration decreases [188].

Adenine nucleotides result from Ado and PRPP in the presence of APRT, requiring ADK and SAH. Thus, the relationship between Ado and Hcy is clear, as represented in figure 33. Seley *et al.* [189], reported that guanosine fleximers, nucleoside analogue molecules, inhibit SAHH, the enzyme that catalyzes SAH into Ado and Hcy, activity.

Glycolysis is a source of Ser for folate cycle and a major source of energy in ATP form, which is essential for cell proliferation and biosynthesis reactions. Recently, it was discovered that this pathway is crucial to angiogenesis and that a partial reduction of this route has favorable effects on inhibition of pathological angiogenesis, like in tumor conditions [89].

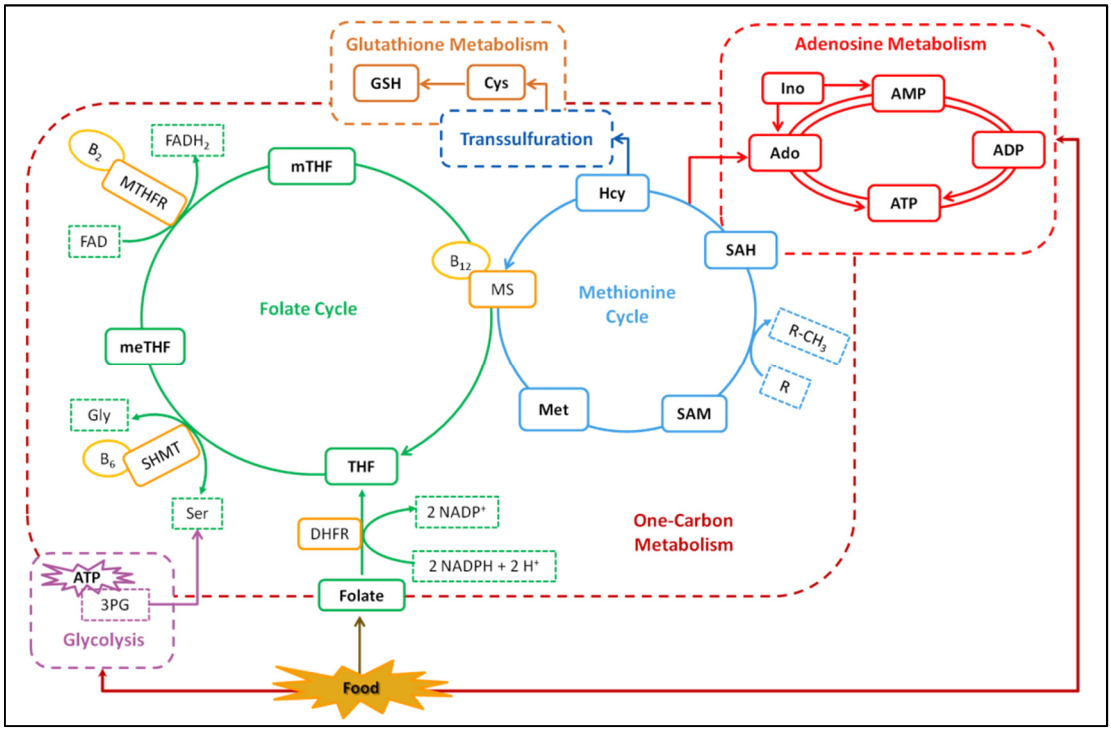

Figure 32 - Schematic representation of the metabolic relationship between the studied compounds.

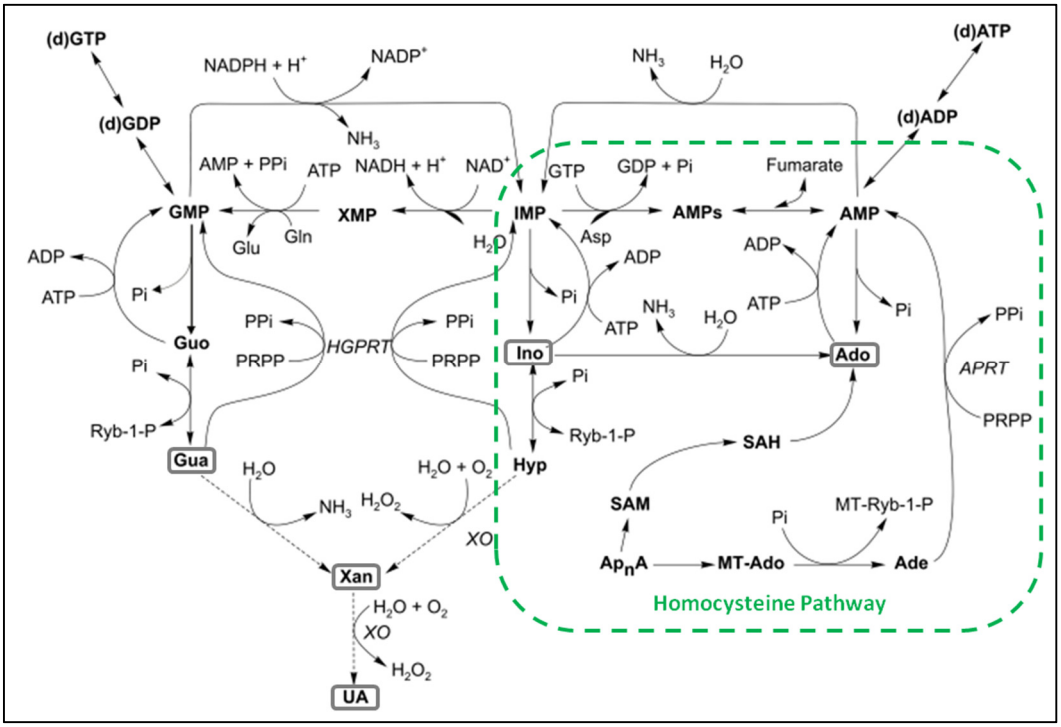

Figure 33 - Purine metabolism emphasizing the link with homocysteine pathway. Adapted from Baranowska-Bosiacka *et al.* [150].

2. Analytical Determination of Thiol Compounds

2.1. Methods for compound identification and quantification

So far, numerous techniques and procedures for qualitatively and quantitatively determination of thiol compounds, have been developed. On the one hand, it reflects the great interest on these compounds for clinical diagnosis. On the other hand, it demonstrates the great difficulty that it is to find an effective method for detecting a metabolic group of thiols. Besides being complex analyses, data interpretation and pathological relationship is not easy [104]. Nowadays, the separation and identification are possible thanks to innovative technologies, precise and with a high resolution, as listed in table 2. Each one has advantages and disadvantages that must be taken into account when choosing the analytical procedure [104]. However, it is important to remember that thiol compounds and their derivatives are oxy-reducible, very unstable and therefore of difficult quantification [190].

Table 2 - Analytical methods for biological markers [190, 193]:

Methods	**Technical Specifications**	
Chromatography	Gas Chromatography-Mass Spectrometry (GC-MS)	
	Liquid Chromatography-Mass Spectrometry (LC-MS)	
	Liquid Chromatography of High or Ultra Performance (HPLC or UPLC)	**Detection:**
		Photometric (PDA)
		Fluorescence (FLR)
		Electrochemical (ED)
		Hydrophilic Interaction (HIC)
	Ionic Exchange	
Capillary Electrophoreses	**Detection:**	
	Photometric	
	Fluorescence	
Immunoassays	Fluorescence Polarization (FPIA)	
	Chemiluminescence (ICL)	
	Enzymatic (EIA)	
Colorimetric enzymatic assays		
Spectroscopic - Nuclear Magnetic Resonance (NMR)		

From the listed methods, mass spectroscopy (MS), used for identifying a wide range of compounds and with high reproducibility, stands out. This technique can be associated with gas chromatography (GC-MS), which is more suitable for the analyses of volatile organic compounds and derivatives of primary metabolites [190]. Also, it can be coupled to liquid chromatography (LC-MS), which is applied in a wide range of semi-polar and polar compounds without derivatization [190, 191].

Capillary electrophoresis is a technique that, just like with chromatography, uses a small sample volume and analyses takes place in a short time, with good resolution and uses little solvent in the run [192]. One disadvantage is the poor sensitivity when ultraviolet (UV) detection is used [193].

Immunoassays, such as ELISA (Enzyme Linked Immunosorbent Assay), do not require such specialized equipment and are of high sensitivity and reproducibility [194]. However, the results are limiting as only metabolically reactive molecules are identified, for instance by antigen-antibody link or by enzymatic reactions. Another problem is the lack of standardization of trials and the high cost of reagents [190].

Nuclear magnetic resonance (NMR) is among the latest methodologies. The main advantage is that the analyzed sample is not destroyed, remaining viable for further analysis but, it has relatively low sensitivity [193].

The above-mentioned diversity of methods is due to the great need to find biomarkers of different pathologies for biochemical and molecular investigation. In these areas of research, the great clinical interest requires simple automated methodologies, very sensitive and accurate, which fit the routine work of the clinical pathology laboratories [195]. Thus, the scientific community has applied these powerful tools, in order to define metabolites and related compounds important in understanding mechanisms and in diagnosis and prognosis of pathologies [193].

2.1.1. Chromatography

Among the several methods that may be used for determination and quantification of thiol compounds, and their metabolic products, chromatographic methods are currently, the most used, since they are very versatile. They allow different forms of sample handling and many analytical conditions can be applied, besides different detection parameters as will be specified further on. A major advantage of this technique is the possibility of detecting multiple compounds in a single analysis, with a high specificity and sensitivity [196]. However, the main difficulty of analysis is associated with physicochemical properties of thiol compounds. They are highly unstable and reactive molecules so, they require cautionary measures throughout sample treatment. Taking all these factors into account, liquid chromatography was considered as one of the best methods to fit the goals of the presented work.

2.1.1.1. History and Evolution

Chromatography was set up at the beginning of the year 1900, describing a colorimetric experience characterized by the separation of a mixture of compounds distributed between two or more immiscible phases (gaseous, liquid or solid).

It is a dynamic method in which the components are separated between two phases, one fixed (stationary phase) and another one that moves relatively to it (mobile phase) [197]. Liquid chromatography is the science that allows separating, identifying and quantifying compounds that are in a liquid sample. This technique involves the injection of a small sample volume into a column packed with porous particles. The individual components carried along the column by a liquid solvent move by gravity or pressure [198]. The components of interest are separated from each other due to the chemical and physical interactions established with

the column packing material. The separated compounds are detected and identified by output external measurement techniques. Since the development of the first chromatographic system, this method became one of the most powerful tools in analytical biochemistry [197, 198].

The first separative chromatography demonstrations date back to 1860, but only in 1902 the botanist Tswett described the technique in his studies. He used a gravitational column (Classic method) and demonstrated its ability to separate different compounds in plants extracts. Years later, in 1941 Martin reported that the most efficient columns must be composed of very small particles and withstand high-pressure differences throughout its length [197]. In the early 1950´s, the first-automated method that enabled the analysis of amino acid mixtures, by ion-exchange chromatography was described, which was considered an important precursor of the high-pressure liquid chromatography (HPLC) [197, 198]. The relevant theoretical foundations of this technology have been established until the 1960´s, which allowed, in the following years, the creation of the commercially known HPLC equipment patented by the company Water Associates, which so far dominates the market [198, 199].

In the pre-HPLC period, the results were obtained after a two-hour run, but the progressive improvement already achieved in 1970, allowed reduction of the run time to 15 minutes. Since 1980 until today, the method has developed so much that in just a few seconds it is possible to obtain a specific chromatogram of a mixture of compounds [198]. The company now renamed Waters Corporation, states that the improvement of results are due to reduction of the separation time and the increase of pressure in the column, a consequence of reducing the size of particles that constitute the column packing material [197]. They claim that the emergence of HPLC results from the urgency in analytical biochemistry results, within the Biotechnology, Pharmaceutical and Agrochemical Industry seeking fast, efficient and economic methods. Therefore, it points out that the technique has been set in order to obtain results in a short time [197, 198]. However, in 2004, the high analytical demands led to the development of ultra performance liquid chromatography (UPLC), a unique technology that combines the best attributes in speed, sensitivity and resolution in a chromatographic system [200]. This new approach involves simultaneously innovation, technology and design in a single instrument, designed to meet and overcome the challenges of laboratory analysis. These characteristics have been achieved by reducing the particle size of the stationary phase (1.7 – 1.8 μM), compatible with many mobile phases and pressure limits up to 1030 bar (15.000 psi). This innovative system achieves ultra-fast analytical separation, with high yield and resolution in less time of analysis [197, 199].

2.1.1.2. Ultra Performance Liquid Chromatography System (UPLC®)

The chromatography equipment used was the ACQUITY UPLC® H-Class of Waters Corporation, shown in figure 34. Its main components are a quaternary manager solvent, a sample flow controller through a needle, tunable detectors and exchangeable separation columns. All this analytical chromatographic system is controled by the software Waters® Empower™.

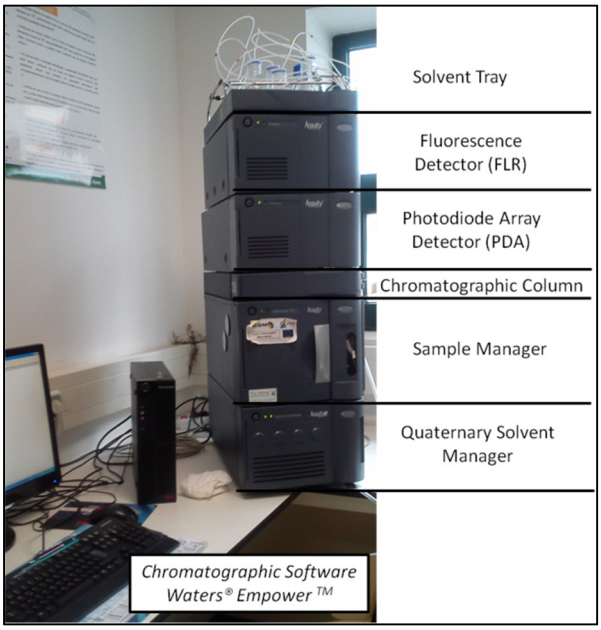

Figure 34 - Characterization of the ultra performance liquid chromatography system ACQUITY UPLC® H-Class of Waters Corporation.

I. UPLC® operation system

This chromatographic system (figure 35) operates effectively, in order that a portion of the sample is pipetted, mixed in the solvent flow and transported to the column through a high-pressure flux. That injected mixture, crosses the column through the porous center and, at the output, the detector identifies fractions of constituents [201]. Subsequently, the signal is received and decoded by the computer system, reflecting it into peaks. The separation of the analytes is possible due to competition between the phases that make up the system. The first detected compounds have higher affinity for the mobile phase, moving faster through the column. The last, have higher affinity for the stationary phase and suffer greater retention [201, 202]. Therefore, the essence of liquid chromatography lies in the travelling speed of compounds, which in turn is determined by the molecular characteristics of the sample, but also by the properties of the column packing material and reagents used as solvents.

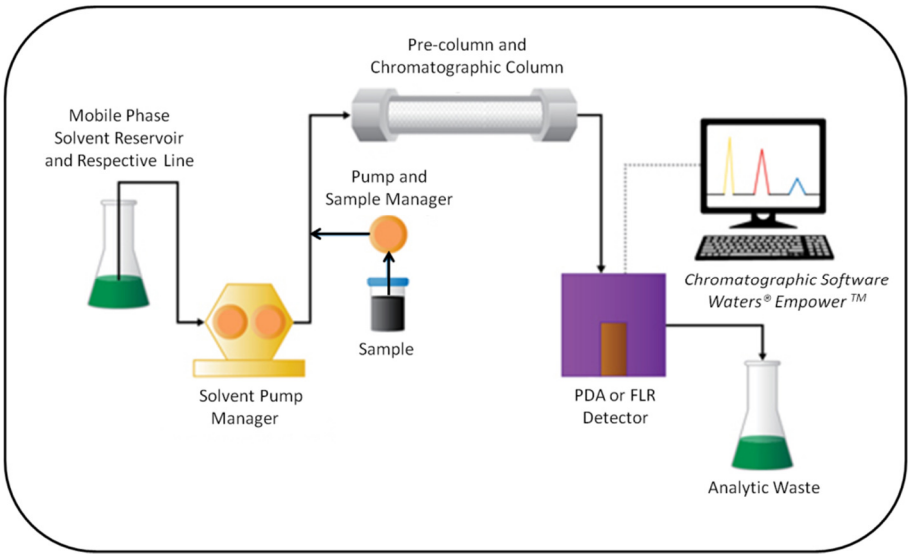

Figure 35 - Schematic representation of the operation system UPLC®. Image obtained from [203].

II. Chromatographic analysis

The separated compounds are identified by external measurement techniques after their exit from the column. As previously mentioned, the specific bands of each analyte are detected and transmitted to the computer system. The detector when receiving that information identifies the band, in contrast with the mobile phase background and sends the signal to the software that translates in a chromatogram, a graphical representation of all chemical compounds that are possible to detect in the sample injected [203]. A band is characterized by two distinct zones, the central part with higher concentration of the compound, and the anterior and posterior edges with lower concentrations. These differences across a band are interpreted and translated at the peak and each peak expresses each compound identified, in a graph that reveals the concentration in arbitrary units relative to the run time [202, 203].

The most important considerations about the chromatographic system UPLC® H-Class are:

A. **<u>Column</u> –** A wide variety of columns can be used in this equipment and the choice is made according to the compounds that the operator wishes to identify. In this case, we opted for the ACQUITY UPLC HSS T3 (2.1 x 100 mm, 1.8 µm particle size) and the ACQUITY UPLC HSS T3 VanGuard™ (2.1 x 5 mm, 1.8 µm particle size) pre-column from Waters Corporation (Figure 36) [204]. It consists of spherical silica particles with 1.8 µm of diameter, forming pores of 100 Å, bonding technology T3 that utilizes a trifunctional C_{18} alkyl phase. Particles have a high compatibility with aqueous mobile phase, perfect to retain and separate a wide range of polar organic compounds. Designed to have a high resistance (HSS – High Strength Silica) and ensure rapid separation with higher resolution. This way, it combines efficiency, robustness and high yield. It operates effectively in solvents at pH between 2 - 8, pressure above

18.000 psi (1241 bar or 124 MPa) and temperature ranging between 20 - 45 °C. The column contains an intelligent chip, which records the history of its performance and, at the same time, calculates the lifetime. It is considered one of the most advanced technologies in liquid chromatography [205, 206].

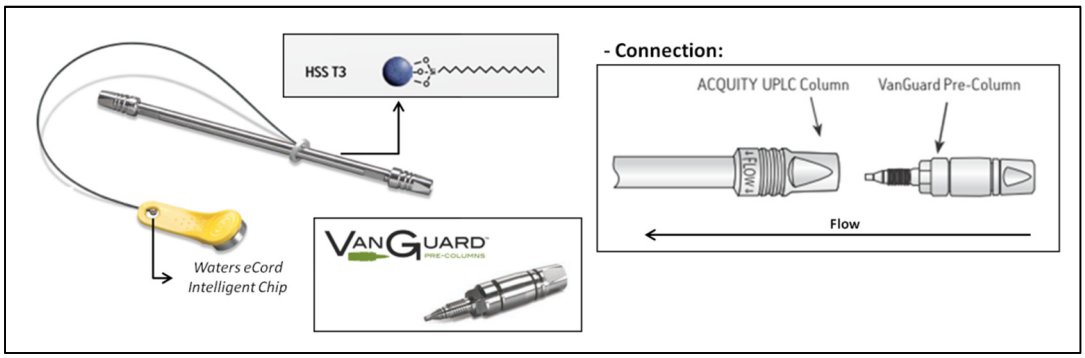

Figure 36 - ACQUITY UPLC HSS T3 (2.1 x 100 mm, 1.8 µm particle size) column linked to ACQUITY UPLC HSS T3 VanGuard™ (2.1 x 5 mm, 1.8 µm) pre-column and representative scheme of their connection. Image obtained and adapted from [204, 207].

B. **Detector -** There are various types of detectors and all have the ability to reveal the presence of a specific compound in the analyzed sample. However, it is necessary to adjust each sample to the method, system conditions and reagents that are used. The detectors for UPLC® system have been designed, taking into consideration the physical and chemical properties of the analytes that can be identified and solvents that can be used [208]. The detectors differ in capture and translation of the received signal. There are fluorescence (FLR), photodiode array (PDA), electrochemical (EC), evaporative light scattering (ELSD), mass spectrometry (MS; SQD – Single Quadrupole Mass Spectrometry Detector and TQD – Quadrupole Mass Spectrometry Detector) and refractive index (RI) detectors, among others [208, 209]. Several features must be taken into account before choice, such as, the need of a rapid response with high sensitivity and reproducibility and, at the same time, to be specific and predictable. The detector should provide a dynamic response and express direct proportionality [208], in particular, the response should increase linearly with the increasing amount of analytes in the sample. The results should be translated in a qualitative and in quantitative way.

Finally, something to keep in mind is that the results are affected by the pH of the sample and solvent, the system temperature, the flow rate and type of mobile phase used [208, 210].

Following, the main characteristics of two of the detectors used in the chromatographic system UPLC® H-Class:

B1. Photodiode array (PDA) – Characterized by ultraviolet and visible light (UV/Vis) spectrophotometer, illustrated in figure 37, that captures information between 190 - 500 nm, with an optical resolution of 1.2 nm, transmitted by a matrix of 512 aligned photodiodes [211]. The flow cell of the column is similar to an optical fiber, coated with Teflon AF amorphous and chemically inert fluoropolymer [212]. When the mixture of mobile phase and sample pass the core of this section, the light from the deuterium lamp (D2 lamp – Hydrogen Isotope) is projected [211]. The refractive index of Teflon AF is lower that the aqueous mixture, so the light beams entering the liquid core are reflected and transmitted throughout its extension. The light coming out of the column through the silica pores is projected to the concave spectrograph (Diffraction Grating), which disperses and projects the received light to the photodiode array [211, 212]. Thus, the detector measures the amount of light that reaches the photodiode array and determines the absorbance of the sample in the flow cell. Each photodiode operates with a capacitor that retain a certain amount of charge, which when struck by light is released [211]. Therefore, the magnitude of the discharge depends on the amount of light that reaches the photodiode [208]. Subsequently, the detector measures the amount of energy needed in charging each photodiode, being proportional to the amount of light transmitted through the flow cell, for a given time interval [208, 211]. Therefore, the chromatographic results are given through a graph, where the absorbance units (AU) and the time (min) of analysis are expressed. In this type of detector, it is important to consider the possibility of some photometric errors. As such, the PDA experts report that to minimize these effects, the maximum spectral absorbance of a compound should never exceed 1 AU [211].

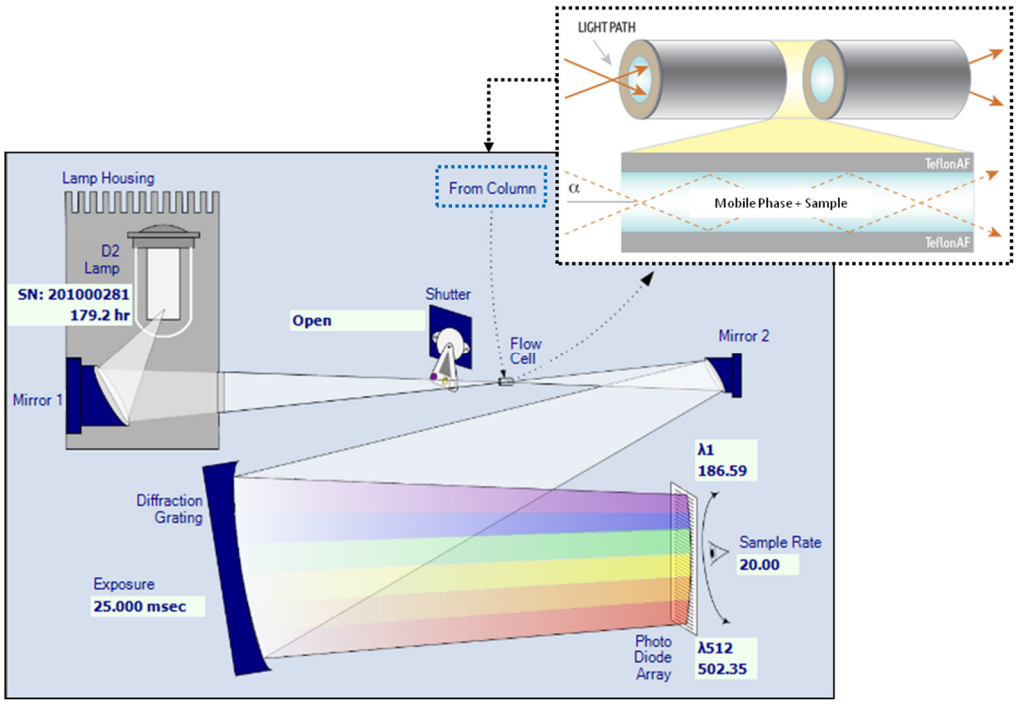

Figure 37 - Schematic representation of the ACQUITY UPLC Photodiode Array Detector operating mode in the UPLC® system. Interactive image taken from the chromatographic equipment used and adapted the information referred to in Waters Corporation publications [208, 212].

B2. Fluorescence (FLR) – It consists in determining the optical light emitted by the sample, which is excited by a specific wavelength [208]. The detection process begins by filtration of the source light from the mercury and xenon lamp (Hg-Xe Lamp), through the excitation monochromator (Excitation grating and parabolic mirror) an optical device with selects the excitation spectrum [213, 214]. Afterwards, the selected wavelength comes into the opening flow cell, where the sample is excited for this high-intensity light. In the axial length of this section, the energy reflected back and forth, doubling the length of the path, which in turn increases the sensitivity of the detected signal [213]. Subsequently, the light is emitted by the sample through the top cell to the emission monochromator (Emission grating and photomultiplier tube (PMT)), where the low energy intensity is captured, filtered and translated into a fluorescent signal [208, 213]. In this detection, schematized in figure 38, it is possible to distinguish two light paths, the optical excitation and the emission monochromator, capable of detecting multiple wavelengths between 200 - 980 nm and 210 - 900 nm, respectively [213, 214]. Therefore, the chromatographic results are given through a graph where the emission units (EU) and the time (min) of analysis are expressed.

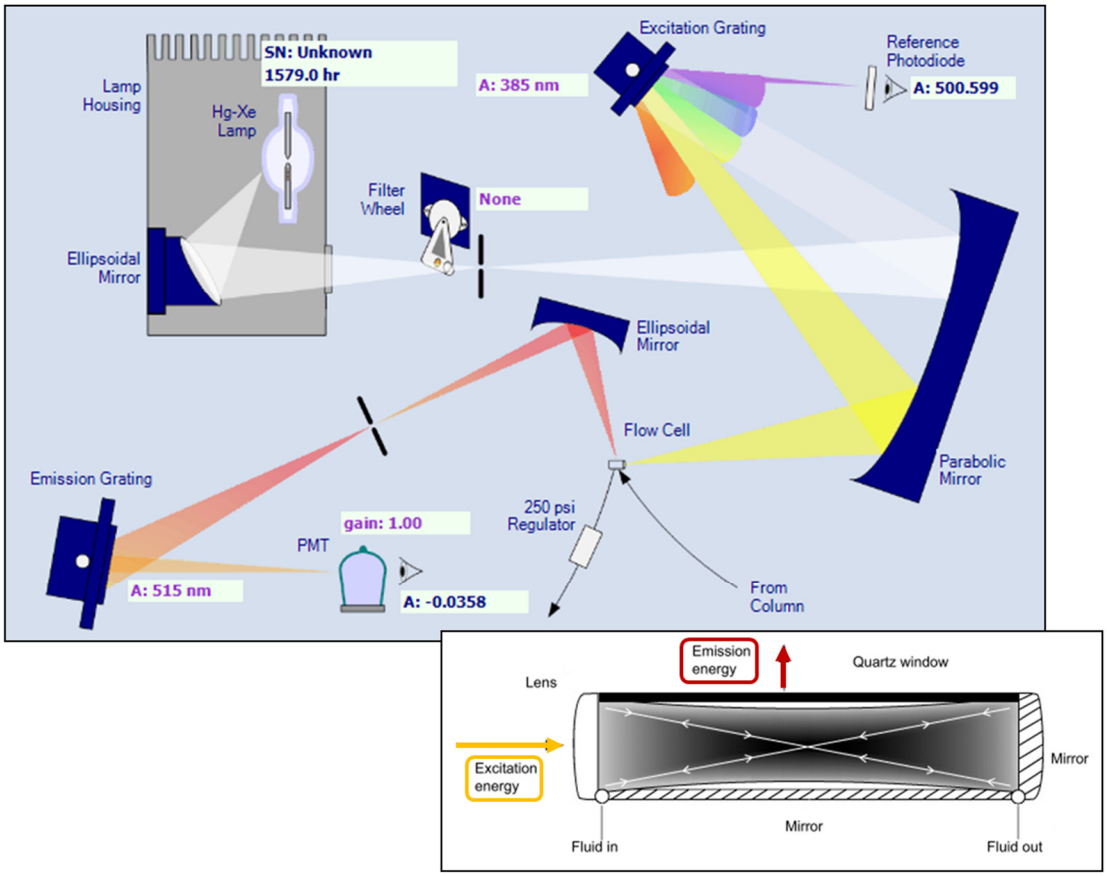

Figure 38 - Schematic representation of the ACQUITY UPLC Fluorescence Detector operating mode in the UPLC®. The yellow light corresponds to the excitation energy and in red the emitted by the sample. Interactive image taken from the chromatographic equipment used and adapted the information referred to in Waters Corporation publications [213].

Summarizing, the UPLC® system is very versatile and allows combining different separation parameters and detection so, it is currently one of the most used technologies. It is widely applied for the detection and identification of metabolite profiles related to various human pathologies and it is a powerful tool that identifies numerous biomarkers. Noteworthy, the UHPLC-PDA|FLR methods provide fast and excellent resolution results using low volume of sample and solvents, and for this reason it is also economic. Therefore, our chromatography assay had low environmental impact.

2.1.2. Pre-analytical considerations

In order to select the best technique, besides the previously referred, one must know well the characteristics of compounds intended to analyze, such as, the polarity (hydrophilic or hydrophobic), the volatility, etc.

Few UHPLC-PDA|FLR methods for thiol compounds identification, in biological fluids, have been reported. Experimental procedures for this type of compounds imply the pre-treatment of samples, such as, deproteinization, reduction reactions and derivatization.

In the preparation of stock solutions of thiol compounds, some studies have reported that the formation of disulfide bonds promoted in basic medium [215]. So, they oxidize easily in alkaline solution. Furthermore, they suffer photo [216] and thermal [217] degradation, as such, throughout all procedures they should be kept protected from light and refrigerated around 0 °C, because they are more stable.

To ensure stability of thiol compounds throughout the process of chromatographic analysis, reduction of disulphide bonds is a crucial step. For that purpose, the addition of a reducing agent was required. Due to its properties and good results presented in numerous reports, tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) ($C_9H_{15}O_6P$·HCl) has been tested [218]. It results from the hydrolysis of the compound tris(2-cyanoethyl)phosphine in an acidic medium. It is reported to be a good reducing agent since it has high specificity for thiol compounds, large reactive capacity, even at low concentrations, is less sensitive to temperature changes and ensures better reproducibility and robustness of the tests [219]. Besides, it is water soluble, stable in acidic or basic medium, resistant to oxidation by air, odourless, non-volatile and has low toxicity. TCEP-HCl is considered more effective than other reagents on the market, such as, dithiothreitol (DTT) and 2-mercaptoethanol [219, 220]. Tributylphosphine ($C_{12}H_{27}P$), also known as TBP, is another reducing agent, which has an identical action to TCEP·HCl mentioned above but it is considered less effective and was also tested. It is a colorless reagent with amine odor [221] and, sometimes, it must be dissolved in dimethylformamide, a toxic solvent [219], which requires great caution.

The derivatization of thiol compounds is an exclusive and crucial step for their detection by FLR. The fluorescence of certain molecules results from light absorption in a specific wavelength that promotes their rise to a higher energy state. Later, when they return to their normal state, the excited molecules release the adsorbed energy in the form of photons [213]. The organic compounds analyzed in this work, do not naturally have that

characteristic, are able to absorb energy, but in the release they do not emit fluorescence. Therefore, it is necessary to perform labelling with a florescent molecule. As such, the derivatization reagent 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid ammonium salt ($C_6H_3FN_2O_4S{\cdot}NH_3$), also colled SBD-F, which specifically reacts with the sulfhydryl group of thiol compounds, was considered as. It is water-soluble and the labeled compounds have an excitation wavelength between 380 - 385 nm and emission at 515 or 510 nm [222].

It is important to consider that some parameters affect the reactive capacity of compounds, such as, pH changes that modify the electronic distribution of compounds with gain or loss of protons, which subsequently determines the intensity of the emitted signal. Furthermore, the fluorescence decreases with temperature increase and presence of oxygen dissolved in the sample [213].

Chapter 2

Experimental

In this chapter, experimental protocols will be described in detail.

2.1. Reagents

During this work, reference compounds of analytical grade: L-glutathione (GSH), DL-homocysteine (Hcy), L-homocystine (2Hcy), L-cysteine (Cys), cysteamine (Cyst), adenosine (Ado), inosine (Ino), as well as, the reducing agents tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) and tributylphosphine (TBP), ammonia, boric acid, and the derivatizing agent 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid ammonium salt (SBD-F) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade solvents: acetonitrile (ACN) and methanol (MeOH) from Fisher Scientific (Lisbon, Portugal), and formic acid (FA) from Panreac Química (Barcelona, Spain).

Water was purified by a Milli-Q Integral purification system from Millipore Corporation (Billerica, MA, USA) under the following conditions: 18 MΩ cm at 25 °C.

2.2. Equipment

UHPLC analysis was carried out in an ACQUITY UPLC® H-Class System, combined with a Waters ACQUITY Quaternary Solvent Manager (QSM), an ACQUITY Sample Manager (SM), a column heater and a degassing system. Two chromatographic columns: the HSS T3 (2.1 x 100 mm, 1.8 µm particle size) and HSS T3 VanGuard™ (2.1 x 5 mm, 1.8 µm particle size) pre-column; the BEH C_{18} (2.1 x 150 mm, 1.7 µm particle size) and BEH C_{18} 1.7 µm VanGuard™ (2.1 x 5 mm column) pre-column, were employed. Two detectors were used: Photodiode Array Detector (PDA) and the Fluorescence Detector (FLR). The whole chromatographic system was driven by Empower Software v2.0, also from Waters Corporation (Milford, MA, USA).

A vacuum filtration system with polytetrafluoroathylene (PTFE) membrane filters (0.22 µm) was used for solvents and syringes with disposable filters of the same type of membrane porosity, for samples. All obtained from Millipore Corporation (Billerica, MA, USA).

2.3. Preparation of solutions

2.3.1. Standard solutions

Stock solutions of GSH (2.00 mM), Hcy (7397 µM and 2.00 mM), 2Hcy (2.00 mM), Cys (100 mM and 2.00 mM), Cyst (2.00 mM), Ado (2500 µM) and Ino (2500 µM). Briefly, 10 mg Hcy was dissolved in 10 mL of acidified Milli-Q water with 0.4% FA [223]. 3.07 mg GSH, 1.21 mg Cys, 2.35 mg Cyst were all dissolved in 5 mL of acidified Milli-Q water with 0.04% FA.

In order to evaluate the stability of standards and to maintain free sulfhydryl groups, several conditions were tested including the use of 0.04% FA and 0.04% boric acid. Standard

solutions used in the elaboration of calibration curves of thiols, were prepared from the above-mentioned stock solutions by dilution in the mobile phase used or in acidified Milli-Q water. The concentrations of GSH, Cys, Hcy and Cyst ranged from 5.00 to 100 µM, and of Ado and Ino from 25.0 to 500 µM.

Ado 500 µM solution resulted from the dilution of a pre-existing 5000 µM stock solution. It was prepared by diluting 500 µL of this stock solution with Milli-Q water, to obtain a 5 mL solution. Ino 2500 µM solution was obtained by weighing 3.35 mg and dissolving with Milli-Q water until obtaining a 5 mL solution. When necessary, a few drops of 0.1 M ammonia solution were added to these purine compounds, in order to achieve a clear solution.

Solutions were all filtered with a PTFE membrane and preserved at -20 °C.

2.3.2. Reducing agent

As described by Valente *et al.* [224], 5 mL of 390 mM TCEP·HCl was prepared in Milli-Q water. Subsequently it was also filtered with a PTFE membrane and aliquots were made and stored in eppendorf tubes at -20 °C [223].

TBP solution was used diluted 1:40 in the sample.

2.3.3. Derivatizing agent

The derivatizing agent used for thiols was SBD-F, prepared at 4.24 mM, weighing 1 g and dissolving in 1 L of Milli-Q water.

2.4. Pre-analytical treatment performed in standard solutions

2.4.1. Standard solutions of purines for UHPLC-PDA analysis

Ado and Ino at different concentrations, were injected directly in the chromatographic system, without any sample pre-treatment.

2.4.2. Standards solutions of thiol compounds for UHPLC-FLR analysis

Thiol compounds GSH, Hcy, 2Hcy, Cys and Cyst were previously labeled with a fluorescent molecule, the derivatizing agent SBD-F.

Derivatization procedure consisted in mixing the standard solution with TBP and incubating for 30 minutes at 4 °C. Then, 100 µL of borate buffer and 50 µL of derivatizing agent SBD-F were added to 50 µL of the previous reduced mixture and it was incubated for 60 minutes in a 60 °C water bath. After this time, the tube was placed in an ice bath for 5 minutes. Finally, 2 µL was injected in the UPLC-FLR system.

The entire procedure was carried out while maintaining and protecting eppendorf tubes from light, by covering them with aluminum foil, since thiols are photosensitive compounds.

2.5. Development and optimization of analytical methods

A method for thiol analysis was developed on a UPLC® system consisting of a quaternary solvent manager, a sample manager and two tunable detectors, PDA and FLR.

The starting point of this research was based on the work previously developed by a former master student, Hugo Câmara in his dissertation [223]. We started using the same UHPLC techniques, which were modified in order to obtain the best results.

Preliminary experiences to optimize chromatographic separation of standard compounds involved testing several UHPLC conditions.

Different mobile phase compositions, such as, Milli-Q water acidified with 0.1% formic acid (H_2O|0.1%FA), ACN without or with FA acidification (ACN|0.1%FA) and MeOH without or with FA acidification (MeOH|0.1%FA), as well as, different pH conditions were assayed. Two chromatographic columns were tested, the HSS T3 and the BEH C_{18}, protected with the respective pre-columns. Various injection volumes were analysed (2, 4, 6 and 8 µL), as well as, sample temperatures (4, 8, 10, 20, 23, 37 °C). Several conditions of flow-rate (0.2, 0.3 and 0.4 mL/min), time of analysis (5, 8 and 10 min) and equilibrating (1, 2, 5 and 10 min), were tested.

2.5.1. Validation of methods

Validation of an analytical method involves several procedures, which are necessary to show that it can be used in quantitative measurement of one or more analytes in a given biological matrix. Moreover, it must be reliable and reproducible [225]. Validation must be documented by the experimental work. Acceptability of an analytical data should be reflected in the parameters of validation [226]. In this case, there was an exploratory and internal research method with the expectation of promissory results, where validation was a requirement.

With the purpose of validating our method, selected concentrations of calibration solutions were chosen according to equipment sensitivity and the concentration usually found in biological samples. In this study, the following analytical parameters were considered:

The **selectivity** was measured by the absence of interfering compounds in analyte's retention time. This ensures that the quantification of analytes is not affected by compounds used in sample treatment or by mobile phase constituents [226]. To measure this, the chromatogram from direct injection of standard solutions must be compared with the chromatogram from biological samples determining the absence of interference of analytes, on the desired retention time. Accordingly, it is considered that a method is specific when it allows discriminating compounds, providing assurance that a certain quantity comes only from the intended analyte [225].

The **linearity** of the method expresses results directly proportional to the concentration of the compound and it is obtained by determining the correlation coefficient [225]. With this purpose, three calibration curves for each analyte, were carried out with six (or more) different concentrations of the compound prepared in acidified water, mobile phase or by derivatizing procedures. Each concentration was injected six times. It was estimated from the concentration range of the calibration curve, the lowest level (Low Level - LL), middle (Medium Level - ML) and higher level (High Level - HL) [226, 227]. The superposition of the different concentrations levels expressed the linearity of the method.

The **sensitivity** was determined by the limits of detection (LOD) and quantification (LOQ) for each tested compound [227]. It is calculated by the lowest concentration level of the analyte in the linearity evaluation. LOD is calculated by 3 x standard deviation (SD), which is three times the noise in the baseline, and LOQ is obtained by 10 x SD, what is ten times greater than the baseline noise [226].

The **precision** allows evaluating the dispersion of results between dependent tests, repeated on the same sample under the same conditions [225]. It is expressed by the relative standard deviation (RSD) obtained by injecting three concentrations - Low, Medium and High level calibration curves. Standards were analyzed six times on the same day (Intra-day) and reproducibility (Inter-day) was determined on three non-consecutive days with replicas of different concentrations [226, 227]. Within this parameter are the repeatability and reproducibility.

Chapter 3

Results and Discussion

Throughout this chapter, the most relevant results obtained during the development of the UHPLC method with FLR detection for identification and quantitation of some thiol compounds, and PDA detection of some purines are presented and discussed.

3.1. Development and optimization of an analytical method for analysis of thiols by UHPLC with FLR detection.

3.1.1. Thiol analysis by UHPLC with PDA detection

In order to find an effective method for the detection of all thiol compounds (GSH, Hcy, 2Hcy, Cys, Cyst), in a single run, some preliminary experiences were performed. Chromatographic conditions were tested in the UPLC® system, concerning the mobile phase composition, type of column, flow-rate, analysis and equilibrating times, injected sample volumes and column temperature. The main results obtained are reported below. Our chromatographic results corresponded to the data provided by the UPLC® system, for each injection performed. The identification of each compounds were expressed by the retention time (min) and by the peak intensity in AU or EU, whether the detection was by PDA or FLR. Thus, the intensity was translated by the peak area (µV°sec) and height (µV) Additionally, the spectrum obtained by PDA helped to confirm the identity of each peak.

Firstly, **thiol analysis by UHPLC with PDA detection** was tried with the chromatographic conditions described by Câmara [223] for Hcy analysis.

The results obtained with the UPLC-PDA method, were unfavourable like featured below in figure 39. The chromatograms showed peaks with poor resolution, low sensitivity and a great interference of the mobile phase, with a high background noise.

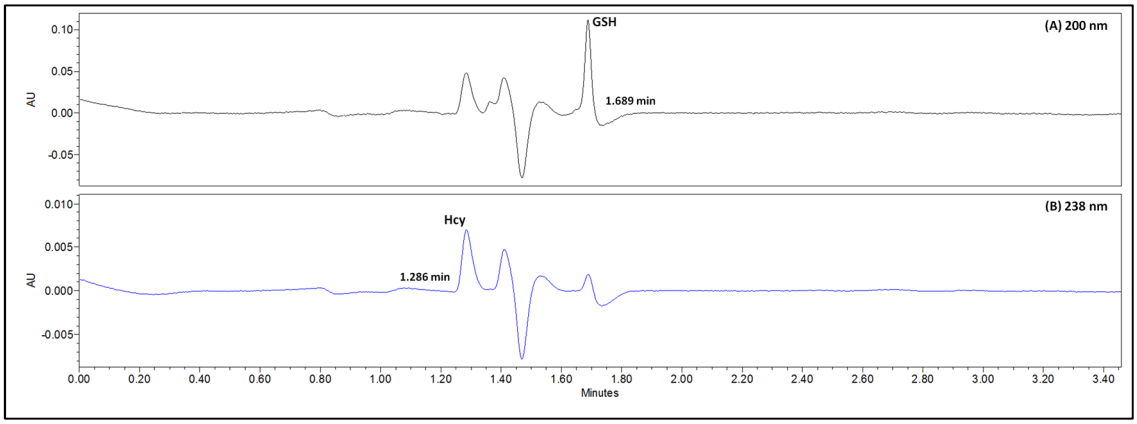

Figure 39 - UHPLC-PDA chromatogram of a mixture of glutathione (100 µM) (A) and homocysteine (7397 µM) (B).

Several gradient conditions of the mobile phase were tested (see below). None gave good results. Peak overlap and doubled peaks were constantly obtained. More, in several

assays the chromatograms acquired with PDA detection evidenced large interferences of the mobile phases (figures 40 and 41) and poor sensitivities.

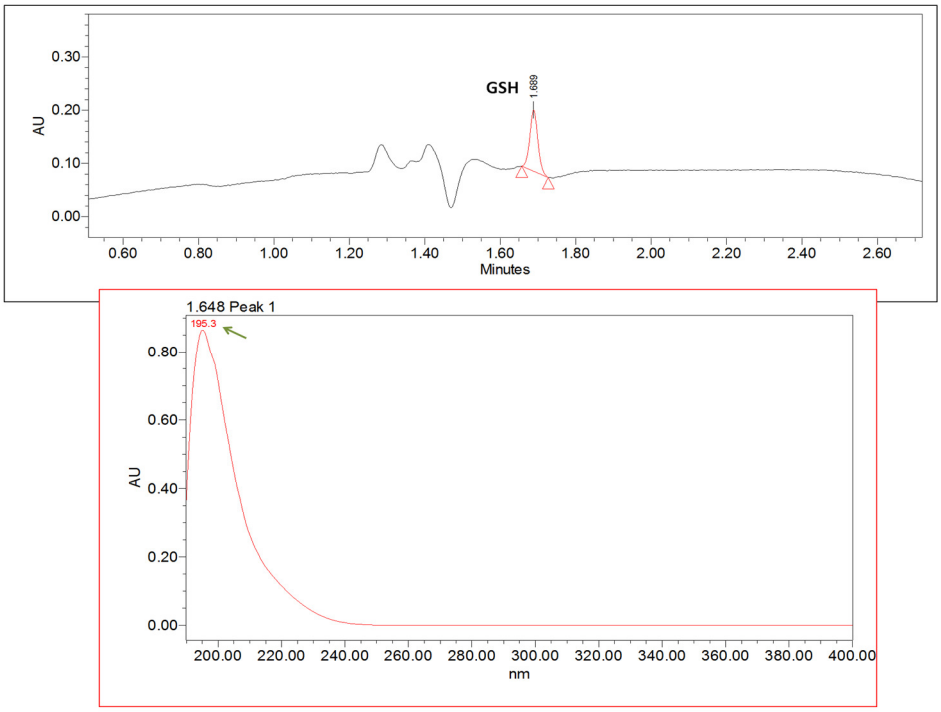

Figure 40 - Spectra of analyzed glutathione (200 ± 4.7 nm) by the UHPLC-PDA method. For additional chromatographic conditions, see table on annex 1.

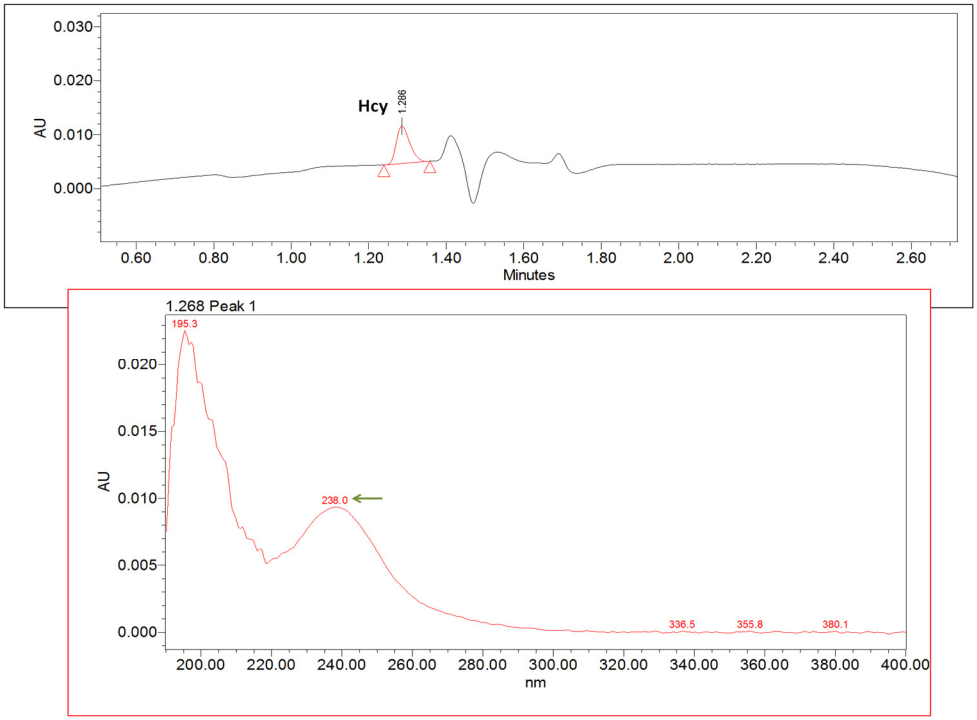

Figure 41 - Spectra of analyzed homocysteine (238 ± 0.0 nm) by the UHPLC-PDA method. For additional chromatographic conditions, see table on annex 1.

I. Sample treatment procedure tested for standard solutions

TCEP·HCl was tested as a thiol reducing agent, according to the work developed by Câmara [223], but using different chromatographic conditions as presented in annex 2. TCEP·HCl 1 mM was also assayed in several different chromatographic conditions, by diluting it in the mobile phase to exclude possible interferences.

These chromatographic conditions showed to be more favourable than those previously tested, based on the conditions of Câmara [223], like featured below in figure 42. The chromatograms showed a better resolution of peaks, higher sensitivity and less interference from the mobile phase.

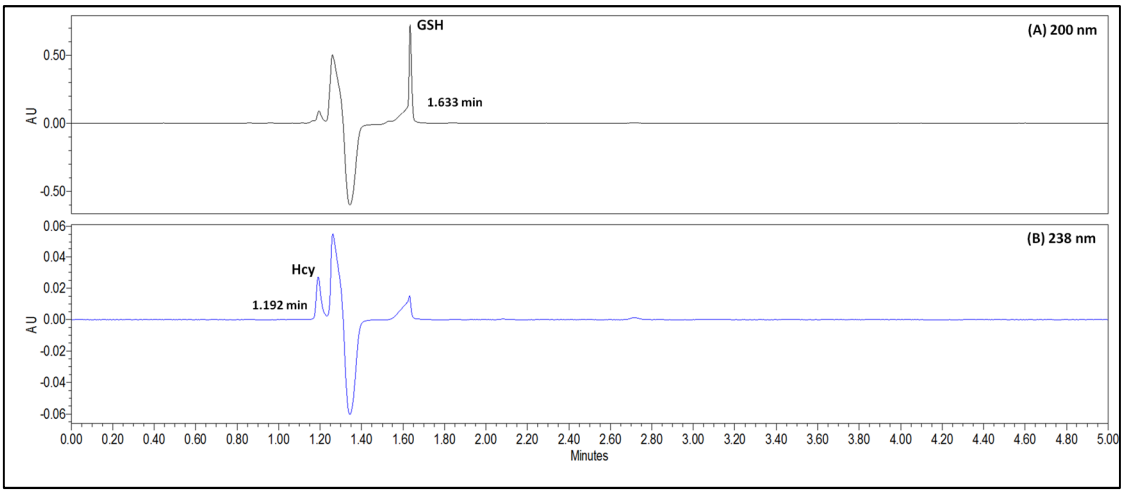

Figure 42 - UHPLC-PDA injection of a mixture of thiol glutathione (100 µM) (A) and homocysteine (7397 µM) (B). Chromatographic conditions presented in annex 2.

However, the interference of the mobile phase and the low sensitivity persisted.

Therefore, we concluded that the best procedure for identification of thiols would be a method with FLR detection, which is more sensitive.

3.1.2. Thiol analysis by UHPLC with FLR detection

Considering the previous results and the low sensitivity of the method, it was decided that FLR detection would be a better approach.

One of the requirements for FLR detection is that the molecule to be analyzed emits fluoresce. Otherwise, it must be labeled with a fluorescent compound. Thiol compounds do not emit fluorescence as was confirmed by injecting standards in the UPLC® system with FLR detection, without derivatization (see the chromatographic conditions in the annex 3). As a matter of fact, these tests proved the need of derivatizating thiol compounds as referenced in [224, 228].

In order to optimize the UHPLC-FLR method, several analytical conditions were tested:

I. Mobile phase composition

Different **mobile phase compositions and pH conditions** were tested as shown in tables 3 and 4.

Table 3 - Different mobile phases tested in isocratic conditions with pH adjustments:

Mobile Phase (%)		Solvent pH*	
		H_2O|0.1%FA	ACN|0.1%FA
90% : 10%		2.60	
90% : 10% NaOH (1 mM)		2.90	
Mobile Phase (%)		**H_2O|0.1%FA**	**ACN**
90% : 10%		2.60	
90% : 10% NaOH (1 mM)	**1º**	3.60	
	2º	4.30	
	3º	5.33	
90% : 10% NaOH (12 mM)		3.00	
95% : 5%		2.70	
95% : 5% NaOH (1 mM)		3.60	
Mobile Phase (%)		**H_2O|0.1%FA**	**MeOH**
90% : 10%		2.80	

Note: (*) Each pH determination was performed tree times; the average (± 0.03 to 0.05 variation) was determined and presented in the table above.

The best approach for thiol separation was the use of a mobile phase in **isocratic conditions**. Following these conditions, several isocratic mobile phases were tested with pH adjustments as shown in the previous table. Methanol revealed to be worse than ACN in the mobile phase organic solvent composition, because it caused greater variations in peak retention times and areas (worse reproducibility). Therefore, ACN was elected as the organic solvent.

Table 4 - Mobile phase conditions tested for chromatographic separation of thiols:

Solvents (%)		Results
H_2O|0.1%FA (%)	**ACN (%)**	
90	10	Figures 43, 44 and 45
95	5	
97.5	2.5	

Pre-treatment and derivatization of standards was performed as described ahead. After derivatization of thiol compounds with SBD-F and UHPLC analysis coupled to FLR detection, as presented in figures 43 and 44, for Cys and Hcy, respectively, good results were

obtained with mobile phases 95:5 and 97.5:2.5 (v/v), with good peak resolutions. For a 90:10 (v/v), mixture, a poor resolution was obtained with emergence of double peaks.

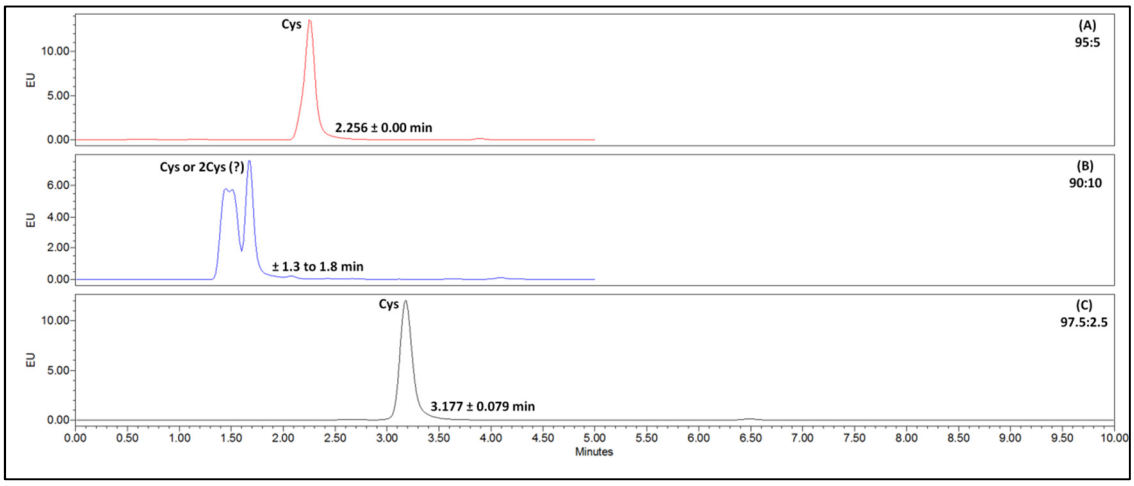

Figure 43 - UHPLC-FLR injections of cysteine (100 µM) with an isocratic mobile phase at (A) 95%H_2O|0.1%FA:5%ACN, (B) 90%H_2O|0.1%FA:10%ACN and (C) 97.5%H_2O|0.1%FA:2.5%ACN.

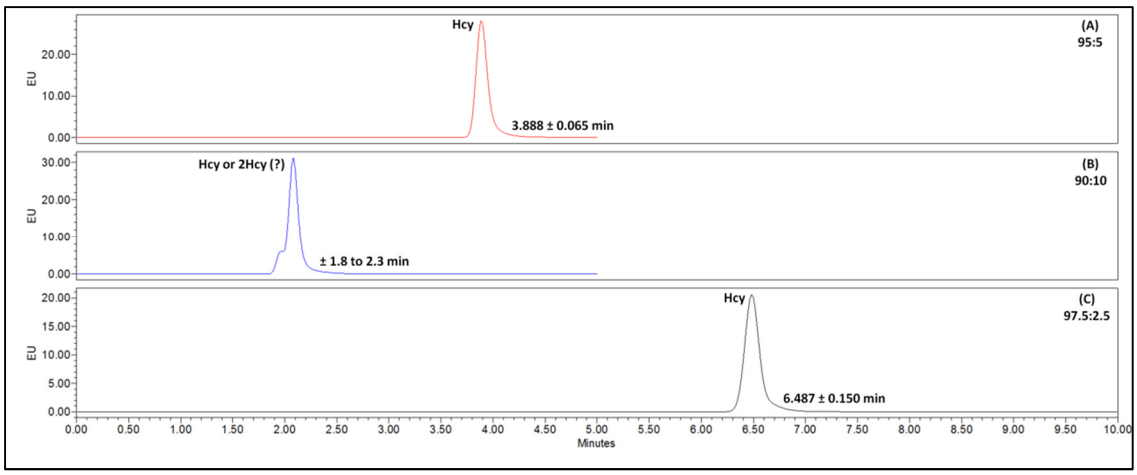

Figure 44 - UHPLC-FLR injections of homocysteine (100 µM) with an isocratic mobile phase at (A) 95%H_2O|0.1%FA:5%ACN, (B) 90%H_2O|0.1%FA:10%ACN and (C) 97.5%H_2O|0.1%FA:2.5%ACN.

However, the assays performed, demonstrated significant changes of peak retention times, heights and areas, both intra and inter-daily what might indicate that the compounds analyzed are very unstable. According to bibliographic sources, we might be dealing with ionogenic analytes, which means that the compounds analyzed have a pKa (see data in table of annex 4) close to the mobile phase pH. This fact, favors the appearance of different forms in solution [229]. This was clearly confirmed for GSH in tests performed with FLR detector. As presented in chromatograms of figure 45, GSH was very unstable when mobile phase composition was 97.5%H_2O|0.1%FA:2.5%ACN.

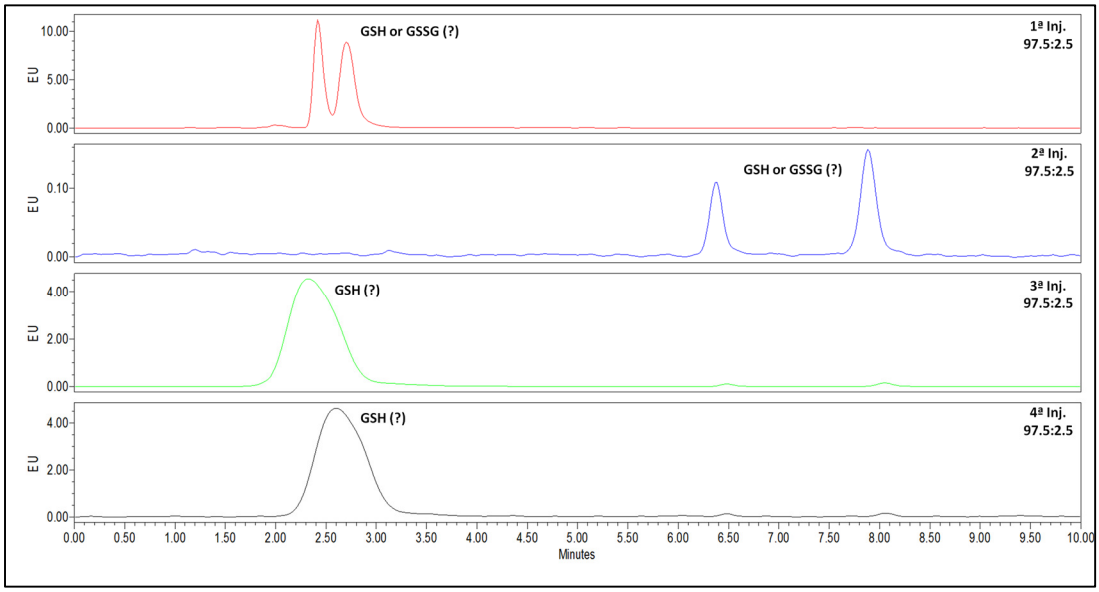

Figure 45 - Four UHPLC-FLR chromatograms of glutathione (100 µM) using an isocratic mobile phase 97.5%H_2O|0.1%FA:2.5%ACN.

The optimum separation conditions were achieved with a mobile phase consisting of Milli-Q water acidified with 0.1% formic acid and ACN in a 95:5 (v/v) ratio, with a pH between 2.6 and 2.7. This choice is in agreement with previous studies, as reported by Valente *et al.* [224], where it was reported that an aqueous phase at pH of 2 maximizes the fluorescence intensity of Hcy and Cys thiol compounds.

II. Stationary phase composition

Two different columns were also tested in order to choose the stationary phase that best fitted the chromatographic separation of our compounds. The HSS T3 with the HSS T3 VanGuard™ pre-column, and the BEH C_{18} with BEH C_{18} VanGuard™ pre-column. Their most relevant characteristics are presented in table of annex 5a.

Results obtained for thiol analysis with the BEH C_{18} column (figure 46) were poor, with split or double, broad and tailing peaks. Therefore, the best chromatographic column was demonstrated to be the HSS T3, which showed good resolution and selectivity characteristics.

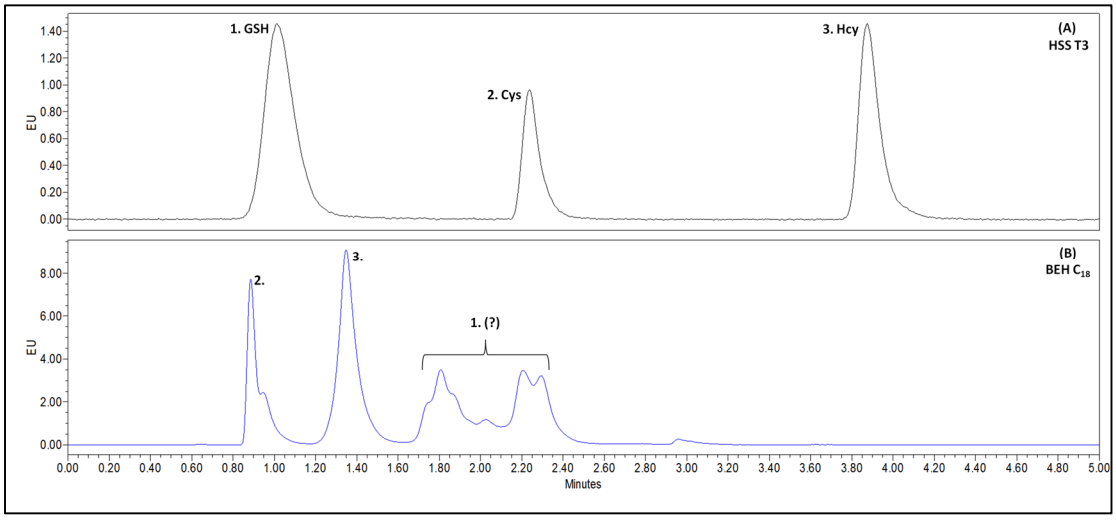

Figure 46 - Separation of mixture of thiols composed of GSH (1), Cys (2) and Hcy (3), (each 100 µM) in two columns (A) HSS T3 and (B) BEH C_{18} (Chromatographic conditions annex 5b).

Table 5 - Chromatographic data obtained from separation of a mixture of thiols in the HSS T3 column:

Data	Retention time		Area		Height	
	min	RSD (%)	µV°sec	RSD (%)	µV	RSD (%)
GSH	1.011 ± 0.002	0.17	135800.67 ± 3336.12	2.46	13841.67 ± 429.26	3.10
Cys	2.238 ± 0.001	0.07	55069 ± 7628.64	13.85	9890 ± 1254.39	12.68
Hcy	3.872 ± 0.002	0.07	99800.33 ± 14351.88	14.38	14886.33 ± 1987.16	13.39

Note: average ± SD from three injections

Our choice was also based on theoretical information, such as, the fact that silica is a material of high mechanical and thermal stability, great rigidity and of high efficiency in chromatographic separations [230]. It only has two limitations, the restricted pH range and the presence of residual silanol groups, which cause peak asymmetry when the injected sample is a basic one [231]. The manufacturers also report that in acidic medium at a pH lower than 2, there are ≡Si-O-Si≡ bonds forming in the skeleton of silica, which are responsible for maintaining organic groups on the silica surface immobilized and they are more susceptible to hydrolysis. In basic medium, above pH 8, the hydroxyl groups (OH^-) react easily with residual silanol, promoting dissolution of silica. As a result, the chromatographic core of the column changes, leading to low efficiency and front peak broadening [231].

The HSS T3 column revealed to be more appropriate because the pH of the mobile phase was within the pH limits of the column.

III. Temperature

The sample manager temperature (°C) is an important parameter, as it ensures the stability of samples intended to be analyzed. In the UPLC-FLR method the conditions tested were 4, 8, 10 and 37 °C. Temperatures of the laboratory fluctuated between 23 °C and 30 °C. This was a critical point mainly for thiol compounds, which are more sensitive to oxidation

processes. In fact, it has been reported that the lifetime of thiol molecules decreases with increasing temperatures and basic pH of the solutions [232]. Taking that into account, the sample manager was set at 10 °C.

The column temperature (°C) is a specific parameter related to the stationary phase composition, whose limit is set by the manufacturer. As presented in table of annex 5a, the maximum temperature for HSS T3 column is 45 °C. This is one of the factors, which determines peak retention times in a chromatographic run. In our study, the HSS T3 column temperature was maintained at 30 °C for all assays. A balance between stability of compounds and laboratory temperature was made. At this temperature, viscosity of the mobile phase decreases, which consequently, reduces pressure in the column and promotes diffusion of the sample originating narrower and higher peaks [233, 247].

IV. Analysis and equilibrating times

Run and equilibrating times were tested and showed similar results in the UPLC-FLR method for thiol compounds and in the UPLC-PDA detection for purines.

The run time (min) parameter is the time taken for a complete chromatographic analysis, for each portion of sample injected. In our method, the run time was tested considering the type of mobile phase used and the retention time expected for every compound. Thus, 5, 8 and 10 minute runs, were tested. In the end, a good resolution was obtained with a fast 5 min analysis time.

The equilibrating time (min) is also an important parameter to take into account in a chromatographic analysis. Considering the type of mobile phase used, 1, 2, 5 and 10 minutes equilibration between injections, were tested.

Suppliers recommend that initial column equilibration should be made with, at least 10 column volumes of the mobile phase used. In this case, the HSS T3 column needs at least 4 mL (4000 µL) [234]. However, by the observation of the obtained results it was assumed that 1 min between injections was an adequate time . It is considered that a complete chromatographic cycle starts when the sample is injected and ends with the restoration of the pre-analytical conditions. Therefore, the run time plus the equilibrating time, totalized 6 minutes. These conditions, allowed us to obtain good chromatographic results.

V. Sample treatment procedure tested for standard solutions

Taking the above results of thiols (Hcy and GSH), previously reduced with TCEP·HCl (1 mM) and analyzed by UHPLC with PDA detection, into account and the need to use FLR detection, it was necessary to previously derivatize standards. The derivatization reaction, transforms molecules that do not have fluorescent properties into fluorophore derivatives. The chosen derivatizing agent was SBD-F according to [235]. Before this action, it was necessary to reduce thiol dimmers and TBP was the chosen reducing agent according to this derivatization agent.

After testing all the procedure steps and optimizing derivatization conditions, the following chromatograms were obtained (figure 47 and table 6) after reduction with TBP followed by derivatization with SBD-F. A good resolution of thiols and sensitivity of the method were obtained.

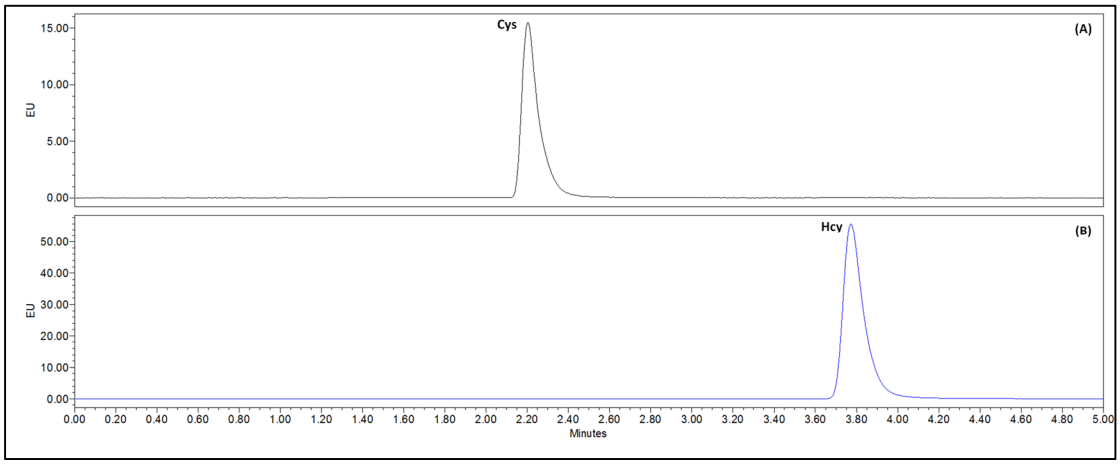

Figure 47 - Chromatographic results of the injection of cysteine (A) and homocysteine (B) at 2.00 mM, after reduction with TBP and derivatization with SBD-F. (See chromatographic conditions in annex 6).

Table 6 - Chromatographic data obtained from homocysteine and cysteine:

Data	Retention time		Area		Height	
	min	RDS (%)	µV*sec	RSD (%)	µV	RSD (%)
Hcy	3.773 ± 0.001	0.02	354870.50 ± 1595617.63	44.96	633295.83 ± 83962.88	13.26
Cys	2.204 ± 0.003	0.12	814253.83 ± 50755.42	6.23	146767.04 ±7732.01	5.27

Note: Average ± SD of six injections

The thiol standard **homocystine** (2Hcy), the dimmer of homocysteine, was also analysed. The results are shown below (figure 48 and table 7).

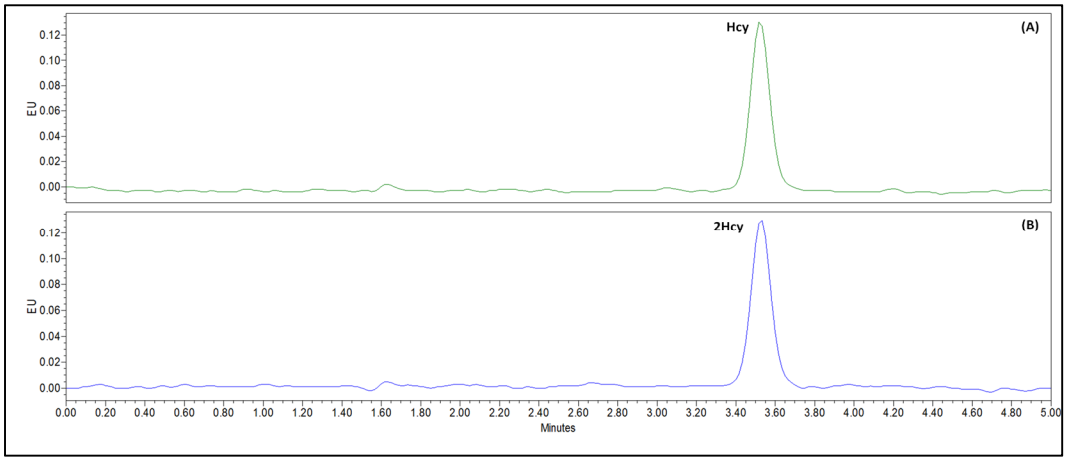

Figure 48 - UHPLC-FLR chromatograms of the thiol standards homocysteine (A) and homocystine (B) at 10 µM. (See chromatographic conditions in annex 6).

Table 7 - Chromatographic data obtained from identification of thiol standard homocysteine and homocystine at 10 µM:

Data	Retention time		Area		Height	
	min	RSD (%)	µV°sec	RSD (%)	µV	RSD (%)
Hcy (A)	3.528 ± 0.005	0.13	8056.16 ± 1128.26	14.00	1230.67 ± 134.26	10.91
2Hcy (B)	3.530 ± 0.002	0.08	7762 ± 938.42	13.24	1148 ± 144.14	13.75

Note: Average of six injections (mean ± SD)

These results confirmed that **reduction of thiols by TBP was effective. Derivatization was done with SBD-F** and posterior UPLC-FLR analysis.

3.1.3. Optimal chromatographic conditions for thiol analysis

The **optimal chromatographic conditions** for the analysis of GSH, Hcy, Cys and Cyst thiols are shown in table 8.

Table 8 - Optimal chromatographic conditions achieved for the determination of thiol compounds:

Optimal chromatographic conditions	
Injection volume	2 µL
Sample manager temperature	10 °C
HSS T3 column temperature (100 mm x 2.1 mm, 1.8 µm particle size)	30 °C
Excitation wavelength	385 nm
Emission wavelength	515 nm
Flow-rate	0.200 mL/min
Analysis time	5 min
Equilibrating time	1 min
Mobile phase - Isocratic pH 2.6 - 2.7	95% H_2O|0.1%FA : 5% ACN

Below, a chromatogram of a mixture of GSH, Cys and Hcy standards, obtained with the above conditions, is shown.

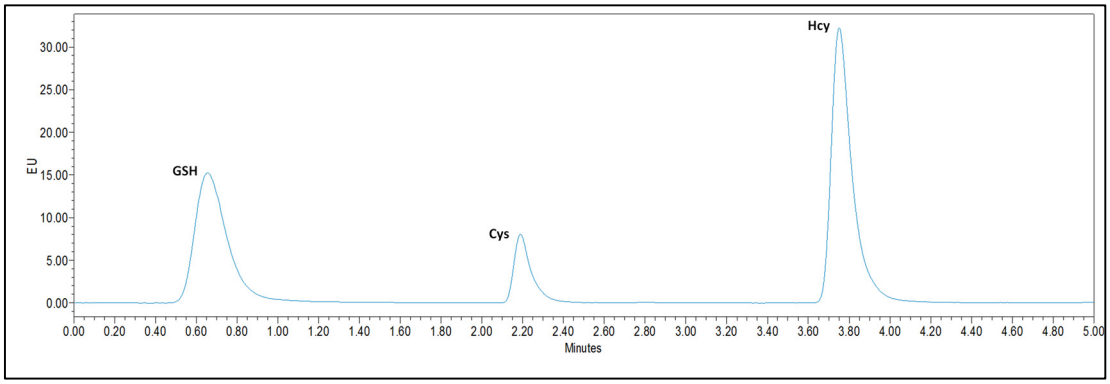

Figure 49 - Optimal conditions for the chromatographic analysis of a mixture of thiols glutathione, cysteine and homocysteine (each 100 µM), with FLR detection.

The precision of the method was evaluated by repeated analysis (n=3) of the standards mixture, as shown in table 9.

Table 9 - Chromatographic data obtained from separation of a mixture of thiols glutathione, cysteine and homocysteine (each 100 µM):

Data	Retention time		Area		Height	
	min	RSD (%)	µV°sec	RSD (%)	µV	RSD (%)
GSH	0.655 ± 0.005	0.26	1501726.33 ± 4355.30	12.05	150467.67 ± 320.58	11.49
Cys	2.189 ± 0.000	0.03	355893.67 ± 7628.64	9.87	69398 ± 1254.05	8.94
Hcy	3.752 ± 0.001	0.03	1926833.33 ± 14351.88	9.06	288050.67 ± 1987.16	8.76

Note: average of three injections (mean ± SD)

3.2. Development and optimization of an analytical method for analysis of purines by UHPLC with PDA detection.

Several tests that proved that the PDA detection would be the best approach for purines were performed, since they are very stable compounds and absorb in the UV/VIS region, being good chromophores.

Purines (Ino and Ado) were not subjected to any sample pre-treatment (see the biochemical and molecular characteristics in annex 8). Stock solutions were diluted in purified water and the desired concentration was directly injected in the chromatographic system.

In order to find an effective method for detection of the purine compounds, in a single run, several conditions were previously tested based on the conditions reported by Câmara [223] and Araújo [236]. The main chromatographic conditions and results obtained with PDA detection are reported below.

Firstly, the UV-Visible absorption spectra (190 to 400 nm) were obtained for both purine compounds, Ado and Ino. The diagram below shows the absorbance units (AU) versus the wavelength (nm), what translates the amount of radiation absorbed by each specific

compound. The highest absorbance value determines a particular wavelength at which chromatograms should be extracted.

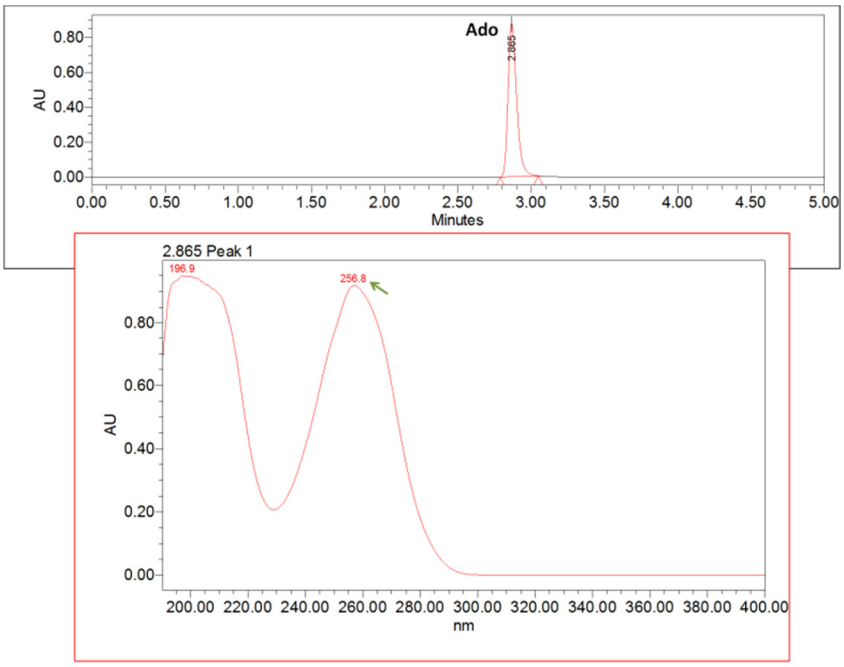

Figure 50 - Verification of the specific wavelength detection and spectrum of adenosine (254 ± 2.8 nm). (Chromatographic conditions used are listed in the table of annex 9).

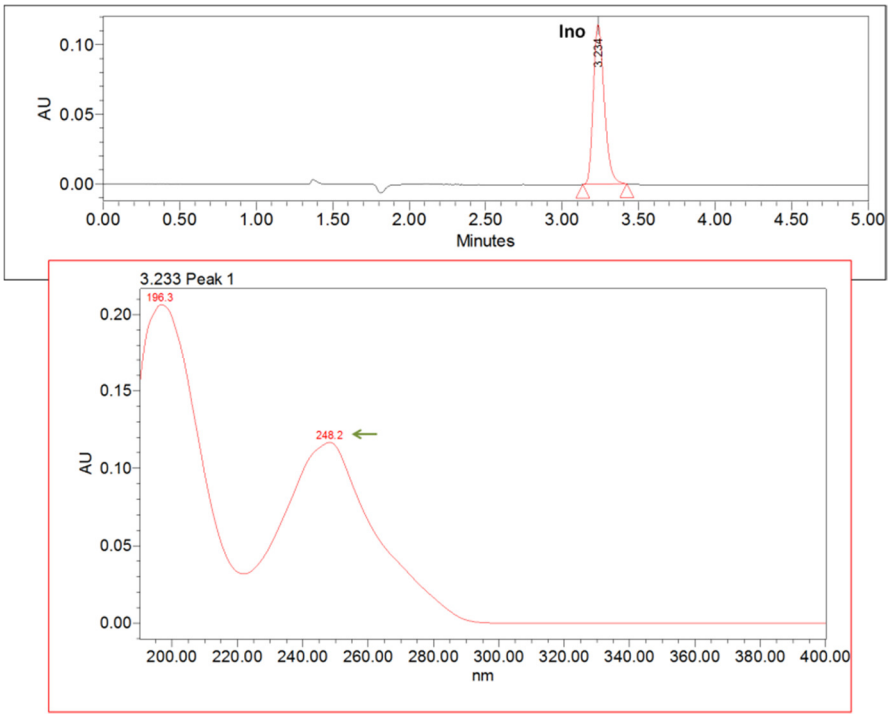

Figure 51 - Verification of the specific wavelength detection and spectrum of inosine (250 ± 1.8 nm). (Chromatographic conditions used are listed in the table of annex 9).

The UV-Visible absorption spectrum is specific for each compound and it is very important for the identification of analytes in a mixture of standards or in biological samples [237]. In the several analyses that were conducted, identification of different compounds was achieved by confirming retention times (min) and absorption spectra (nm).

I. Mobile phase composition

Initially, purine analysis by UHPLC with PDA detection was tested using the chromatographic conditions described by Câmara [223], for Hcy analysis. The results were unfavorable although without mobile phase interferences (see annex 10). Several mobile phase compositions were tested both in gradient and isocratic conditions (table 10), as well as, pH variations (table 11) until the best chromatographic separation of compounds was obtained.

Table 10 - Mobile phase conditions tested for chromatographic separation of purines:

Solvents (%)		**Results**
H_2O|0.1%FA : ACN|0.1%FA Different Gradients		See annex 11
90% H_2O|0.1%FA : 10% ACN 95% H_2O|0.1%FA : 5% ACN Isocratic with pH variations		Table 11
H_2O|0.1%FA (%)	**ACN|0.1%FA (%)**	
90	10	Data not shown
H_2O|0.1%FA (%)	**ACN (%)**	
90	10	Data not shown
95	5	Figure 52
97.5	2.5	Data not shown
80	20	Data not shown
70	30	
50	50	
H_2O|0.1%FA (%)	**MeOH (%)**	
90	10	Figure 53

Table 11 - Different mobile phases tested in isocratic conditions with pH adjustments:

Mobile Phase (%)		**Solvent pH***		**Results**
		H_2O|0.1%FA	**ACN|0.1%FA**	
90% : 10%		2.60		Data not shown
90% : 10% **NaOH (1 mM)**		2.90		
Mobile Phase (%)		**H_2O|0.1%FA**	**ACN**	**Results**
90% : 10%		2.60		Data not shown
90% : 10% **NaOH (1 mM)**	**1º**	3.60		
	2º	4.30		
	3º	5.33		
90% : 10% **NaOH (12 mM)**		3.00		
95% : 5%		2.70		Figure 52
95% : 5% **NaOH (1 mM)**		3.60		
Mobile Phase (%)		**H_2O|0.1%FA**	**MeOH**	**Results**
90% : 10%		2.80		Figure 53

Note**:** (*) Each pH determination was performed tree times; the average (± 0.03 to 0.05) was determined and presented in the table above.

The best approach for purine separation was also in isocratic mode. Following these conditions several isocratic mobile phases were tested with pH adjustments, as shown in the previous table.

Figure 52 shows the UPLC-PDA chromatographic results of Ado using a mobile phase of 95% H_2O|0.1% FA : 5% ACN, at two different pH: 2.70 and 3.60. The small increase in the mobile phase pH caused a quite greater retention of the analyte on the column, what obliged to increase the run time. This would be disadvantageous, since the method should be fast and efficient, with results obtained in the shortest time. In accordance, the lowest pH was chosen for the mobile phase.

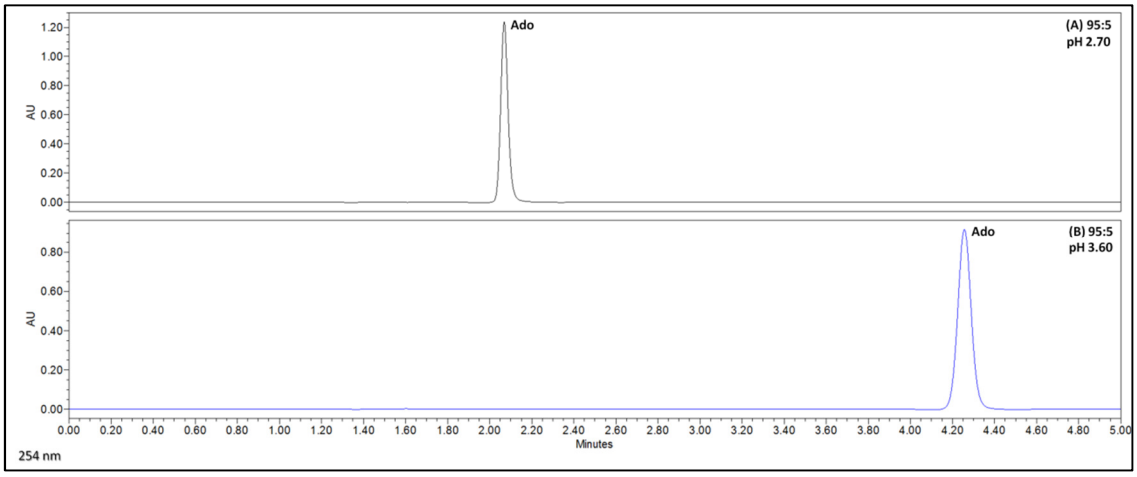

Figure 52 - UHPLC-PDA chromatograms of adenosine (150 µM), using an isocratic mobile phase 95%H_2O|0.1%FA : 5%ACN at pH of 2.70 (A) and 3.60 (B).

Table 12 - Chromatographic data obtained from separation of adenosine in isocratic mobile phase 95%H_2O|0.1%FA : 5%ACN with pH variation:

Data	Retention time		Area		Height		Maximum absorbance	
	min	RSD (%)	µV°sec	RSD (%)	µV	RSD (%)	AU	RSD (%)
Ado (A)	2.070 ± 0.000	0.03	2954632 ± 214576.11	1.22	1231716.51 ± 116309.89	0.02	1.28 ± 0.10	0.32
Ado (B)	4.257 ± 0.005	0.13	4028040.33 ± 52799.88	1.31	914433.33 ± 5396.15	0.59	0.98 ± 0.01	0.97

Note: average of three injections (mean ± SD)

Methanol was tested as the organic solvent in mobile phase composition. However, it revealed to be worse than ACN, causing greater variations in peak retention times and areas. The results, as presented in figure 53. Chromatograms of standards showed no reproducibility (at least five injections) concerning peak retention times, areas and heights. Therefore, ACN was the elected organic solvent.

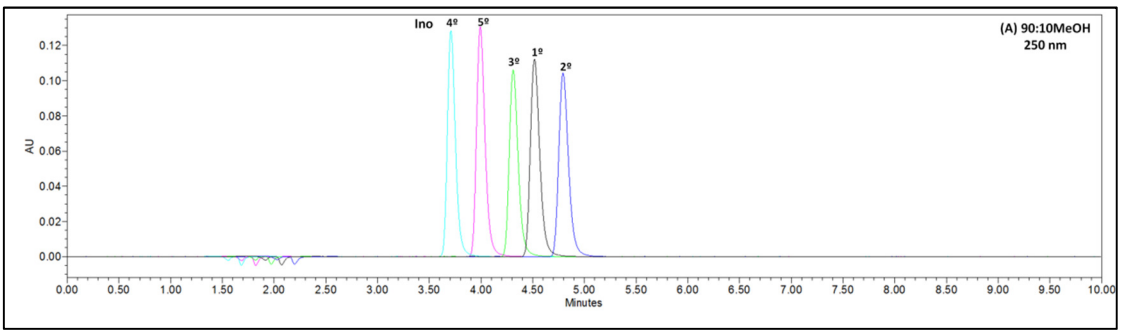

Figure 53 - Overlap of inosine (125 µM) chromatograms using an isocratic mobile phase with methanol. (See chromatographic conditions in annex 12).

After testing the various mobile phase compositions it was considered that the following solvent composition: 95% H_2O|0.1%FA : 5% ACN (v/v) was the best choice for our analytical procedure.

II. Stationary phase composition

Two different columns were also tested in order to choose the one that best fitted the chromatographic separation of compounds: the HSS T3 and the BEH C_{18}. Their most relevant characteristics are presented in table of annex 5.

Both columns were appropriate although the results obtained indicated that the BEH C_{18} was even more suitable for purines detection as shown in figure 54, 55 and 56 for Ino, Ado and their mixture, respectively. A good resolution and sensitivity were obtained. Additionally, the column suitability was based on the theoretical information mentioned above for thiol compounds as the standards Ado and Ino presented a pH of 10.3 and 10.6, respectively. However, the HSS T3 was chosen, once it was being used for analysis of thiol compounds.

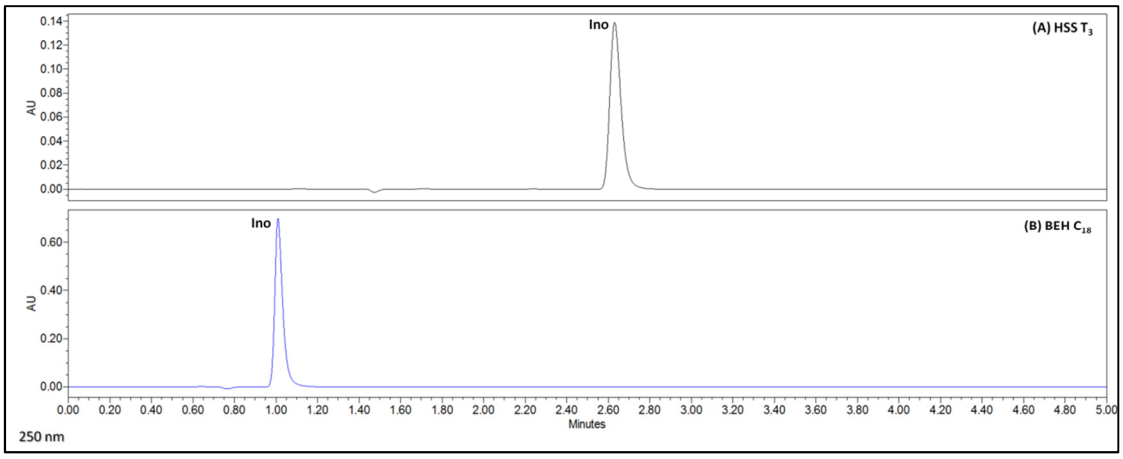

Figure 54 – UHPLC-PDA chromatograms of inosine (250 µM) with (A) HSS T3 and (B) BEH C_{18}. (See chromatographic conditions in annex 13).

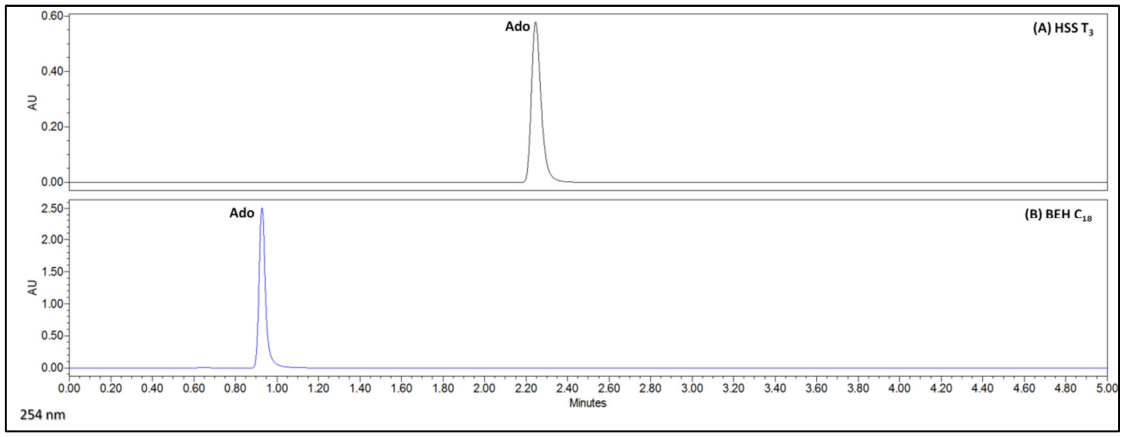

Figure 55 – UHPLC-PDA chromatograms of adenosine (250 µM) with (A) HSS T3 and (B) BEH C_{18}. (See chromatographic conditions in annex 13).

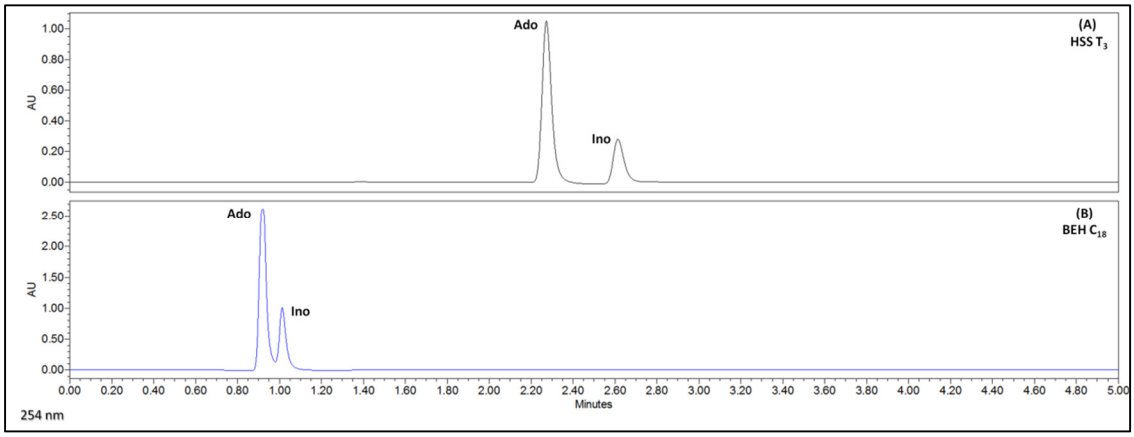

Figure 56 – UHPLC-PDA chromatograms of adenosine and inosine (500 µM) with (A) HSS T3 and (B) BEH C_{18} both at 254 nm. (See chromatographic conditions in annex 13).

Table 13 - Chromatographic data obtained from separation of adenosine and inosine (500 µM) with (A) HSS T3 and (B) BEH C_{18}:

Data	Retention time		Area		Height		Maximum absorbance	
	min	RSD (%)	µV°sec	RSD (%)	µV	RSD (%)	AU	RSD (%)
Ado (A)	2.245 ± 0.044	0.00	168297 ± 4579.33	1.53	530499 ± 3065.7	2.96	0.97 ± 0.01	0.64
Ino (A)	2.630 ± 0.108	0.02	473153 ± 2109.62	1.44	128595 ± 5453.14	3.15	0.23 ± 0.01	2.53
Ado (B)	0.928 ± 0.000	0.06	5339191.67 ± 12828.04	0.24	2510863 ± 5794.17	0.23	2.54 ± 0.00	0.06
Ino (B)	1.015 ± 0.001	0.10	2527239.67 ± 451336.85	17.86	1218950.33 ± 24461.39	2.01	1.05 ± 0.01	0.74

Note**:** average of three injections (mean ± SD)

III. Injection volume

The injection volume (µL) of samples to be analyzed by UPLC should be proportional to the volume of the column used. This parameter is important for the stability of the chromatographic column and it was tested considering the type of sample and mobile phase used. As presented, injections of 2, 4, 6 and 8 µL of sample, were tested.

What should be taken into account, before defining the injection volume is the void volume of the column [238], which corresponds to the amount of space that is not taken up by the peaking material. It is theoretically calculated using the equation represented in figure 57. The calculation considers the dimension of the column. The HSS T3 column of 2.1 x 100 mm, has a void volume equal to 240 µL (or 0.24 mL). It is assumed that an average value for the pore volume is 0.70 (70 %) for a bare silica support [238].

Void volume, Vm:

$$V_m = 0.7 \times \pi \times r^2 \times L$$

Figure 57 - Theoretical equation for the calculation of the empty space of a chromatographic column [238]. Alternatively, it is presented as: Column Void Volume (µL) = (d^2(mm)*Pi*L(mm)*0.7)/4 [239].

Regarding HSS T3 column, 240 µL is the empty volume that can be occupied by solution. Considering the run and equilibrating times already defined for thiol compounds, the 5 min run plus 1 min of equilibrating and a constant flow-rate of 0.200 mL/min, it is estimated that 1200 µL is the total mobile phase volume that crosses the column during a complete chromatographic cycle. This estimate is important to realize the amount of solvent and sample, which should pass through the column during the chromatographic analysis. Therefore, it is important not to overload the column with sample and ensure that the running and rebalancing times are enough to clean and stabilize the chromatographic system for a new injection.

Chromatograms, obtained through analysis by UHPLC with PDA detection for the standard inosine can be seen below. It was observed that the increase of injected volume, affected proportionally and directly the area and height of the peaks without changing the retention time. The concentration of the compound in the sample is always constant but, increasing the injected volume means that a greater amount of molecules are introduced into the column and are present in the analyzed portion so, resulting in greater intensity of peaks. The increase of the injected volume is reflected in broader peaks, what affects the chromatographic separation.

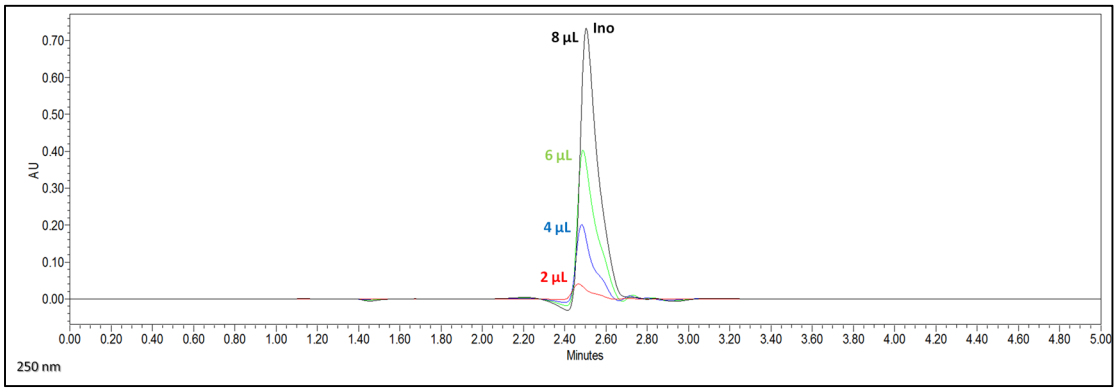

Figure 58 - Overlap of chromatograms of different injected volumes, 2 (red), 4 (blue), 6 (green) and 8 µL (black), of inosine (500 µM) detected at 250 nm. (See chromatographic conditions in annex 13).

To better understand how the injected sample volume affected the chromatographic results, data were treated (table 14), and the results are presented in figure 59. As expected, the results showed that the mean ± SD peak area and height, and absorbance increased proportionally to the increase of volume.

Table 14 - Variation of injection volume for standard inosine:

Ino volume (µL)	Retention time		Area		Height		Maximum absorbance	
	min	RSD (%)	µV°sec	RSD (%)	µV	RSD (%)	AU	RSD (%)
2	2,465 ± 0.001	0.06	231374,67 ± 108.16	4.67	39632,33 ± 1830.74	4.62	0,08 ± 0.00	4.75
4	2,482 ± 0.000	0.02	1186041 ± 77004.48	6.49	208615,67 ± 12108.64	5.80	0,40 ± 0.02	5.26
6	2,488 ± 0.000	0.00	2541412,33 ± 136770.16	5.38	420419,67 ± 2911.59	6.95	0,76 ± 0.05	5.95
8	2,504 ± 0.000	0.02	4200363,67 ± 131754.08	3.14	730176,67 ± 24104.01	3.30	1,13 ± 0.01	1.02

Note**:** Average of tree Injections (mean ± SD)

The graphical representations in figure 59, allow us to compare the different results obtained for the four volumes tested. The absorbance and the intensity of the peaks increased approximatelly 0.3 AU as the injection volume rised.

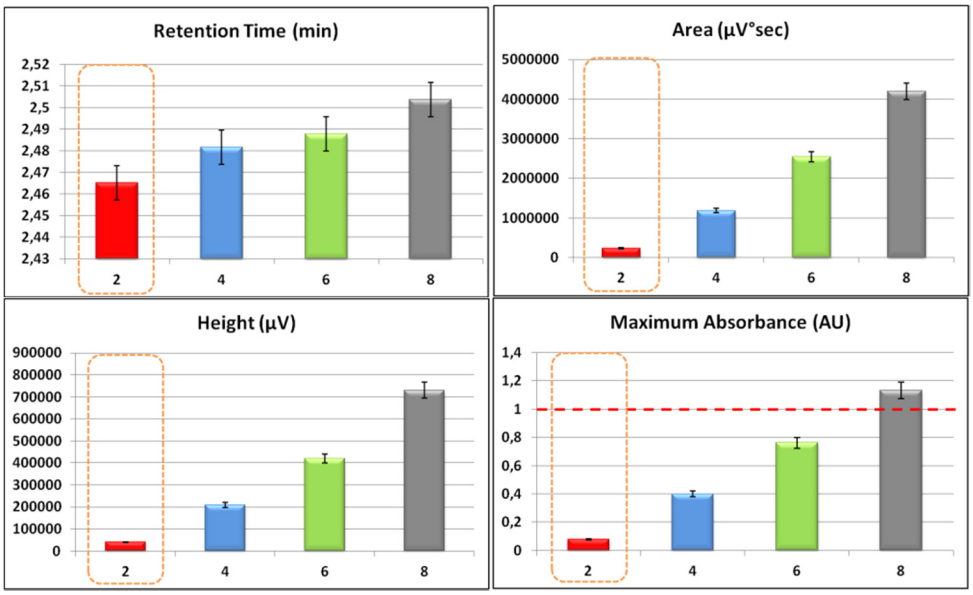

Figure 59 - Graphical representation of the chromatographic data obtained for inosine when testing injection volume.

Considering the above results it was assumed that the most suitable sample volume of injection for this analysis, was 2 µL**.** It was enough to achieve good results, without overloading the column and maintaining the system pressure stable.

IV. Temperature

The sample manager temperature (°C) ensures the stability of samples as mentioned above for thiol compounds. In the UHPLC-PDA method for purine compound analysis, the conditions tested were 4, 8, 10, 20, 23 and 37°C. Purine compounds showed to be quite stable in this range of temperatures.

3.2.1. Optimal chromatographic conditions for purine analysis

The optimal chromatographic conditions for the analysis of purines were achieved using PDA detection and are shown in Table 15. A chromatogram of Ado and Ino is presented in figure 60 showing results obtained with this method

Table 15 – Optimal chromatographic conditions achieved for the analysis of purines and related compounds:

Optimal chromatographic conditions		
Injection volume		2 µL
Sample manager temperature		10 °C
HSS T3 column temperature (100mm x 2.1mm, 1.8µm particle size)		30 °C
Specific UV wavelength detection	**Ado**	254 nm
	Ino	250 nm
Flow-rate		0.200 mL/min
Analysis time		5 min
Equilibrating time		1 min
Mobile phase - Isocratic pH 2.6 – 2.7		95% H_2O|0.1%FA : 5% ACN

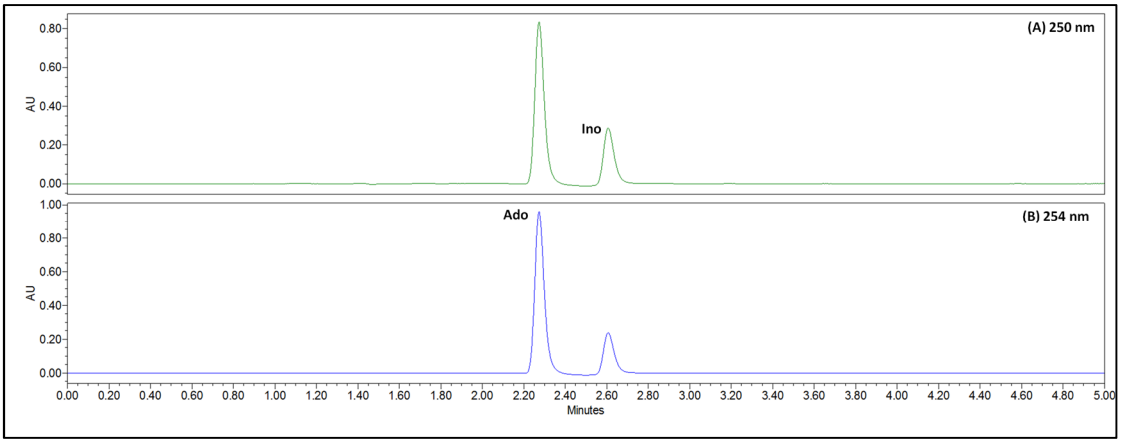

Figure 60 - Optimal conditions for the chromatographic analysis of inosine and adenosine (500 µM) with PDA detection at 250 and 254 nm, respectively.

Table 16 - Chromatographic data obtained from separation of inosine and adenosine:

Data	**Retention time**		**Area**		**Height**		**Maximum absorbance**	
	min	**RSD (%)**	**µV°sec**	**RSD (%)**	**µV**	**RSD (%)**	**AU**	**RSD (%)**
Ado	2.249 ± 0.001	0.06	2201479.75 ± 134283.82	6.99	688259.51 ± 44416.44	6.45	0.88 ± 0.05	5.34
Ino	2.584 ± 0.005	0.01	708557.75 ± 47006.22	6.63	203003.75 ± 14224.91	7.01	0.38 ± 0.03	6.85

Note: average of six injections (mean ± SD)

3.3. Difficulties encountered during the optimization procedures of UHPLC-PDA and UHPLC-FLR analytical methods

A recurrent problem of our analyses was an absence of intra or inter-day precision in the obtained results. After testing and excluding possible interferences in all the analytical conditions, which could be related to pre-analytical procedures, the chromatographic results obtained continued to demonstrate constant variations in peak retention time, areas, height and intensities, both in PDA and FLR methods.

All the pre-analytical procedures were tested and samples were injected using, at least, six replicates. The intra-assay and inter-assay precision were evaluated and results are shown below:

- **Intra-day results**

Mixtures of thiol standards, previously derivatized by the procedure already specified above, were injected. In some cases, a good reproducibility was evidenced but not in other cases.

Variations in peak areas and heights were observed as shown in figure 61 for three injections of the same standard of cysteamine,. The overlap of chromatograms shows the great variation of peak height and areas despite the stability of derivatives.

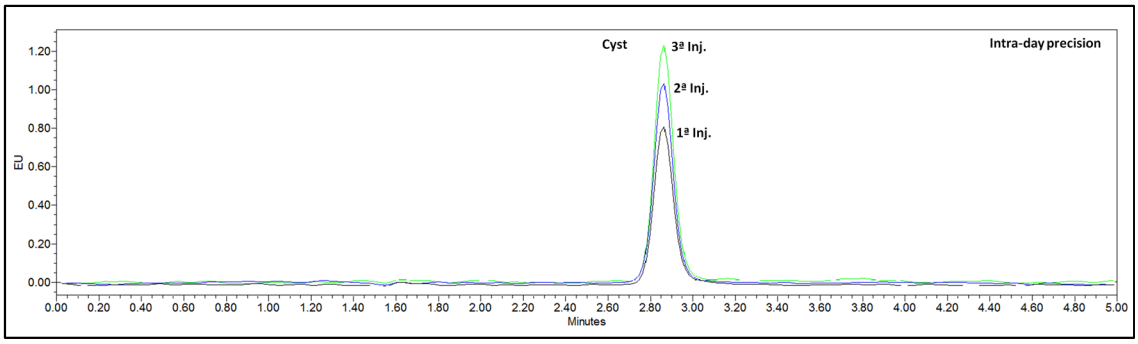

Figure 61 - Chromatographic results of three injections of cysteamine standard (42 µM) showing no intra-day precision.

Table 17 - Chromatographic data obtained from separation of cysteamine showing no inter-day precision:

Data	Retention time		Area		Height	
	min	RSD (%)	µV°sec	RSD (%)	µV	RSD (%)
Cyst	2.863 ± 0.001	0.03	20206 ± 3943.44	19.52	3207.67 ± 635.81	19.82

Note: average of three injections (mean ± SD)

Similar results were obtained for the UHPLC-PDA method used in purine analysis, where in addition to variations in peak areas and heights, there were also fluctuations in peak retention times as shown in an inosine chromatogram featured in figure 62. For comparative

purposes, we present also the relationship with the inter-day tests, which will be mentioned later on.

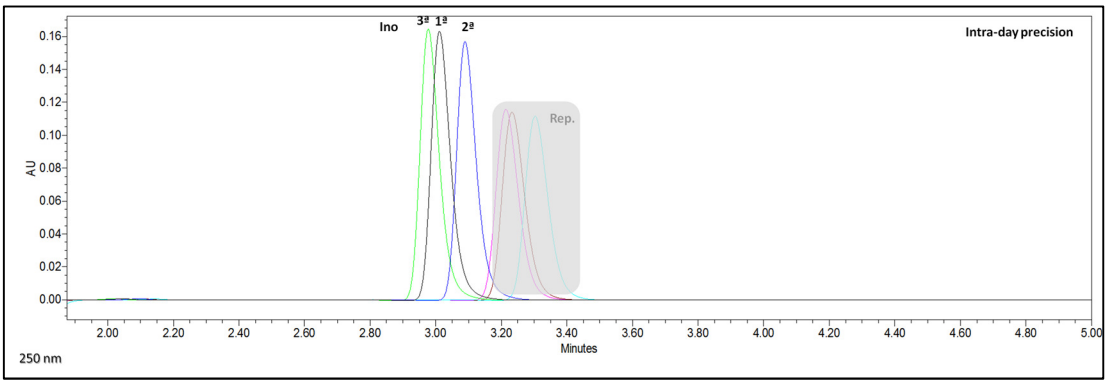

Figure 62 - Overlap of chromatographic results of three injections of inosine standard (100 µM), showing no intra-day precision.

Table 18 - Chromatographic data obtained from separation of inosine without precision:

Data	Retention time		Area		Height		Maximum absorbance	
	min	RSD (%)	µV°sec	RSD (%)	µV	RSD (%)	AU	RSD (%)
Ino	3.607 ± 0.044	1.44	566846.67 ± 7255.33	1.97	122360 ± 3065.67	3.12	0.22 ± 0.07	2.85

Note: average of three injections (mean ± SD)

- **Inter-day results**

The inter-day precision of purine compound analysis, by UHPLC with PDA detection, presented similar results to the intra-day precision, as featured in figure 63. These chromatograms indicated that the UPLC® system was not functioning properly, since this method was already applied by HPLC [236] with very good results.

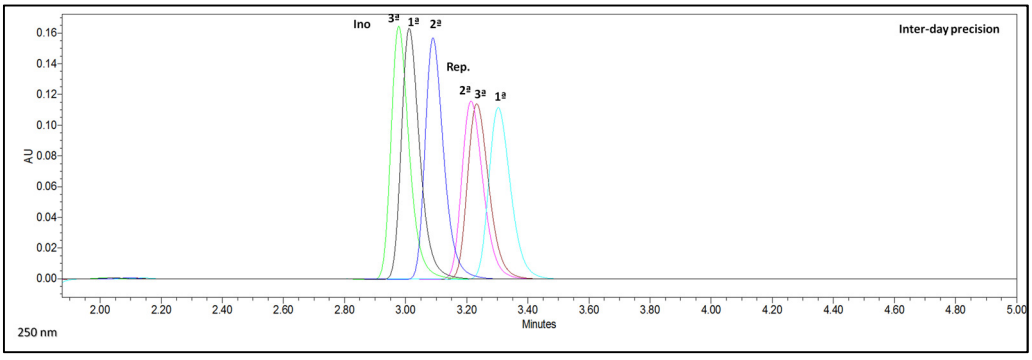

Figure 63 - Chromatographic results of three injections of inosine standard (100 µM) showing no inter-day precision.

Table 19 - Chromatographic data obtained from separation of inosine showing no inter-day precision:

Data	Retention time		Area		Height		Maximum absorbance	
	min	RSD (%)	µV°sec	RSD (%)	µV	RSD (%)	AU	RSD (%)
Ino	3.159 ± 0.108	3.42	564109.24 ± 8173.67	1.45	118048.88 ± 5453.14	4.62	0.22 ± 0.01	5.24

Note: average of sex injections (mean ± SD)

The inter-day precision of thiol analysis was evaluated. Results are shown in figure 64 below.

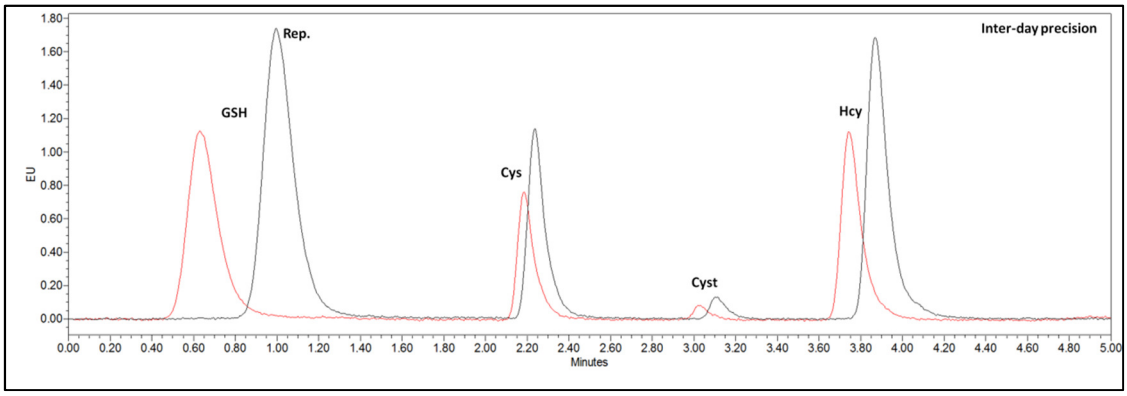

Figure 64 - Chromatographic results of two injections of a mixture of standard thiols (100 µM) identifying GSH, Cys, Cyst and Hcy.

When calculating the precision (table 20) of the method, we verified that the coefficients of variation (CV or RSD) were too high [240]. The CV determines if the analytical method is precise for the individual measurements of an analyte, when the procedure is applied repeatedly to multiple aliquots of a single homogeneous sample [227]. CV values must be below 15%.

Table 20 - Chromatographic results of the inter-day precision of a mixture of standards:

Inter-day precision (%)	Retention time		Area		Height	
	min	RSD (%)	µV°sec	RSD (%)	µV	RSD (%)
GSH	0.82 ± 0.242	29.49	1989449 ± 826555.54	41.55	194873.80 ± 77662.67	39.85
Cys	2.215 ± 0.036	1.63	329806.76 ± 93277.11	28.28	63799.53 ± 18323.67	28.72
Cyst	3.071 ± 0.054	1.77	8112.76 ± 1503.14	18.53	4393 ± 2796.91	63.67
Hcy	3.814 ± 0.085	2.24	1884839 ± 555265.16	29.46	278089.22 ± 78875.62	28.36

Note: average of sex injections (mean ± SD)

This method of analysis of thiol compounds, with previous derivatization with SBD-F, has been reported as being a precise method by HPLC in Minniti *et al.* [235], Akgül *et al.* [241] and Ferin *et al.* [242]. However, some studies report the addition of stabilizer agents, such as

hydrochloric acid [243], sodium phosphate [244] or acetic acid, to stop the derivatization reaction.

Some more tests were carried out to exclude the hypothesis of instability of thiol compounds, even after reduction and derivatization, as presented in the chromatograms from figure 65. Conflicting results were obtained. While it seemed that, over time, thiol compounds suffered degradation translated by the appearance of double peaks (the case of cysteine) and disappearance of GSH, posterior injections of the same derivatized mixture resulted perfectly, what made us suspect of malfunction of the UPLC® system.

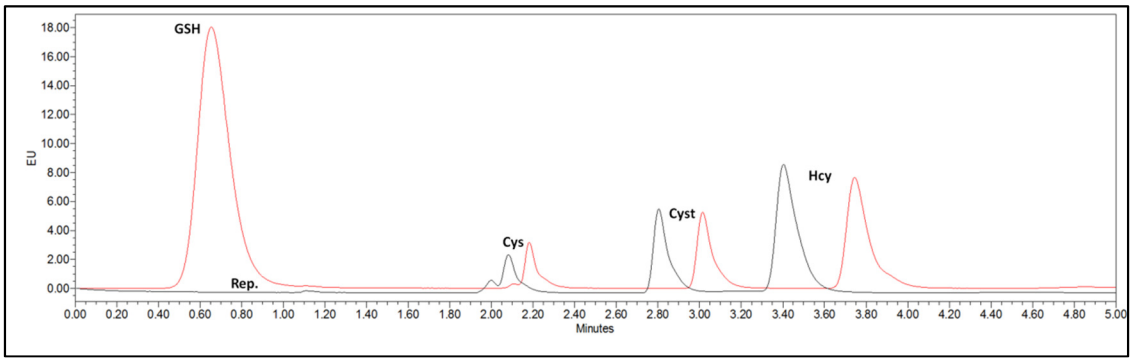

Figure 65 - Chromatographic results of inter-day injections of a mixture of standard thiols (100 µM) identifying GSH, Cys, Cyst and Hcy.

The UHPLC-FLR method showed poor reproducibility (table 21), which was attributed to UPLC® problems.

Table 21 - Chromatographic result of the inter-day precision of a mixture of standard:

Inter-day precision (%)	Retention time		Area		Height	
	min	RSD (%)	µV°sec	RSD (%)	µV	RSD (%)
GSH	---	---	---	---	---	---
Cys	2.131 ± 0.071	3. 37	85245.42 ± 6562.79	7.70	24752 ± 4470.33	18.06
Cyst	3.284 ± 0.380	11.57	258320.87 ± 32883.67	12.73	53810.3 ± 4947.76	9.19
Hcy	3.574 ± 0.245	6.85	541529.33 ± 88228.52	16.29	79926 ± 10081.87	12.61

Note: average of six injections (mean ± SD)

Despite having changed to a new HSS T3 column as a final attempt to overcome the described difficulties and, besides having obtained very well resolved peaks, precision was inconsistent and it was difficult to find the source of the problem.

Also important to mention is that result variations were not constant. They occurred randomly, what made them even more difficult to identify. Exhaustive tests were always conducted, in order to identify the origin of these problems.

Subsequently, loss of linearity was observed as expressed in the calibration curve in figure 66.

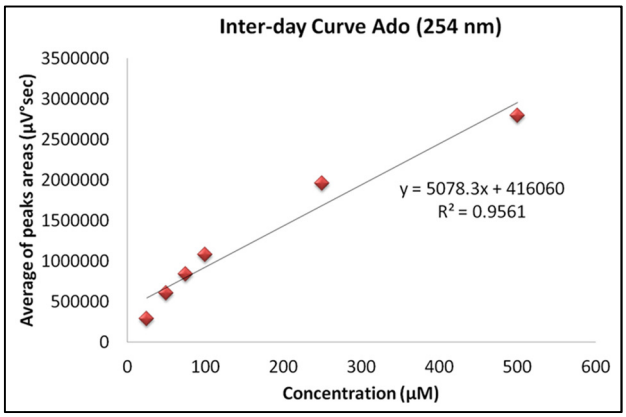

Figure 66 - Graphical representation of the inter-day calibration curves of adenosine (n=6).

3.4. Validation of the analytical methods

Validation of an analytical method implies performing several tests to show that a particular procedure can be used for the measurement of one or more compounds in a given biological matrix. Moreover, it must be reliable and reproducible for the intended use, like clinical evaluation, for example [225]. The acceptability of data results depends directly on the criteria defined for the validation of the methods [226], as the parameters presented below.

In this work, an exploratory research was performed, where validation was an important requirement. Therefore, an extensive laboratory work was done to verify the performance of the developed analytical method.

In order to demonstrate the applicability of both UHPLC methods (with PDA and FLR detection), qualitative and quantitative analyses were performed in an attempt to validate them as presented below.

- **Linearity of the UHPLC-FLR method**

It was possible to obtain good calibration curves with the UHPLC-FLR method developed for thiol analysis, as presented in figure 67 and 68 for glutathione, cysteine and homocysteine in a concentration range of 5, 10, 25, 50 and 100 µM. However, the validation was not completed because reproducibility was inconsistent.

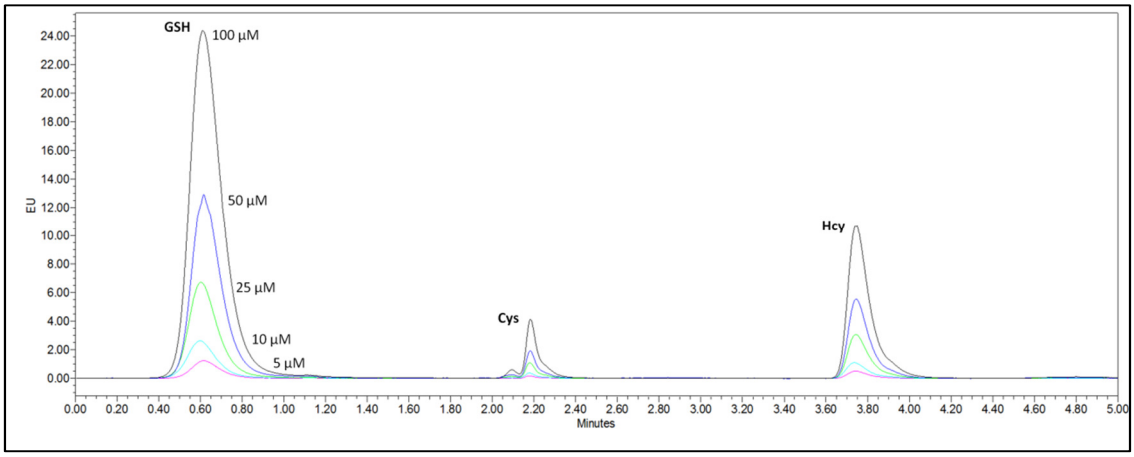

Figure 67 - Chromatograms obtained for the calibration curves of the mixture of thiol standards glutathione, cysteine and homocysteine.

The calibration curves of glutathione, cysteine and homocysteine, built with mean peak areas extracted from chromatographic data (average of three injections for each concentration), showed good correlation coefficients as presented in figure 68.

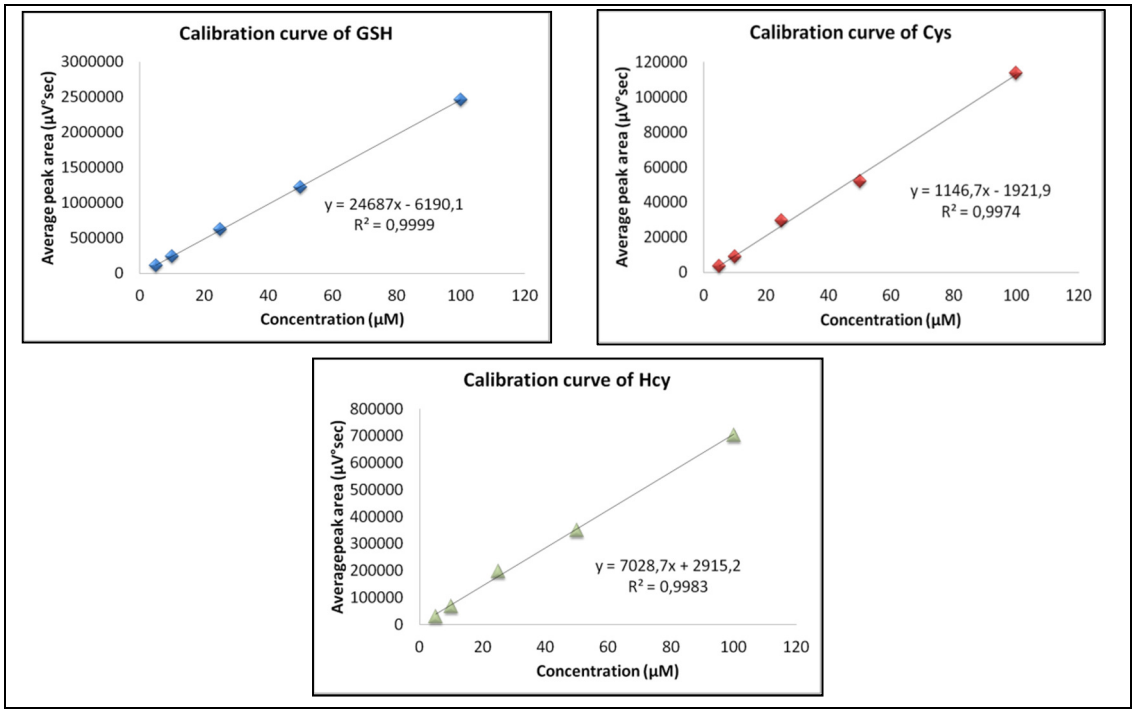

Figure 68 - Graphical representation of the calibration curves of glutathione, cysteine and homocysteine (n=3).

- **Linearity of the UHPLC-PDA method**

The concentrations of the calibration solutions were selected according to the sensitivity of the equipment and concentration normally found in biological samples.

Linearity is obtained by calculating the correlation coefficient of calibration curves for each analyte [225].

Calibration curves from six replicas of six (or more) different concentrations were built for each standard. Finally, the superposition of the curves expressed the linearity of the methods.

This validation parameter was only partially achieved because equipment failure did not allow concluding all the analysis needed.

It was possible to obtain good calibration curves with the UHPLC-PDA method of purine analysis, as presented in figure 69 and 70 for inosine and adenosine, in a concentration range of 25, 50, 75, 100, 250 and 500 µM. However, the validation was not completed, because some results were not consistent.

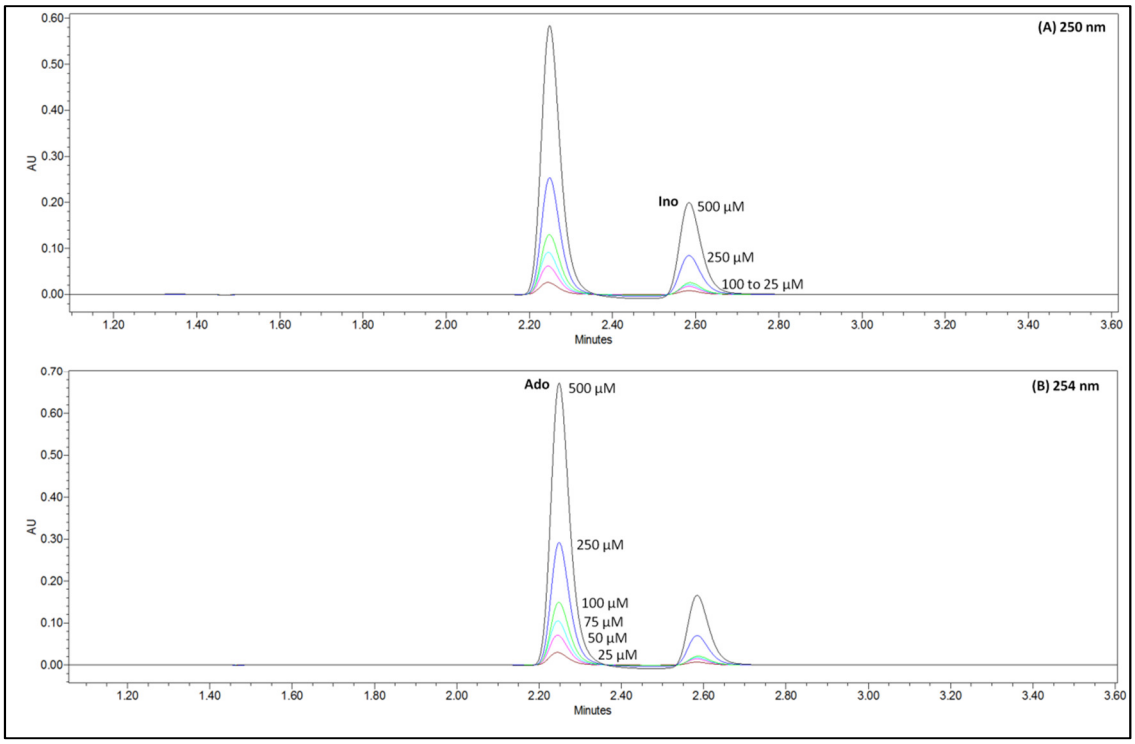

Figure 69 - Chromatograms obtained for the calibration curve of a mixture of standard (A) inosine and (B) adenosine.

The calibration curves of Ado and Ino, built with mean peak areas extracted from chromatographic data (average of six injections for each concentration), showed good correlation coefficients as presented in figure 70.

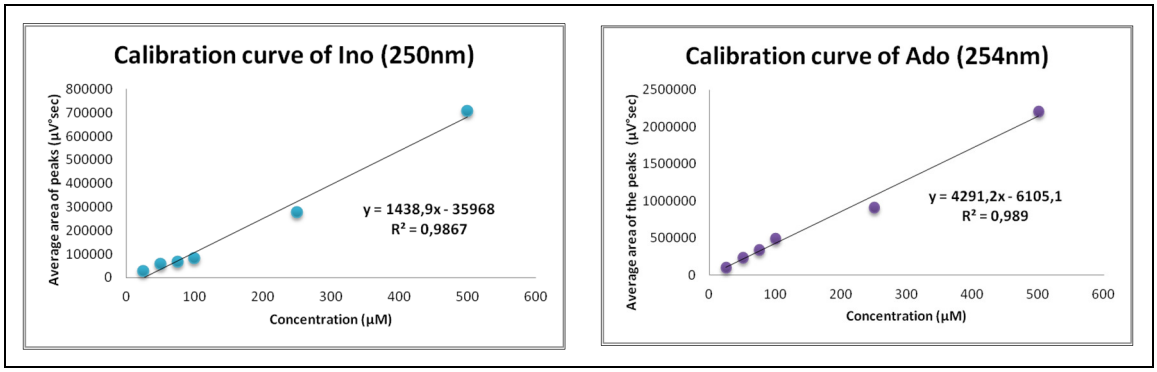

Figure 70 - Graphical representation of the calibration curves of inosine and adenosine (n=6).

- **Selectivity**

Methods are considered specific when they enable discriminating compounds providing assurance that a certain quantity comes only from the intended analyte [226].

The absence of interference from other compounds is measured for each analyte in their expected retention time. Selectivity ensures that the quantification of every analyte is not affected by the reagents used in sample pretreatment, in our case it was TBP and SBD-F, or by the mobile phase constituents, ACN and FA. This parameter was confirmed for both chromatographic methods, since there was no interference in chromatograms obtained from the analyzed standards.

As validation was not concluded, no biological samples were analyzed. Therefore interferences from samples were not checked.

- **Sensitivity**

It is determined by the LOD and LOQ of each compound tested. These parameters are calculated by the lowest concentration that was used in the calibration curve. The LOD is the product obtained by multiplying the SD by three, which is three times the noise of the baseline. The LOQ is the product obtained by multiplying SD by ten what is ten times greater than the baseline noise [226].

Therefore, for thiols the LOD was calculated based on the lowest concentration (5 µM and 25 µM, average of tree injections) of the calibration curve and the regression equations obtained. LOD and LOQ are presented in table 22.

Table 22 - Detection and quantification limits for standards detection with the established methods:

Thiol compounds UHPLC-FLR	Regression equations	LOD (µM)	LOQ (µM)
GSH	y=24687x-6190.1	0.35	1.17
Cys	y=1146.7x-1921.9	1.21	4.04
Hcy	y=7028.7x+2915.2	0.54	1.81
Purine compounds UHPLC-PDA	**Regression equations**	**LOD (µM)**	**LOQ (µM)**
Ado	y=4291.2x-6105.1	0.20	0.68
Ino	y=1438.9x-35968	1.44	4.82

These parameters were quite higher than expected and need to be re-evaluated after maintenance of the UPLC® system.

- **Accuracy**

This parameter, allows evaluating dispersion of results between dependent tests, when repeated for the same sample and under the same chromatographic conditions. It is expressed by the CV or RSD obtained by injecting three different concentrations, the low, medium and high levels, of the calibration curves. Samples must be analyzed six times on the same day (Intra-day) and reproducibility on three non-consecutive days (Inter-day) [245], and the calculations with the replicate data of the different concentrations must be performed. Additionally, accuracy of an analytical method increases as the value of R^2 is closer to 1, obtaining a good linear regression [246].

Within this parameter are the repeatability and reproducibility of methods. In the present work and, as reported, it was not possible to obtain them.

- **Matrix effect and Recovery**

The matrix effect is determined by the percentage of the ratio between control and fortified sample. The recovery can be calculated by the following equation [245]:

Percent Recovery = 100 x ([Standard Fortified] - [Standard]) / [analyte added]

This study was not conducted, because it was not possible to conclude validation of the developed chromatographic methods, neither apply the developed methods to biological samples.

An analytical method is also considered applicable when it is specific, selective, and when the recovery tests are closer to 100% [226]. More, the calibration curve, the analytical linearity and sensitivity of the technique express quantification. Moreover, the method is

robust if it proves to be practically insensitive to small variations that may occur when performed [224].

In our work we were not able to completely validate the methods due to instability of the chromatographic system translating into a high variability in peak retention times, areas and heights both for the method developed for thiol compounds and for the method for purine analysis. Following the specialized technical instructions we eliminated all the possible causes presented below [233, 247]:

i. Incompatibility of the mobile phase composition and the samples to be analyzed;	✓ The solvents used have been widely reported as compatible with the intended analysis. ✓ Several solvents at different ratios were tested, the 95% H_2O|0.1%FA : 5% ACN being chosen, was the most compatible.
ii. The system is not equilibrated;	✓ The system was allowed to equilibrate even up to 2 hours and the results were the same.
iii. Pump pressure or mechanical problems;	✓ In the first instance, an expert evaluated this possibility and found no problem in the pump or the system. Further, it was found that the chromatographic equipment presented problems in the pumps system, requiring specialized maintenance.
iv. Air trapped in the pumps or a leak in the system;	✓ It was not observed any external leakage. Only an expert in UPLC equipment could confirm this.
v. Temperature variations due to temperature of the laboratory;	✓ This may have caused small variations, because the laboratory has no cooling system and it was not easy to completely stabilize the temperature of the sample manager, especially on warmer days. However, it does not explain the poor reproducibility obtained.
vi. Column overloading, as the retention time usually decreases as the mass of the solute injected exceeds the column capacity;	✓ It was not considered a problem, because it the injection of highly concentrated standard samples was always avoided.
vii. Degradation or contamination of the column, that gradually decreases the retention time;	✓ Tests to check the column status were held with good results. See more below.
viii. Unstable flow-rate;	✓ Flow rate changes were not observed. However, it could have happened without being identified or reported by the computer system.

As noted above, some tests were held in order to check the column status using a quality control solution, supplied by Waters Corporation. The results performed indicated no problem with the HSS T3 column used. Also a mixture of certified standards composed by acetone, naphthalene and acenaphthene, had been tried previously. The chromatographic

results were reasonable in terms of plate count, resolution and tailing factor. See results annexed in annex 14.

In our results, it was possible to verify large peak area fluctuations, when the same sample was injected multiple times. There may be many different reasons for this. Nearly every part of the UPLC® equipment can conceivably contribute to changes in peaks areas. Problems in the injector, which may cause any air bubble formation, result in a random variation of the injection volume, from injection to injection. Decreases in peak area from run to run, can be caused by temperature changes, but the effect is rather small, under 2% and not significant [229]. Random variations of flow-rate can also be a problem, by malfunctioning of the check valves and air or cavitations in the pump head. The detector also may contribute to peak area variations, but in an indirect way [229]. At one point we questioned, what reproducibility of the peak area was reasonably expected? The theoretical information indicates that the relative standard deviation of the peak area, for repetitive injections, should be less than 1%, where values between 0.2% and 0.5% are excellent [229].

The theoretical reproducibility can be increased by using an **internal standard** and calculating the ratio of the peak retention time (or area or height) to the one of the internal standard, for each analyte [248]. This was tested in the UHPLC-FLR method, as presented below in the table 23 and figure 71 using cysteamine as internal standard.

Table 23 - Ratio of peak retention time, area and height for each thiol analyte with the internal standard cysteamine:

Ratio	Relative standard deviation (%) of the retention time	Relative standard deviation (%) of the area	Relative standard deviation (%) of the height
GSH/Cyst	1.17	10.33	7.76
Cys/Cyst	0.09	7.89	4.51
Hcy/Cyst	0.08	6.94	2.78

Unfortunately, results were mostly above 1%, mainly for peak area and height, confirming the above-mentioned problems with the UPLC® system.

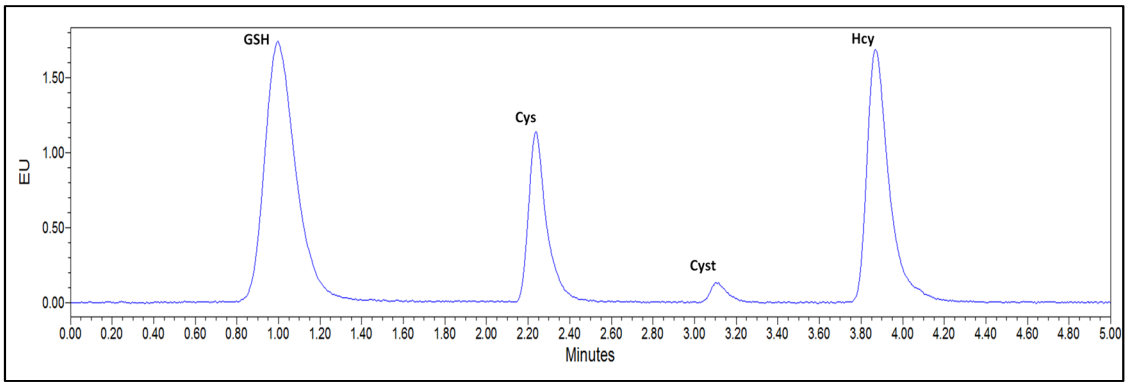

Figure 71 - Chromatographic results of injection of a mixture of standard thiols, glutathione, cysteine, cysteammine and homocysteine (each 100 µM).

4. Conclusions and Future Perspectives

Thiol compounds are important biomarkers of oxidative stress, and some purines are also involved in these processes. However, analysis of thiols constitutes a great challenge due to their high instability.

Since Hopkins discovery, the scientific community has developed numerous methods to identify and quantify thiol compounds and their derivatives, in several biological fluids and cell extracts. The stability of thiol compounds has been one of the major concerns, since they oxidize easily.

In our work we tried to adapt HPLC conditions from previously reported methods of analysis of thiol and purine compounds, to UHPLC. A method with fluorescence detection for thiol compounds and another method with photodiode array detection for purine compounds.

One of our limitations was the avoidance of buffered solutions currently used in HPLC, in order to prevent UPLC® technical problems. The use of buffered mobile phase solutions is important, mainly for thiols, and it has been reported that these compounds remain more stable as they ensure the protection of the sulfhydryl groups, preventing their oxidation [232]. Nonetheless, we succeeded in finding a pre-analytical treatment and chromatographic conditions, which provided good chromatogram resolutions for the compounds of interest.

We developed two procedures that are simple, fast and sensitive but they were not accurate. Therefore, the validation of these methods has not been completed and the reasons were not clear. After several attempts to disclose the problems we came to the conclusion that the UPLC® system was having pump problems, what certainly explains the lack of precision of our methods. Still, some more time was needed to clarify that matter.

Optimization of the methods was accomplished. Sample pre-treatment, namely derivatization of thiols for UHPLC analysis with fluorescence detection, was successfully optimized.

The mobile phase compositions seemed to be appropriate for the separation of thiol and purine compounds but validation needs further analyses to be concluded. Consequently, it was still not possible to apply these methods to analysis of biological samples or cell lysates.

As future perspectives, completing validation of both methods and testing biological samples, are the main goals. To find biomarkers that are effective in preventing and anticipating pathological conditions related to oxidative stress is another major target.

In conclusion, besides the difficulties, this work was considered to be a real profit since, it allowed the acquisition of a broad knowledge on analytical methods like liquid chromatography, which is widely used in several scientific areas of laboratory diagnosis.

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6. Annexes

Annex 1 – Chromatographic conditions tested in the UHPLC analysis with PDA and FLR detection for the wavelength detection assays:

UHPLC system testing conditions	
Injection volume	2 µL
Sample manager temperature	10 °C 37 °C
HSS T3 column temperature	30 °C
Flow-rate	0.200 mL/min
Analysis time	5 min
Equilibrating time	1 min
Mobile phase - Isocratic	95% H_2O|0.1%FA : 5% ACN 90% H_2O|0.1%FA : 10% ACN

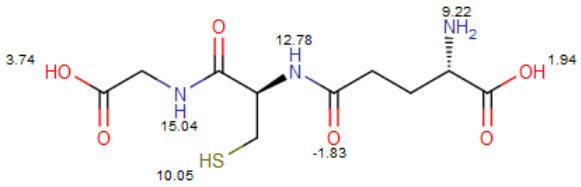
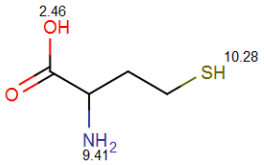
Annex 2 - Testing the reducing agent TCEP·HCl on thiol compounds and UHPLC-PDA analysis:

UHPLC-PDA testing conditions		
Injection volume		2 µL
Sample manager temperature		37 °C
HSS T3 column temperature		30 °C
Specific UV Wavelength detection	**GSH**	200 nm
	Hcy	238 nm
Flow-rate		0.200 mL/min
Analysis time		5 min
Equilibrating time		1 min
Mobile phase - Isocratic		90% H_2O|0.1%FA : 10% ACN

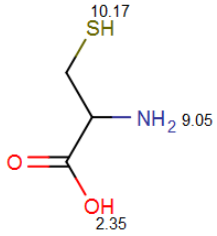
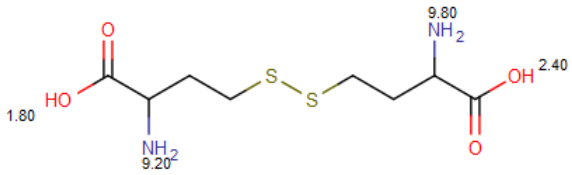
Annex 3 – Thiol compounds are not fluorophores:

UHPLC-FLR system testing conditions	
Injection volume	2 µL
Sample manager temperature	37 °C
HSS T3 column temperature	30 °C
Excitation wavelength	385 nm
Emission wavelength	515 nm
Flow-rate	0.200 mL/min
Analysis time	5 min
Equilibrating time	1 min
Mobile phase – Isocratic pH 2.90	90% H_2O|0.1%FA : 10% ACN

Annex 4 - Biochemical and molecular characteristics of thiol compounds **[**Consulted online in: ChemSpider - Search and share chemistry http://www.chemspider.com and PubChem - Open Chemistry Database página online http://pubchem.ncbi.nlm.nih.gov, last entry 27/07/2016**]**:

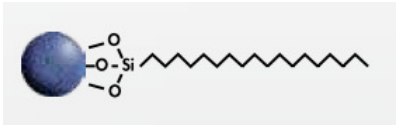
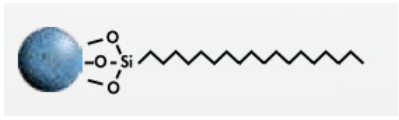
L-Glutathione (GSH)	
Molecular Formula	$C_{10}H_{17}N_3O_6S$
IUPAC Name	(2S)-2-amino-5-[[(2R)-1-(carboxymethylamino)-1-oxo-3-sulfanylpropan-2-yl]amino]-5-oxopentanoic acid
Molecular Weight	307.32348 g/mol
Hydrogen bond donor count	6
Hydrogen bond acceptor count	8
pKa	
DL-Homocysteine (Hcy)	
Molecular Formula	$C_4H_9NO_2S$
IUPAC Name	2-amino-4-sulfanylbutanoic acid
Molecular Weight	135.18476 g/mol
Hydrogen bond donor count	3
Hydrogen bond acceptor count	4
pKa	

Annex 4 (cont.) - Biochemical and molecular characteristics of thiol compounds **[**Consulted online in: ChemSpider - Search and share chemistry http://www.chemspider.com and PubChem - Open Chemistry Database página online http://pubchem.ncbi.nlm.nih.gov, last entry 27/07/2016**]**:

L-Cysteine (Cys)	
Molecular Formula	$C_3H_7NO_2S$
IUPAC Name	(2R)-2-amino-3-sulfanylpropanoic acid
Molecular Weight	121.15818 g/mol
Hydrogen bond donor count	3
Hydrogen bond acceptor count	4
pKa	
Cysteamine (Cyst)	
Molecular Formula	C_2H_7NS
IUPAC Name	2-aminoethanethiol
Molecular Weight	77.14868 g/mol
Hydrogen bond donor count	2
Hydrogen bond acceptor count	2
pKa	
L-Homocystine (2Hcy)	
Molecular Formula	$C_8H_{16}N_2O_4S_2$
IUPAC Name	2-amino-4-[(3-amino-3-carboxypropyl)disulfanyl]butanoic acid
Molecular Weight	268.35364 g/mol
Hydrogen bond donor count	4
Hydrogen bond acceptor count	8
pKa	

Annex 5 – Chromatographic columns and pre-columns tested

Annex 5a- Specifications of the column and pre-column tested, the HSS T3 and BEH C_{18} from Waters Corporation:

	HSS HIGH STRENGTH SILICA	BEH Technology™
Name	**ACQUITY UPLC® HSS T3 (2.1 x 100 mm) column and ACQUITY UPLC® HSS T3 VanGuard™ (2.1 x 5 mm) 1.8 µm particle size**	**ACQUITY UPLC® BEH C_{18} (2.1 x 50 mm) column and ACQUITY UPLC® C_{18} VanGuard™ pre-column (2.1 x 5 mm) 1.7 µm particle size**
Part Number	186003539 186003976	186002350 186003975
Lot Number	0167351071 0166351181	0234340521 0233340421
% Carbon Load	11%	18%
Bonding Technology	T3	---
Brand	AQUITY UPLC	
Chemistry	C18	
End capped	Yes	
End Fitting Type	Waters Direct-connect	
Format	Column VanGuard Pre-column	
ID	2.1 mm	
Length	100 mm 5 mm	50 mm 5 mm
Mode	Reversed-Phase	
Particle		
Particle Shape	Spherical	
Particle Size	1.8 µm	1.7 µm
Particle Substrate	Silica	Hybrid
Pore Size	100 Å	130 Å
Silanol Activity	Low Medium	Low Low
Surface Area	230 m^2/g	185 m^2/g
Ligand Density	1.6 µmol/m^2	3.1 µmol/m^2
System	UPLC/UHPLC	
Technology	HSS	BEH
USP Classification	L1	
Units in Package	1/pkg 3/pkg	
pH Range	2 - 8	1 - 12
Temperature Limits	Low pH 45 °C and High pH 45 °C	Low pH 80 °C and High pH 60 °C

Note: Consulted online in: www.waters.com; http://www.waters.com/waters/partDetail.htm?partNumber=186003539 ; http://www.waters.com/waters/partDetail.htm?locale=119&partNumber=186002350, last entry 27/07/2016.

Annex 5b – Assay to choose the optimal chromatographic column for the best separation of thiol compounds by UHPLC-FLR method:

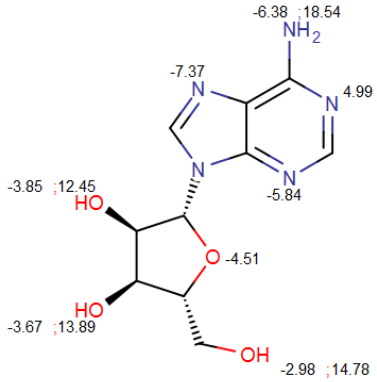
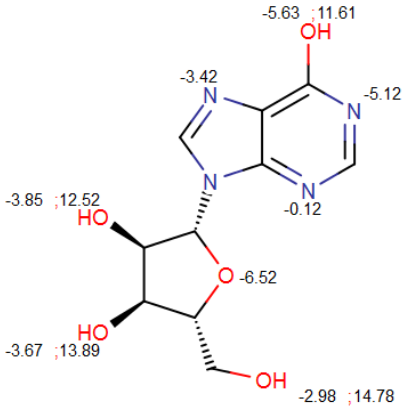
UHPLC-FLR testing conditions	
Injection volume	2 µL
Sample manager temperature	10 °C
HSS T3 column temperature	30 °C
BEH C_{18} column temperature	
Excitation wavelength	385 nm
Emission wavelength	515 nm
Flow-rate	0.200 mL/min
Analysis time	5 min
Equilibrating time	1 min
Mobile phase - Isocratic	95% H_2O|0.1%FA : 5% ACN

Annex 6 – Assay for obtaining optimal chromatographic separation of cysteine, homocysteine and homocystine in the UHPLC-FLR method:

UHPLC-FLR testing conditions	
Injection volume	2 µL
Sample manager temperature	4 °C
HSS T3 column temperature	30 °C
Excitation wavelength	385 nm
Emission wavelength	515 nm
Flow-rate	0.200 mL/min
Analysis time	5 min
Equilibrating time	5 min
Mobile phase – Isocratic pH 2.90	95% H_2O|0.1%FA : 5% ACN

Development and optimization of an analytical UHPLC-PDA method for purines analysis by UPLC® system

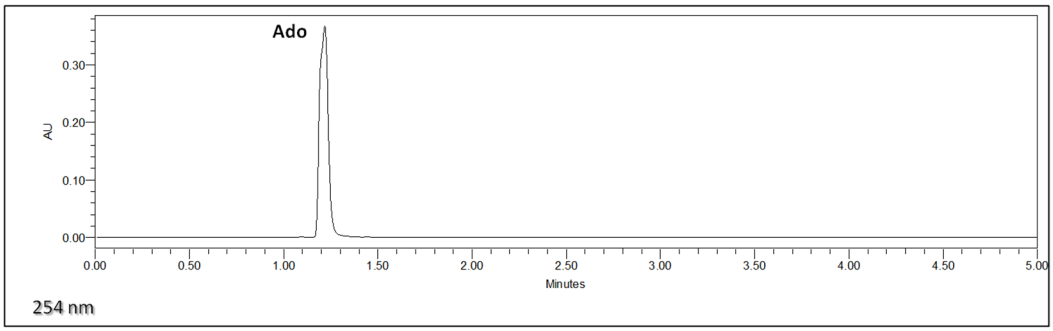
Annex 8 - Biochemical and molecular characteristics of the analyzed purine compounds **[**Consulted online in: ChemSpider - Search and share chemistry http://www.chemspider.com and PubChem - Open Chemistry Database online http://pubchem.ncbi.nlm.nih.gov, last entry 27/07/2016**]**:

Adenosine (Ado)	
Molecular Formula	$C_{10}H_{13}N_5O_4$
IUPAC Name	(2R,3R,4S,5R)-2-(6-aminopurin-9-yl)-5-(hydroxymethyl)oxolane-3,4-diol
Molecular Weight	267.24132 g/mol
Hydrogen bond donor count	4
Hydrogen bond acceptor count	8
pKa	
Inosine (Ino)	
Molecular Formula	$C_{10}H_{12}N_4O_5$
IUPAC Name	9-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-3H-purin-6-one
Molecular Weight	268.22608 g/mol
Hydrogen bond donor count	4
Hydrogen bond acceptor count	6
pKa	

Annex 9 – Conditions tested in the UHPLC analysis by PDA detection:

UHPLC system testing conditions		
Injection volume		2 µL
Sample manager temperature		10 °C
HSS T3 column temperature		30 °C
Flow-rate		0.200 mL/min
Analysis time		5 min
Equilibrating time		1 min
UV Wavelength specific detection	**Ado**	254 nm
	Ino	250 nm
Mobile phase - Isocratic		95% H_2O|0.1%FA : 5% ACN

Annex 10 - Gradient mobile phase tested in adenosine detection on UHPLC-PDA system [223]:



Annex 10a - Chromatogram of UHPLC-PDA detection of adenosine (100 µM) at 254 nm. See chromatographic conditions in annex 1a.

Annex 10b - Chromatographic data obtained from separation of adenosine:

Data	**Retention time**		**Area**		**Height**		**Maximum Absorvance**	
	min	**RSD (%)**	**µV°sec**	**RSD (%)**	**µV**	**RSD (%)**	**AU**	**RSD (%)**
Ado	1.217 ± 0.000	0.12	233908.52 ± 823.67	0.35	74305 ± 275.67	0.37	0.513 ± 0.000	0.49

Note: average of two injections.

Annex 11 - Gradient mobile phase tested in the UHPLC-PDA system:

Run time (min)	H_2O|0.1%FA (%)	ACN|0.1%FA (%)
0	90	10
0.5 - 1.5	95	5
2	98	2
3	90	10
4 - 5	90	10
Run time (min) [*]	**H_2O|0.1%FA (%)**	**ACN|0.1%FA (%)**
0	96	4
0.5	96	4
2.5	90	10
5	72	28
5.1	5	95
6.1	5	95
6.2	96	4
7.5	96	4
Run time (min) [**]	**H_2O|0.1%FA (%)**	**ACN|0.1%FA (%)**
0	99.9	0.1
2	98	2
3.5	70	30
3.51	5	95
4.79	5	95
4.8	99.9	0.1
6	99.9	0.1

Note: Gradient mobile phase tested consulted at: [*] Gray N and Plumb R. A Validated Assay for the Quantification of Amino Acid in Mammaian Urine. Waters Corporation© 2014, 720005189EN AG-PDF. [**] New L and Chan E. Evaluation of BEH C18, BEH HILIC and HSS T3 (C18) Column Chemistries for the UPLC-MS-MS Analysis of Gluthione, Glutathione Disulfide and Ophthalmic Acid in Mouse Live and Human Plasma. Journal of Chromatographic Science 2008, 46, 209-214.

Annex 12 – Testing methanol as solvent in mobile phase for purine by UHPLC-PDA detection:

UHPLC-PDA testing conditions		
Injection volume		2 µL
Sample manager temperature		10 °C
HSS T3 column temperature		30 °C
Flow-rate		0.200 mL/min
Analysis time		5 min
Equilibrating time		1 min
Specific detection UV Wavelength	**Ado**	254 nm
	Ino	250 nm
Mobile phase - Isocratic		90% H_2O|0.1%FA : 10% MeOH

Annex 13 – Assay to choose the optimal chromatographic column for the best separation of purines compounds by UHPLC-PDA method:

UHPLC-PDA testing conditions		
Injection volume		2 µL
Sample manager temperature		10 °C
HSS T3 column temperature		30 °C
BEH C_{18} column temperature		
Flow-rate		0.200 mL/min
Analysis time		5 min
Equilibrating time		1 min
Specific detection UV Wavelength	**Ado**	254 nm
	Ino	250 nm
Mobile phase - Isocratic		95% H_2O|0.1% AF : 5% ACN

Annex 14 - Quality Control Test on the Chromatographic Column HSS T3

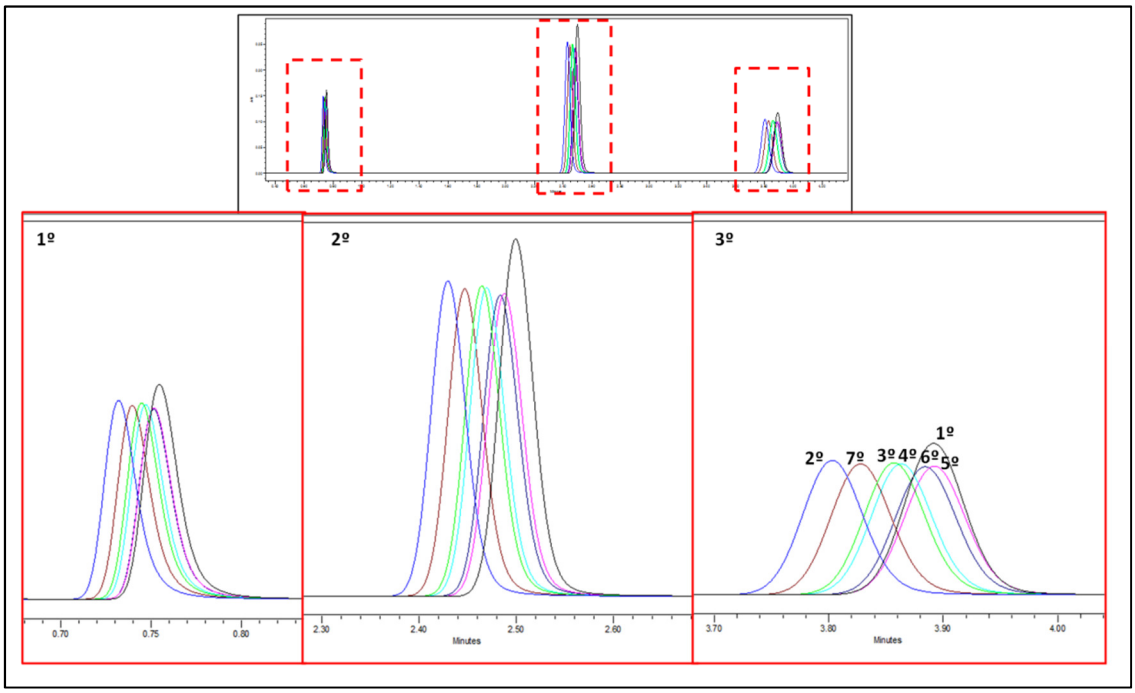
The goal of this test, it was to indicate that the chromatographic system was functioning as expected or outside of expectation.

The Certificate of Analysis, Waters Neutrals QC References Material (Number 186006360), composed by acetone (10 µL/mL), naphthalene (0.25 mg/mL) and acenaphthene (0.4 mg/mL). This mixture of standards was injected in the chromatographic system seven times, under the following standardized conditions by which the quality of the column is measured.

Annex 14a - Chromatographic conditions for determining the state of the HSS T3 column:

UHPLC-PDA Analytic Conditions	
Injection volume	1.2 µL
Sample manager temperature	off
HSS T3 column temperature **(100mm x 2.1mm, 1.8µm particle size. Without pre-column)**	30 °C
UV Wavelength detection	254 nm
Flow-rate	0.450 mL/min
Analysis time	6 min
Rebalancing time	1 min
Mobile phase - Isocratic	35% H_2O : 65% ACN

- **Chromatographic results:**

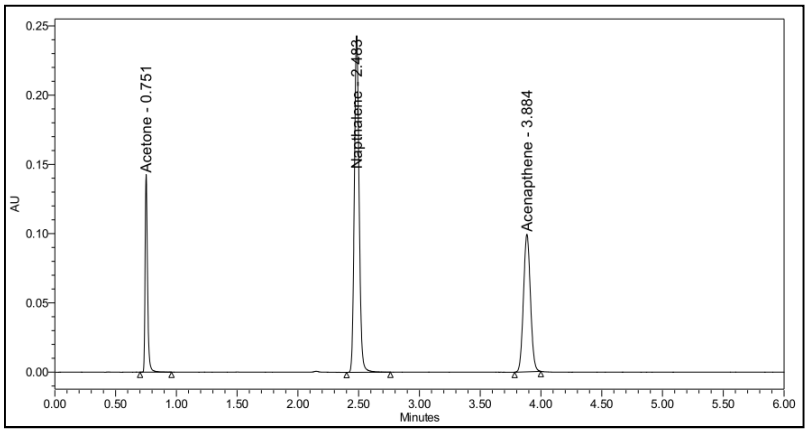


Annex 14b - Overlap of the seven injections performed with the quality control composed by acetone (1º peaks), naphthalene (2º peaks) and acenaphthene (3º peaks).

It was possible to check with these results, that there is a wide variation in the retention time of each compound. The Waters experts report, if the retention time shifts it should be investigated problems in the pumping system, in the mobile phase or even some column instability, such as loss in bonded phase (Consulted at Waters Corporations. Neutrals Quality Control Reference Material. Waters The Sciences of What´s Possible.™ 2013, 18 pages, Ref.: 720004432EN Rev B IH-KP-PDF).

Annex 14c - Chromatographic data obtained in the HSS T3 column status data obtained though automatic of integration method:

Average of 7 injections	Acetone	Napthalene	Acenapthene
Retention time (min)	0.751	2.483	3.884
Area (µV°sec)	198936	661402	395312
Area (%)	15.84	52.67	31.48
Height (µV)	142734	242947	99202
USP Plate Count	7.307760e+003	1.972874e+004	2.175556e+004
USP Resolution	---	3.206020e+001	1.564278e+001
USP Tailing	1.364202e+000	1.111123e+000	1.050123e+000



The number of theoretical plates (N), also known as column efficiency, is a measure of the dispersion of a peak. The supplier refers that the columns do not have fixed number of plate counts. For a given column, plate counts depend on the flow-rate, the viscosity of the solvents and the molecular weight of the analyte. The column manufacturer has set up the standardized chromatographic conditions were the mobile phase used contain a high amount of organic solvent, so was low viscosity [229]. However, in our case, we are dealing with polar molecules, which require mobile phase with higher water content, thus higher viscosity. For this reason, it is natural that in our chromatographic analysis the plate count is lower, under the practical conditions establish than under column testing conditions.

Observation - Some molecular structures were fulfilled through the HyperChem Release 8.0 Professional Software, applying the basic knowledge acquired in Molecular Modelling. It was used Amber99 programs as Molecular Mechanics force field, which best fits the simulation of proteins. Additionally were performed Molecular Dynamics mode to optimize the molecular structure.