Use of Novel Biomarkers (Homocysteine, Vitamin B6, B9 and B12) on the Assessing the Progression of Cardiovascular Disease

MASTER DISSERTATION

Hugo Miguel de Sousa Câmara
MASTER IN APPLIED BIOCHEMISTRY

UNIVERSIDADE da MADEIRA
A Nossa Universidade
www.uma.pt

September | 2013

Use of novel biomarkers (homocysteine, vitamin B_6, B_9 and B_{12}) on the assessing of the progression of cardiovascular disease

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

2012/2013

Hugo Sousa Câmara

Study performed under the supervision of:

Prof. Doctor José de Sousa Câmara

and co-supervised by:

Doctor Jorge Pereira

Funchal, Portugal

"The whole of science is nothing more than a refinement of everyday thinking".

Albert Einstein

Acknowledgments

The preparation of this thesis would not be possible without the support and guidance of various intermediaries in my academic life. So I come here to express my deep gratitude and appreciation to all that contributed decisively in my training, whether at academic and professional level, as well as personal level.

My special thanks to my supervisor Prof. Doctor José de Sousa Câmara and my co-supervisor Doctor Jorge Pereira for all the support, understanding, availability, experience, professionalism and opportunity.

I also would like to thank the Madeira Chemistry Research Centre (CQM) for the conditions provided to perform this work, and especially the colleagues of the Laboratory of Chromatography, Aldónio Figureira, Berta Mendes, Catarina Luís Silva, Fátima Rodrigues, Rosa Perestrelo and Vera Alves, for all the support, collaboration and availability.

I also would like to thank the collaboration of the Department of Health of the Autonomous Region of Madeira (SESARAM, E.P.E.) and the hospital Dr. Nélio Mendonça, the availability and collaboration in the samples collection.

I must acknowledge the support of the FEDER (2007-2013 MAC Transnational Cooperation Program) project **VinSaudeMAC (MAC/1/M105)**, the Foundation for Science and Technology (FCT) for funding the project PEst-OE/QUI / UI0674/2011 (CQM, Portuguese Government funds) and the Portuguese NMR Network (PTNMR-REDE/1517/RMN/2005-POCI2010/FEDER).

And finally I must thank deeply to my family and friends for all they have contributed to my academic and personal development.

Research Work

The development of this work was an excellent opportunity to me to learn how to access and analyze scientific information, schedule and perform research work, interprete experimental data and communicate science. Moreover, it has contributed to the improvement of my skills in analytical chemistry, especially the extractive and chromatographic techniques.

The development of this work also provided me the participation in two conferences (one national and one international) through the submission of three poster communications:

1. **Câmara H.**, Pereira J., Câmara, J.S. A simplified and sensitive strategy for the determination of plasma total homocysteine and metabolic related vitamins B6, B9 and B12 using MEPS-UHPLC-PDA Analysis. HPLC 2013, Amsterdam, 16-20 June 2013.
2. **Câmara, H.**; Pereira, J.; Figueira, P.; Câmara, J.S. Optimization of digitally controlled microextraction by packed sorbent combined with UHPLC-PDA for quantification plasma Homocysteine. Comparison with direct extraction procedure. 8º Encontro Nacional de Cromatografia, Covilhã, Portugal. 2, 3 and 4 December 2013.
3. **Câmara, H**.; Pereira, J.; Câmara, J.S. A simplified and sensitive strategy for the determination of the metabolic-related vitamins B6, B9 and B12 based on MEPS combined with UHPLC-PDA analysis. 8º Encontro Nacional de Cromatografia, Covilhã, Portugal. 2, 3 and 4 December 2013.

Resumo

Atualmente relacionam-se os níveis elevados de homocisteína (Hcys) com uma maior incidência no desenvolvimento de doenças cardiovasculares (CVDs), em especial a aterosclerose. De igual forma, associa-se o baixo nível das vitaminas B_6, B_9 e B_{12} a uma instabilidade no ciclo da metionina com excessiva produção de Hcys. Desta forma, as ciências biomédicas têm procurado encontrar uma metodologia analítica para a quantificação em ambiente clínico e hospitalar destes compostos, que seja mais barata, rápida, precisa e exata, face às já existentes. Este constituiu assim o objetivo deste estudo, tendo-se utilizado um procedimento que integrava a microextração em seringa empacotada (MEPS) controlada digitalmente (eVol®) como técnica extrativa e um sistema de cromatografia líquida de ultra eficiência (UPLC) acoplado a um detetor de fotodiodos (PDA) como forma de identificar e quantificar a Hcys e as vitaminas B_6, B_9 e B_{12}.

No seguimento do trabalho experimental, não foi possível combinar a Hcys e as vitaminas num mesmo procedimento analítico, tendo-se procedido à otimização de dois procedimentos diferentes, um para Hcys e outro para as três vitaminas, que apenas divergiam na constituição do gradiente da fase móvel e do volume injetado. Verificou-se que a MEPS não trazia benefícios para a quantificação da Hcys no plasma, procedendo-se assim à validação de um método alternativo que usa a injeção direta de plasma previamente tratado (reduzido e precipitado). Este mesmo método foi avaliado em termos de seletividade, linearidade, limite de deteção (LOD), limite de quantificação (LOQ), efeito matriz e precisão (intra e inter-dia) e aplicado na determinação da Hcys num grupo constituído por pacientes com doença cardiovascular. Obtiveram-se bons resultados em termos de seletividade, de linearidade (R^2> 0,9968), de LOD (0.07 μmol/L) e LOQ (0.21 μmol/L). A precisão intra-dia (1.23 – 3.32 %), precisão inter-dia (5.43 – 6.99 %) e a taxa de recuperação (82.5 a 93.1%) deste método foram satisfatórios. Obteve-se, porém, um efeito matriz superior a 120%, bastante mais elevado que o desejável. Quanto à aplicação prática desta metodologia, foi possível determinar a quantidade de Hcys nos indivíduos-alvo deste estudo. Em relação à metodologia desenvolvida para as vitaminas, apesar da otimização da técnica extrativa e das condições cromatográficas, constatou-se que que os níveis normalmente presentes no plasma estão muito abaixo da sensibilidade do aparelho, pelo que será necessário otimizar ainda mais o procedimento experimental.

Como conclusão, parte dos objetivos deste estudo foram concretizados, tendo sido possível validar um método rápido, simples e barato para quantificação da Hcys em plasma.

Palavras-chave: Homocisteína (Hcys); Aterosclerose; Doenças cardiovasculares; Cromatografia líquida de alta eficiência (UPLC); Validação;

Abstract

A large number of evidences correlate elevated levels of homocysteine (Hcys) with a higher cardiovascular diseases (CVDs) risk, especially, atherosclerosis. Similarly, abnormal low levels of the vitamins B_6, B_9 and B_{12} are associated to an instability in the methionine cycle with an over production of Hcys. Thus, biomedical sciences are looking forward for a cheaper, faster, precise and accurate analytical methodology to quantify these compounds in a suitable format for the clinical environment. Therefore the objective of this study was the development of a simple, inexpensive and appropriate methodology to use at the clinical level. To achieve this goal, a procedure integrating a digitally controlled (eVol®) microextraction by packed sorbent (MEPS) and an ultra performance liquid chromatography (UPLC) coupled to a photodiode array detector (PDA) was developed to identify and quantify Hcys vitamins B_6, B_9 and B_{12}.

Although different conditions were assayed, we were not able to combine Hcys with the vitamins in the same analytical procedure, and so we proceeded to the optimization of two methods differing only in the composition of the gradient of the mobile phase and the injected volume. It was found that MEPS did not bring any benefit to the quantification of the Hcys in the plasma. Therefore, we developed and validate an alternative method that uses the direct injection of treated plasma (reduced and precipitated). This same method was evaluated in terms of selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), matrix effect and precision (intra-and inter-day) and applied to the determination of Hcys in a group composed by patients presenting augmented CVD risk. Good results in terms of selectivity and linearity (R^2> 0.9968) were obtained, being the values of LOD and LOQ 0.007 and 0.21 mol / L, respectively. The intra-day precision (1.23-3.32%), inter-day precision (5.43-6.99%) and the recovery rate (82.5 to 93.1%) of this method were satisfactory. The matrix effect (>120%) was, however, higher than we were waiting for. Using this methodology it was possible to determine the amount of Hcys in real plasma samples from individuals presenting augmented CVD risk. Regarding the methodology developed for vitamins, despite the optimization of the extraction technique and the chromatographic conditions, it was found that the levels usually present in plasma are far below the sensitivity we obtained. Therefore, further optimizations of the methodology developed are needed.

As conclusion, part of the objectives of this study was achieved with the development of a quick, simple and cheaper method for the quantification of Hcys.

Keywords: homocysteine (Hcys), cardiovascular diseases (CVDs), atherosclerosis, ultra performance liquid chromatography (UPLC); validation;

Index

Figures index

Tables index

List of abbreviations

Symbols	**%**	Percentage
	ºC	Degrees Celsius
	λ	Wavelength
	min	Minute
	s	Second
	cm	$1x\ 10^{-3}$ metres
	nm	1×10^{-9} metres
	mg	1×10^{-3} grams
	µg	1×10^{-6} grams
	ng	1×10^{-9} grams
	pg	1×10^{-12} grams
	mL	1×10^{-3} litres
	µL	1×10^{-6} litres
	mmol	1×10^{-3} moles
	µmol	1×10^{-6} moles
	nmol	1×10^{-9} moles
	pmol	1×10^{-12} moles
A	**ABCA1**	ATP-binding cassette A1
	ABCG1	ATP-binding cassette transporter G1
	ACAT-1	AcylCoA: cholesterol transferase A1
	ACN	Acetonitrile
	ADMA	Assymmetric dimethylargigine
	ADP	Adenosine Diphosphate
	APO	Apolipoprotein
B	**BIN**	Barrel Insert and Needle
C	**CBS**	Cysthathionine β-synthase
	CM	Chylomicron
	CL	Cystathionine ϒ-lygase
	CHD	Coronary heart disease
	Cp	Ceruloplasmin
	CVD/CVDs	Cardiovascular diseases/cardiovascular disease

D	**DCs**	Dendritic cells
	DNA	Deoxyribonucleic acid
	dTMP	Deoxythymidine monophosphate
E	**EC**	Electrochemical detection
	ECM	Extracellular matrix
	eNOS	Endothelial Nitric Oxide synthase
	ET-1	Endothelin 1
F	**FA**	Formic acid
	FFA/FFAs	Free fatty acid/ free fatty acids
G	**GAG**	Glycosaminoglycan
	CG	Gas Chromatography
	GP1HOB1	glycosylphosphatidylinositol-anchored HDL-binding protein 1
	GPX-1	Gluthation peroxidise
H	**Hcys**	Homocysteine
	HDL	High-density lipoprotein
	HHcys	Hyperhomocysteinemia
	HIV	Human immunodeficiency virus
	HMGCR	3-hydroxy-3-methylglutaryl coenzyme A reductase
	HPLC	High pressure liquid chromatography
	HSP-60	Heat stock protein
	HTL	Homocysteine-thiolactone
I	**ICAM-1**	Inter cellular adhesion molecule-1
	IDL	Intermediate-density lipoprotein
	INF-ϒ	Interferon-ϒ
L	**LCAT**	Lecithincholesterol acyltransferase
	LC	Liquid chromatography
	LDL	Low-density lipoprotein
	LPL	Lipoprotein lipase
	LIPG	Endothelial lipase
	LPS	Lipopolyssacharide
	Lys	Lysine
M	**mBrB**	Monobrominane
	MCP-1	Monocytes chemoattractant protein-1

M	**MCSF**	Macrophage colony stimulating factor
	MEPS	Microextraction by packed sorbent
	5mTHF	5-methyltetrahydrofolate
	MetS	Methionine synthase
	MIP-3 α	Macrophage inflammatory protein 3-α
	MIPs	Molecular imprinted polymers
	mmLDL	Minimally oxidized LDL
	MMPs	Metalloproteinases
	MPO	Myeloperoxidase
	MTHFR	Methylenetetrahydrolate reductase
	MS	Mass spectrometry
N	**NADH**	Nicotinamide Adenine dinucleotide (Reduced form)
	NaOH	Sodium hydroxide
	NCD/NCDs	Non-communicable Disease/Non-communicable Diseases
	NKT	Natural Killer T
O	**O_2**	Oxygen
	$OONO^{\cdot}$	Oxidant peroxynitrate
	OPA	O-phtaldialdehyde
P	**PACM-1**	Platelet-endothelial cell adhesion molecule-1
	PAI-1	Plasminogen activator inhibitor
	PARP	Poly(ADP-ribose)polymerase
	PAF	Platelet activating factor
	PDA	Photodiode array
	PGL2	Prostacyclin
	pp	Protein precipitation
	PS-DVB	Polystyrene-divinylbenzen copolymer
	PTFE	Polytetrafluorethylene
Q	**QSM**	Quaternary solvent manager
R	**RAM**	Restricted access material
	RHD	Rheumatic heart disease
	RNA	Ribonucleic acid
	ROS	Reactive Oxygen species
S	**SAM**	S-adenosylmethionine
	SAH	S-adenosylhomocysteine
	SCX	Strong cation exchanger

S	**SM**	Sample manager
	SPE	Solid phase extraction
	SOD	Superoxide dismutase
	SR-A	Scavenger receptor type A
	SRB1	Scavenger receptor class B type I
	SREBP	Serol regulatory element-binding protein
T	**TECP**	Tris(2-carboxyethyl)phosphine hydrochloride
	TG	Triglyceride
	THF	Tetrahydrofolate
	TF	Tissue factor
	TLR4	Toll-like receptor 4
	TM	Inhibition of thrombomodulin
	TNF-α	Tumour necrosis factor-α
	TxA2	Thromboxane A2
U	**UPLC**	Ultra Performance Liquid Chromatography
	UV/Vis	Ultraviolet/Visible
W	**WHO**	World Health Organization
V	**VCAM-1**	Vascular cell adhesion molecule-1
	VLDL	Very low-density lipoprotein
	vWF	Von Willebrand factor

Aims

Given the crucial role of Hcys and vitamin B-cofactors for the onset of several diseases such as atherosclerosis, as well as the great interest in the development of a simple, cheaper and reliable analytical methodology for the quantification of these compounds, suitable for the clinical quantification, the aim of this study was the development of a method to quantify the total Hcys and vitamin B-cofactors through the use of Microextraction by packed sorbent (MEPS) followed Ultra Performance Liquid Chromatography (UPLC) coupled with Photodiode Array (PDA) detector.

MEPS was chosen as the extraction technique according to the quality of extraction it provides at a relatively cheap price. The use of UPLC coupled with PDA detector was also selected taking in account the price and the quality of the quantification when compared to others chromatographic assays such as Liquid Chromatography/Mass spectrometry (LC/MS) and different immunoassays possibilities, which allows only total Hcys quantification.

Chapter I: Bibliographic revision

Throughout this chapter some topics considered essential for the understanding of the purpose of this study will be discussed.

1.1. Cardiovascular diseases

The Cardiovascular diseases (CVDs) include a group of disorders of the heart and blood vessels that actually represent the leading cause of death by illness worldwide. They include coronary heart disease (CHD), ischemic strokes, rheumatic heart disease (RHD), cardiomyopathy, and other heart diseases such as congenital heart disease, deep vein thrombosis or pulmonary embolism [1-3]. Although most CVDs in the world are due atherosclerosis (CHD and ischemic strokes), other CVDs due to infection (RHD, Chagas' heart disease, cardiomyopathy from human immunodeficiency virus (HIV) infection, cerebrovascular complications of malaria and among others) remain common in many regions of the developing world [1].

According with Word Heath Organization (WHO) of a total 57 million deaths occurring in 2008, 36 million (63%) were due Non-communicable Diseases (NCDs), mainly cardiovascular disease, diabetes, cancers and chronic respiratory diseases. Of these deaths, an estimated of 17 million deaths were due CVDs (48% of NCDs), namely 7.3 million for CHD and 6.2 million for ischemic stroke. Furthermore, over 80% of these CVD deaths took place in low and middle-income countries and occurred almost equally in men and women [2, 3].

By population growth and increase of the life expectancy, it is envisaged an increasing number and proportions of older people, verifying the emerging of a population more ageing as a significant trend in many parts of the world. So as population's age increase, annual NCD deaths are projected to rise substantially to 52 million in 2030, and concerning to annual CVD mortality, it is expected an increase by 6 million to a total value of 23 million in 2030 [3, 4].

Word Heart Federation considers that the major part of CVDs is caused by risk factors that can be controlled, treated or modified, such as high blood pressure (hypertension), tobacco use, raised blood glucose (diabetes), physical inactivity, unhealthy diet, raised blood cholesterol/lipids, overweight and obesity [5]. However, there are also some major CVD risk factors that cannot be controlled such as the age, gender and familiar history. Regarding to the attributable of deaths by risk factors worldwide, the leading CVDs risk factor is hypertension (13 per cent of global), followed by tobacco use (9 per cent), diabetes (6 per cent), physical inactivity (6 per cent) and overweight and obesity (5 per cent) [3, 5]

1.2. Atherosclerosis

Atherosclerosis is a complex pathological process in the walls of blood vessels that develops over many years, being the responsible for a large proportion of CVDs and the most common cause of death in Western societies [3]. It is a result of blood vessels ageing, with initial lesion developing before age 30 (Figure 1) [6].

Atherosclerosis is recognized as a local inflammation originated by the formation of subintimal plaques in the innermost layer of an artery (*intima*). This layer is, composed by endothelial cells, elastic connective tissue and percytes, and the plaques formed can reduce or obstruct blood flow through the vessel [7, 8]. The process continues with the accumulation of plasma low-density-lipoproteins (LDL) in the walls of medium and large arteries, such as the coronaries, carotids and the aorta [9]. An atherosclerotic symptomatic event manifests itself clinically as myocardial infarction and stroke and their respective precursor disorders, angina pectoris and transient ischaemic attacks of the brain [10].

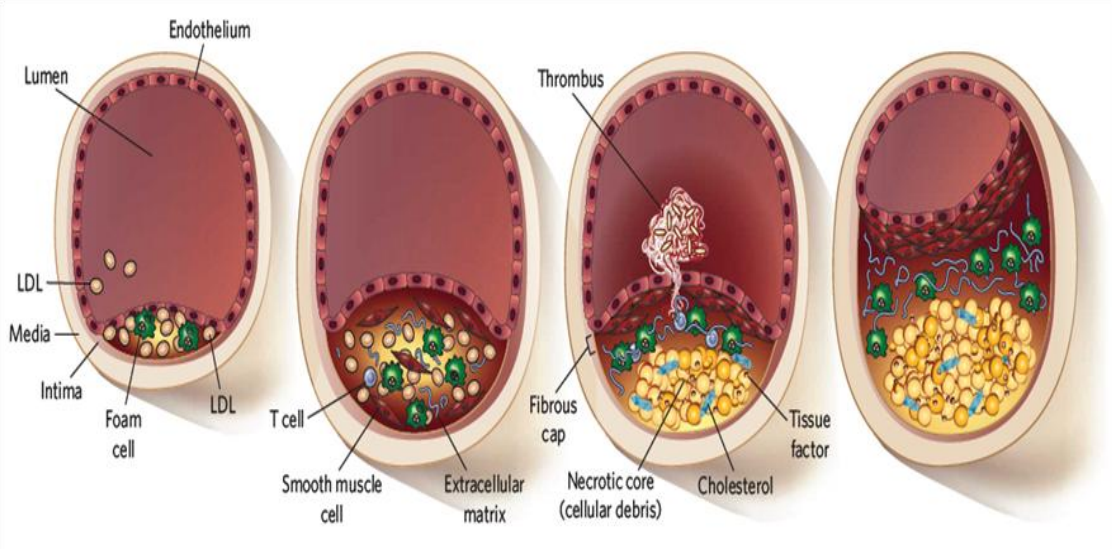


Figure 1 - Evolution of atherosclerosis. Image obtained from Rader *et al* [6].

WHO defines three kinds of risk factors at the rear of the atherosclerosis development [3]:

- **Behaviour risk factors:** tobacco use; physical inactivity; unhealthy diet (rich in salt, fat and calories); harmful use of alcohol.
- **Metabolic risk factors:** raised blood pressure (hypertension); raised blood sugar (diabetes); raised blood lipids (hypercholesterolemia); overweight and obesity.
- **Other risk factors:** poverty and low education status; advancing age; gender; inherit genetic disposition; psychological factors (stress, depression); and excess of homocysteine.

Among these risk factors, elevated levels of serum cholesterol are probably the unique factor capable to being sufficient to drive the development of atherosclerosis, even in the absence of other known risk factor. However, the presence of others risk factors together with hypercholesterolemia contribute for a faster development of the disease, once the main pathophysiological mechanism in atherosclerosis is an endothelium dysfunction that, despite hypercholesterolemia, can be developed by some of these other factors and so facilitate an increased entry and accumulation of LDL into artery walls [10, 11].

Atherosclerotics lesions (*atheromata*) are asymmetric focal thickenings of cells, connective-elements, lipids and debris. Blood-borne inflammatory agents (microbes, autoantigens and other molecules) and immune cells constitute an important part of an *atheroma* (one single atherosclerotic lesion), while the remainder are vascular endothelial and smooth muscle cells. The *atheroma* is preceded by a fatty streak, an accumulation of lipid-laden cells (foam cells) beneath the endothelium, wherein the most of these cells are macrophages, together with T cells [9].

Once an atheromatous process prevents blood flow through the artery it was believed that an ischemia and infarction's event occurs. However, some recent angiographic studies proved that acute myocardial infarction and stroke do not occur generally by stenosis or progressive luminal narrowing from the continued growth of smooth muscle cells in the plaque, but essentially, by the activation of the plaque and the formation of an occluding thrombus on the surface of these vessels [12].

In this context, atherosclerosis is regarded as an event in chain, which includes a set of key steps that begins with: **1)** an endothelial dysfunction: **2)** the retention and oxidation of LDL in the arterial wall; **3)** an immune response and formation of a fatty streak lesion and **4)** the subsequent rupture of the plaque and the formation of an occlusive thrombus [6, 9-11]. Each step will be explained in more detail in the following subtopics.

1.2.1. Endothelial dysfunction

The endothelium is the major regulator of the vascular wall homeostasis. Endothelium maintains the balance between vasodilation and vasoconstriction; regulates vascular permeability to plasma constituents, platelet and leukocyte adhesion and aggregation; inhibits and stimulates smooth muscle cell proliferation and migration, thrombogenesis, fibrinolysis; and maintains low levels of oxidative stress by releasing mediators such as nitric oxide (NO), prostacyclin (PGI2) and controlling endothelin (ET-1) and local angiotensin-II activity. Nevertheless, this balanced endothelial regulation can be altered by a number of conditions, causing damage to an arterial wall [13, 14].

In response to a variety of noxious stimuli, the endothelium undergoes phenotypical modulation to a non-adaptive state (endothelial dysfunction), which is characterized by the loss or deregulation of the homeostatic mechanisms (balance between vasoconstriction and vasodilation mediators) that operate in healthy endothelial cells. This pathophysiological condition (Figure 2) is associated with the increased of the endothelial permeability, an increased expression of adhesion molecules, platelet aggregation, the synthesis of pro-inflammatory and pro-thrombotic factors, the increased of the oxidative stress, and the abnormal modulation of vascular tone by decreased production or activity of NO, in which all of these changes may lead to functional manifestations that includes impaired endothelium dependent vasodilation [13-15].

According with the Figure 2, the CVD risk factors promote an increased production of Reactive Oxygen Species (ROS), which in turn, deregulate the endothelium homeostasis by a decreased production of NO. This is caused by the inactivation of the enzyme endothelial NO synthase (eNOS) and by the formation of ET-1, thromboxane A2 (TxA2) and others contractive molecules such as angiotensin-1 and angiotensin-2. Once produced in significant amount, these molecules trigger an inflammatory response that involves several processes:

- Fibrinolysis is inhibited by the action of plasminogen activator inhibitor (PAI-1);

- Platelet aggregation occurs by the action of TxA2 and von Willebrand factor (vWF) or diminished action of NO and PGL2, which inhibit this process;

- Leukocyte and platelets adhesiveness and migration occur through the action of some adhesion molecules: E-selectin, P-selectin, Intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and platelet-endothelial cell adhesion molecule-1 (PCAM-1);

- Coagulation begins with the endothelium production of tissue factor (TF), inhibition of thrombomodulin (TM) production, and a diminished presence of glycosaminoglycans (GAG) [14-19].

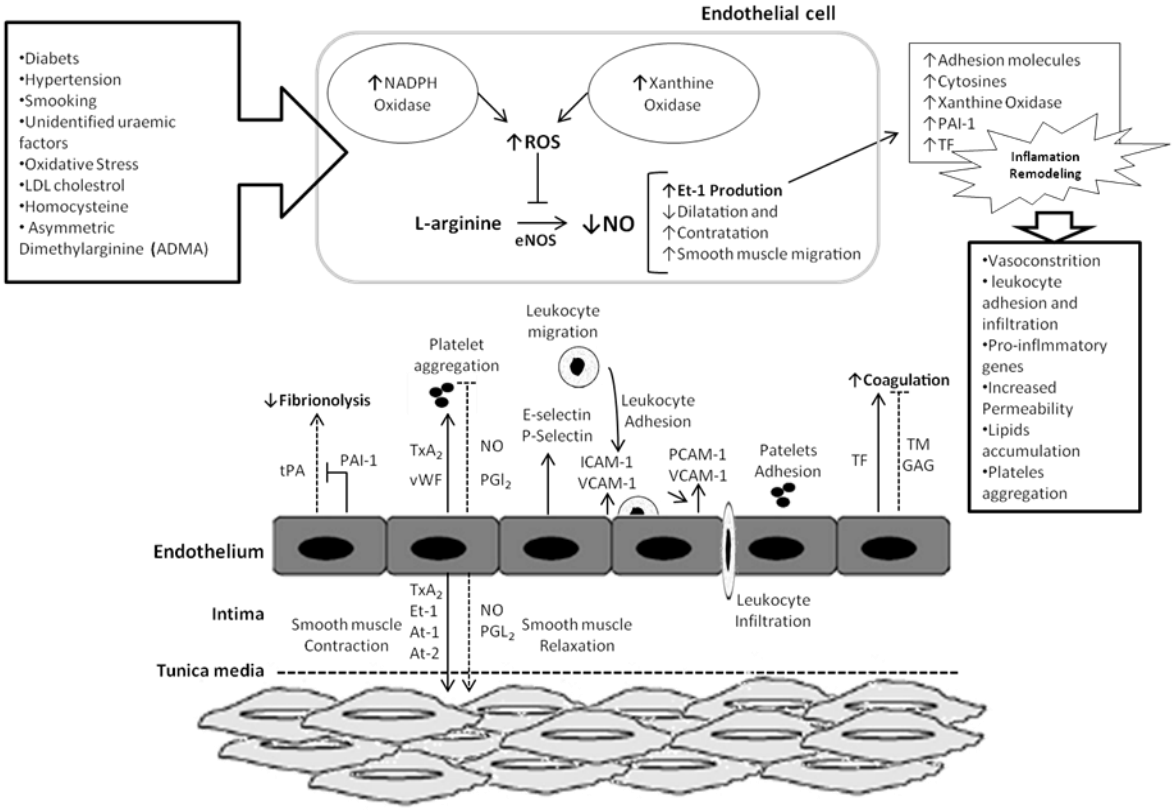


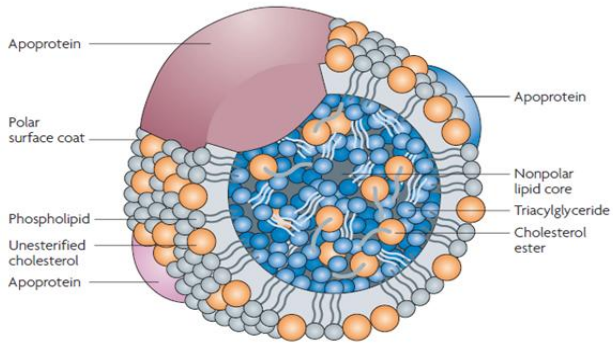
Figure 2 – Endothelium dysfunction mechanism. Legend: ROS| Reactive Oxygen Species; **NO|** Nitric Oxide; **eNOS|** Enzyme NO synthase; **ET-1|** Endothelin-1; **TxA_2|** thromboxane A2; **PAI-1|** Plasminogen activator inhibitor; **vWF|** Von Willebrand factor; **PGL_2|** Prostacyclin; **ICAM-1|** Intercellular adhesion molecule-1; **VCAM-1|** Vascular cell adhesion molecule-1; **PCAM-1|** Platelet-endothelial cell adhesion molecule-1; **TF|** Tissue factor; **TM|** Thrombomodulin; **GAG|** Glycosaminoglycans**.** Image adapted from [14-19].

Current evidence suggests that endothelial dysfunction occurs early in the process of the atherogenesis and contributes to the formation, progression, and complications of atherosclerotic plaque. It is so considered an early marker for atherosclerosis, preceding even angiographic or ultrasonic evidence of an atherosclerotic plaque. Several studies have shown that patients with CVDs risk factors without any clinical signs of atherosclerosis are affected by endothelial dysfunction, through their impaired response to endothelial vasodilators such as acetylcholine and bradykini, suggesting that endothelial dysfunction is a common mechanism between atherosclerotic risk factors and the development of atherosclerosis, and the risk factor score (the total number of risk factor in a given patient) as potent independent predictor of the endothelial dysfunction. Furthermore, some studies have shown that the endothelial dysfunction is an independent predictor of future CVDs event in patients with atherosclerotic risk factors, or stable ischemic heart disease, or acute coronary syndromes. Therefore, endothelial dysfunction seems to be a systemic vascular process that not only mediates the development of an atherosclerotic plaque, but may also modulate its clinical course [13].

1.2.2. LDL retention and oxidation into artery walls and activation of the immune cells

1.2.2.1.Lipoprotein metabolism

The most important plasma lipids are cholesterol and triglyceride (TG). Cholesterol has various roles: it is a component of cell membranes; the precursor for the steroid hormones, vitamin D, oxysterols and bile acids; and it is required for the activation of neuronal signalling molecules. Only a small amount of the circulating cholesterol is originated from the diet and around 80% is derived from endogenous synthesis, which 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) catalyses the rate-limiting step. Most cholesterol in the circulation is esterified, with free cholesterol constituting a minor fraction. TG is a key energy source that is made up of free fatty acids (FFAs) that are ester-linked to a glycerol backbone. It is synthesized in intestinal and liver cells and is then transported through the plasma and, after lipolysis, at the endothelial surface, where delivers FFAs to peripheral cells for β-oxidation or storage. Owing the insolubility of cholesterol and TG in plasma, they are transported in steroidal macromolecules called lipoproteins, which have a hydrophobic core containing phospholipid, fat-soluble antioxidants and vitamins, cholesteryl ester, and a hydrophilic coat that contains free cholesterol, phospholipid and apolipoprotein molecules. Lipoproteins are specifically targeted to cells by distinct apolipoproteins (Apo) on their surface, which enables tissue recognize and take up the lipoprotein particle via specific cell receptors. The main TG-carrying lipoproteins are chylomicron (CM) and very low-density lipoprotein (VLDL). The main cholesterol-carrying lipoproteins are LDL and high-density lipoprotein (HDL). Lipoproteins are distinguished from each other by size, density, electrophoretic mobility, composition and function (Figure 3) [20-22].



Characteristics	Chylomicrons	Very low-density lipoproteins	Low density lipoproteins	High density lipoproteins
Density (g per mL)	< 0.95	0.95–1.006	1.019–1.063	1.063–1.210
Particle diameter (nm)	> 75	30–80	18–25	5–12
Protein composition (% dry weight)	1–2	8–10	20–25	52–60
Triacylglycerol composition (% dry weight)	80–88	45–53	5–9	2–3
Cholesterol composition (% dry weight)	2–4	17–27	43–50	12–25
Phospholipid composition (% dry weight)	7–9	17 – 19	19–21	17–24
Function	Transport of exogenous triacylglycerol and cholesterol	Transport of endogenous triacylglycerol	Cholesterol transport to all tissues	Reverse cholesterol transport

Figure 3 - General Structure and physical proprieties of human plasma lipoproteins. Adapted from Wasan *et al* [22].

Lipoprotein metabolism is a complex network of assembly, secretion, processing and catabolism that involves the transport of lipids, particularly cholesterol and TG in the blood (Figure 4). Briefly, the intestine absorbs dietary fat via free fatty acid (FFA) transporters and packages it into chylomicrons, which are transported to peripheral tissues through the blood. In the capillaries of the muscle and adipose tissue, chylomicrons bind to glycosylphosphatidylinositol-anchored HDL-binding protein 1 (GPIHPB1) and interact with the enzyme lipoprotein lipase (LPL), which it is in turn held by the proteoglycans on the endothelium surface. The enzyme then breaks chylomicrons and FFA enters these tissues. The chylomicron remnants are subsequently taken by the liver. In liver, cholesterol is recycled or synthesized again with HMGCR. Some part of it is esterified with FFAs via cholesterol-acyltransferase to form colesteryl ester, which is then stored together with TG inside the nascent VLDL that contains an apolipoprotein apoB100 in its structure. The nascent VLDL leaves the liver to the periphery. In its course to target-tissues, nascent VLDL becomes a mature VLDL, after receive from HDL the apolipoproteins apoC2 and apoE. Already in the capillaries of the peripherals tissues, the apolipoproteins apoB100 and apoE bind to the VLDL receptors at the endothelial cell, and apoC2 activates the LPL that is still stimulated by insulin, to hydrolyze the triacylglicerol inside de VLDL, producing fatty acids and glycerol. This procedure of deliver leads to the formation of the intermediate-density-lipoprotein (IDL) from VLDL, which is very rich in cholesterol. After, the exclusion of the apoE from IDL by hepatic lipases, LDL is produced, which, in turn, leaves the circulatory system and delivers the cholesterol toward the binding of apoB100 with LDL receptors in liver as well as endothelium cells from peripheral

tissues. By the contrast, HDL mediates the reverse cholesterol transport. It is generated by the intestine and the liver through the secretion of lipid-free apoA-1. It promotes the cellular efflux of cholesterol from tissues through the interaction of apoA-1 with ATP-binding cassette A1 (ABCA1), ATP-binding cassette transporter G1 (ABCG1) and Lecithincholesterol acyltransferase (LCAT), which esterifies cholesterol so it can be used in HDL cholesterol. And it leaves the circulatory system by the hepatocytes via scavenger receptor class B type I (SRB1), through the remodelling by cholesterol ester transfer protein and by endothelial lipase (LIPG) [6, 20-23].

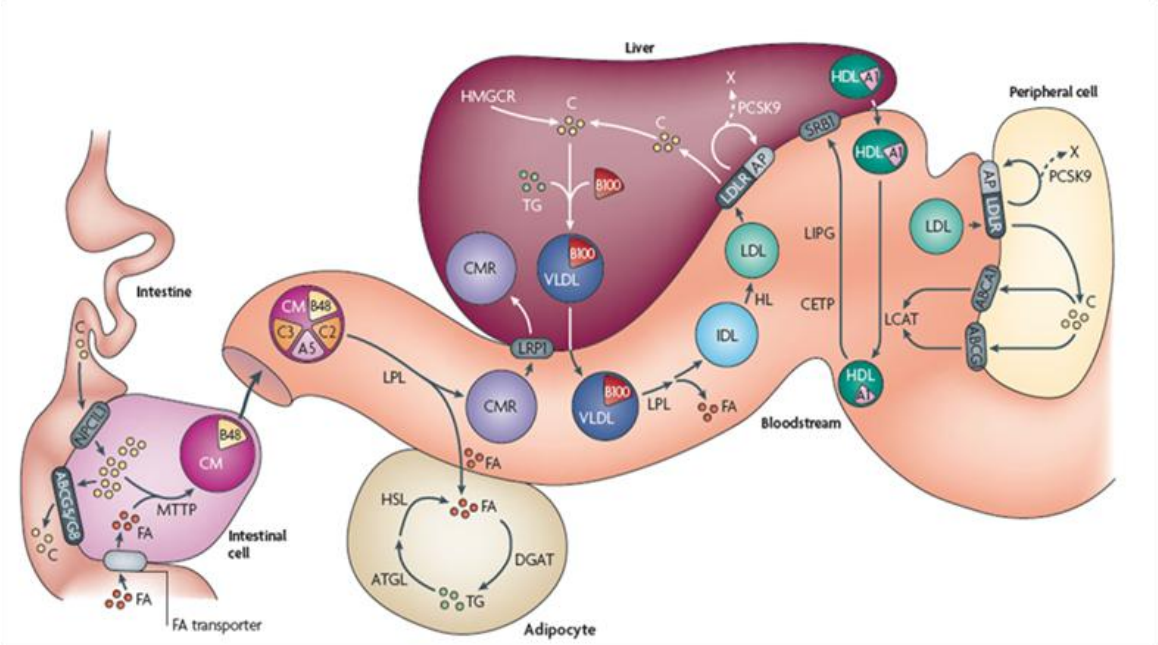


Figure 4 - lipoprotein metabolism. The details regarding of the chylomicrons binding with endothelial cells to release fatty acids, the maturation of the nascent VLDL as well as the modification of the IDL to LDL and the formation of HDL are not shown in this illustration. **Legend: C**| Cholesterol; **NPC1L1**| Niemann-Pick C1-like; **ABCG5/G8**| Heterodimeric ATP-binding cassette transporter G5/G8; **FA**| Fatty acid; **MTTP**| Microsomal TG-transfer protein; **CM**| Chylomicron; **B48**| Apolipoprotein B48; **C2**| Apolipoprotein C2; **A5**| Apolipoprotein A5; **C3**| Apolipoprotein C3; **LPL**| Lipoprotein lipase; **DGAT**| Acyl-CoA:diacylglycerol acyltransferase; **ATGL**| Adipose triglyceride lipase; **HSL**| Hormone sensitive lipase; **CMR**| Chylomicron remnants; **LRP1**| LDL Receptor –related protein -1; **HMGCR**| 3-Hydroxy-3-methylglutaryl coenzyme A reductase; **B100**| Apolipoprotein B100; **VLDL**| Very-low-density-lipoprotein; **IDL**| Intermediary-density-lipoprotein; **HL**| Hepatic lipase; **LDL**| Low-density-lipoprotein; **LDLR**| LDL receptor; **AP**| Adaptor protein; **PCSK9**| Proprotein convertase subtilisin/kexin type 9; **X**| Degradation; **HDL**| High-density-lipoprotein; **A1**| Apolipoprotein A1; **ABCA1**| ATP-binding cassette A1; **ABGC**| ATP-binding cassette transporter G1; **LCAT**| Lecithincholesterol acyltransferase; **CETP**| Cholesterol ester transfer protein; **LIPG**| endothelial lipase; **SRB1**| Scavenger receptor class B type I. Image obtained from Rader *et al* [6].

1.2.2.2. LDL and the beginning of an atherosclerotic lesion

As discussed previously, atherosclerotic lesion develops from an increase accumulation of LDL particles at the connective tissue basement membrane that constitutes the intima layer, due an endothelium injury. Since cardiovascular risk factors, especially hypercholesterolemia, can promote an endothelial dysfunction and a consequent increase of permeability, blood LDL molecules, as they are the smallest lipoproteins (18-25 nm), can easily infiltrate the endothelium of an artery and reach the subendothelial space in significantly amount. Once there, LDL can be trapped due an undesired bond between apoB100 with some proteoglycans that constitute the extracellular matrix (ECM) [24].

The first determinants steps for an atherosclerotic event are the rate of LDL entry and exit from the *intima* layer to the plasma, through the endothelium. Once in the *intima*, the particles may continue into the muscular media and may be processed by cells or may be sequestered by interactions with ECM. However, in normal endothelium, neither apoB100 lipoproteins nor their lipids accumulate with time in the normal *intima*. It seems that in normal conditions, most particles return to plasma from the fluid phase, and those that are associated with the matrix are taken by cells and degraded *in situ* [25].

The proteoglycans are all composed of a core protein and one or more covalently attached GAGs, which are linear polysaccharides consisting of repeating disaccharide units (heparin sulphate, chondroitin sulphate, dermatan sulphate and keratin sulphate). There are five types of proteoglycans: biglycan, decorin, perlecan, versican and syndecan-4. Proteoglycans are negatively charged due to the acidic sugar groups on the GAG polysaccharide backbone and the addition of negatively charged sulphate groups. ApoB100 binds to negatively charged GAG chains on proteoglycans (Figure 5). Chondroitin sulphate proteoglycans, such as versican, are the main structural proteoglycans of the ECM and are considered important atherogenic elements [24, 26].

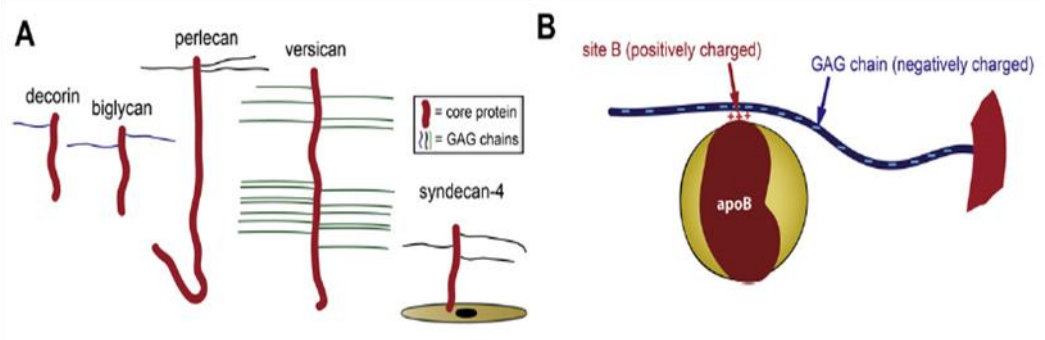


Figure 5 - Proteoglycans structure and interaction with LDL. A| The most common types of proteoglycans in an arterial wall. **B|** The LDL – proteoglycan interaction. Clusters of positively charged amino acids on apolipoprotein B bind to negatively charged glycosaminoglycan (GAG) chains on proteoglycans. Image adapted from Fogelstrand *et al* [24].

Lipid oxidation induces disturbance of the fine structures, alteration of integrity, and permebility, and function loss of biomembranes, modifies LDL to pro-atherogenic and pro-inflammatory forms, and generates potentially toxic (mutagenic and carciongenic) products [27]. The oxidation process followed of an activation of immune cells goes after the binding of the LDL with proteoglycans. LDL particles trapped in ECM are susceptible to undergo modification processes (oxidized LDL and aggregated LDL particles) when they interact with ECM components, because of the action of certain oxidants (lipoxygenases, myeloperoxidase, free radicals, etc.) and/or because of proteolytic enzymes (kinase, tryptase, metalloproteinases, thrombin, etc.) lipolytic enzymes (sphingomyelinase, phospholipase A2, phospholipase C, etc.) and hydrolytic enzymes (esterases), and then they trigger the expression of adhesion molecules and the secretion of chemokines by endothelial cells, which, together with the deposition of platelet-derived chemokine, drive intimal immune cell infiltration [24, 26, 28]. Lipid peroxidation on LDL molecules occurs, either by enzymatic processes or by free radical species, as a chain mechanism, which an initiator oxidize many molecules of lipids, changing their original form. The Figure 6 shows an example of lipid modification by free radicals [27] .

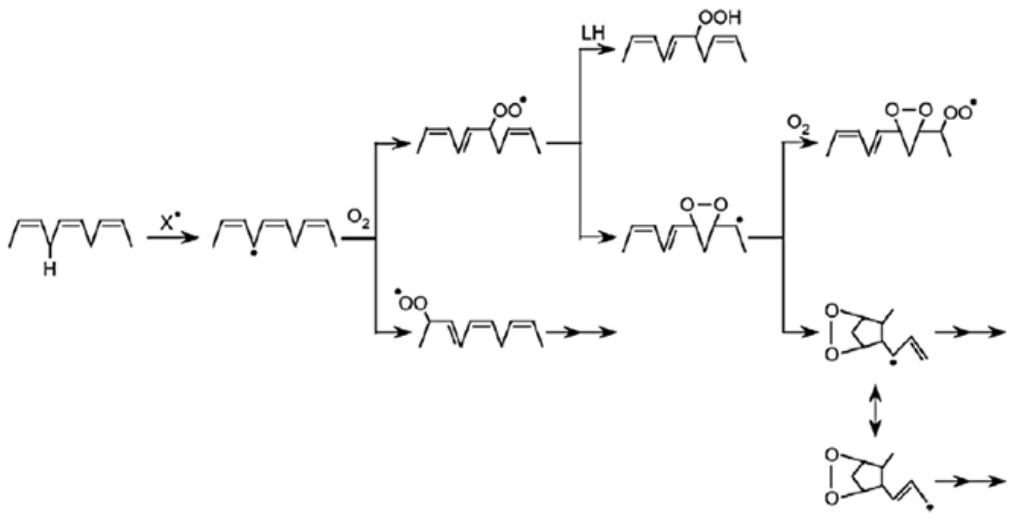


Figure 6 – Pathways of free radical-mediated lipid peroxidation. Legend: X•| Free radical specie**; LH**| Polyunsatured lipids. Image obtained from Niki *et al* [27].

LDL undergoes various degrees of oxidation and such diversity of LDL oxidation provides different atherogenic effects to the vascular cells (Figure 7). Oxidative modifications of LDL can occur in the absence of changes or little changes in apoB100. Such modified LDL is called minimally oxidized LDL (mmLDL), which retains the affinity to the LDL receptor. It has a little negative charge, activates anti-apoptotic signalling, and induces inflammatory changes with increased chemokines and cytokines. On the other hand, the recruitment of inflammatory cells results in a huge variety of enhanced cytokines and the continued oxidation of LDL.

Consequently, LDL constituents are further oxidized and the LDL protein constituents are also modified, leading to a loss of recognition by the LDL receptor and a shift to recognition by scavenger receptors (oxidized LDL receptors), leading to the development of macrophage foam cells [29]. According with the Figure 7, LDL that enters in the artery may be oxidized by some the action of certain oxidants. Minimally oxidized LDL stimulates adhesion molecules and chemokines but has low affinity to macrophages scavenger receptors, and thus, can be recycled into blood circulation and can be detected as a serum oxidized LDL. Extensively oxidized LDL can be taken up by macrophages through the scavenger receptor, leading to the formation of foam cells. However, either extensively or minimally oxidized LDL enhance macrophages scavenger receptors with various modulations of cytokines [29].

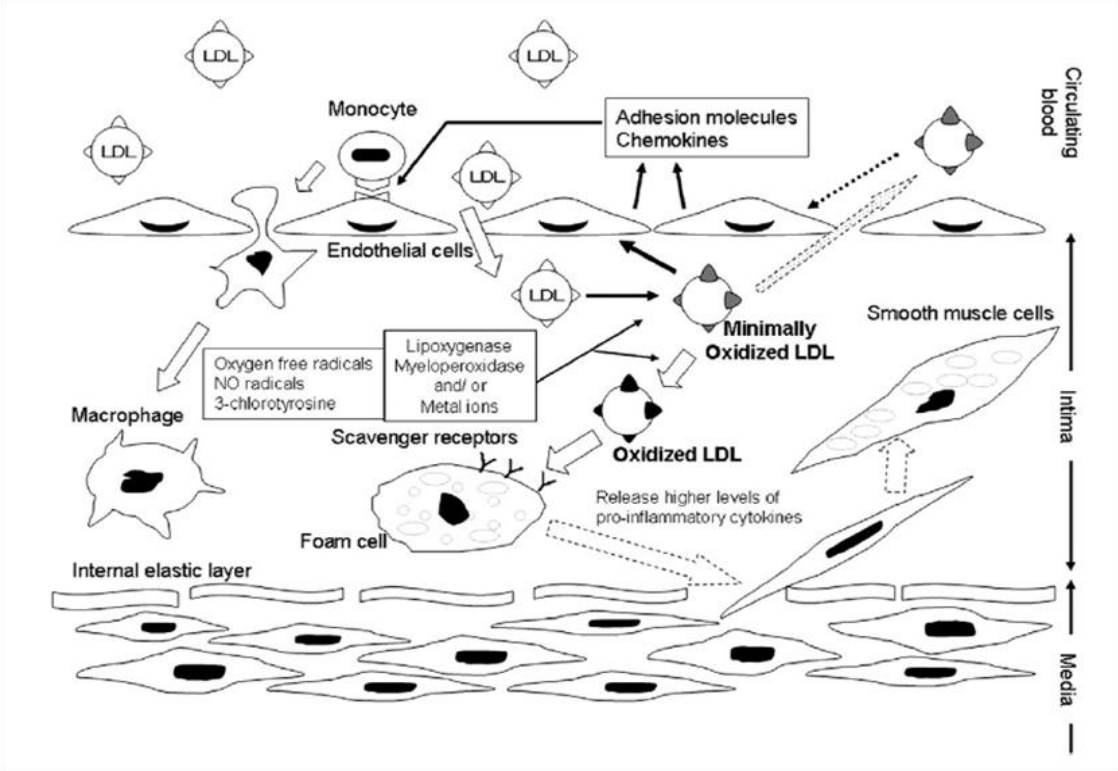


Figure 7 - Mechanism of LDL oxidation and atherogenic effects. Image obtained from Yoshida *et al* [29].

1.2.3. Inflammatory response, formation of a fatty streak lesion and lesion progression

A critical step for the development of atherosclerotic lesions is the infiltration of circulating leukocytes into the intravascular space (Figure 8). The recruitment of mononuclear cells (monocytes and T lymphocytes) as a specialized inflammatory response to modified LDL exposure characterizes the initiation phase of the formation of an atherosclerotic lesion. Specific

adhesion molecules such as vWF, the selectins, and VCAM-1, expressed on the surface of the activated vascular endothelial cells, mediate the leukocyte adhesion. Once adhered, the mononuclear cells enter into the artery wall guided by the chemoattractant chemokines such as monocytes chemoattractant protein-1 (MCP-1). When circulating peripheral monocytes migrate from the vascular to the extravascular space, occurs a parallel process that involves the maturation of these cells to macrophages. This differentiation process provides the cells ready for active participation in the inflammatory and immune response, and it is in turn amplified, by macrophage colony stimulating factor (MCSF), by lipopolyssacharide (LPS) via receptor CD14 in conjugation with toll-like receptor 4 (TLR4), by the heat stock protein (HSP-60) via CD14, and through platelet activating factor (PAF) and cytokines released from macrophages in an autocrine loop [30].

According with the Figure 8, hemodynamics forces play a major role in the determination of the locals of the lesion predilection. These forces, aggravated by cardiovascular risk factors, increase the endothelial permeability and consequently the entrance of LDL into the *intima*. Consequently, LDL is retained as a result of the interaction with the matrix components and oxidized by the interaction with ROS and/or others oxidant agents. This process stimulates the endothelial cells to produce several molecules, such as MCSF and MCP-1, and express variety of cellular adhesion molecules including ICAM-1 and VCAM-1, which participate in the recruitment of monocytes into the vessel wall. Likewise, oxidized LDL decreases the bioavailability of NO. Activated monocytes and T cell express important adhesion molecules, including integrins (VLA4 and β2) and PCAM-1 that binds to the endothelial surface. Within the vessel wall, MCSF stimulates the proliferation and differentiation of macrophages, and together with tumour necrosis factor-α (TNF-α) and interferon-ϒ (INF-ϒ) promote the expression of several scavenger receptor (for instance CD36, CD38 and scavenger receptor A [SR-A]). Those scavenger receptors recognize the highly oxidized and aggregated LDL, formed by the action of the ROS and some enzymes lipase and myeloperoxidase (MPO). This fact allows the rapid uptake of LDL particles by macrophages leading to the formation of foam cells. Foam cells will die, forming a mass of extracellular lipids and other cell debris within the vessel wall [31].

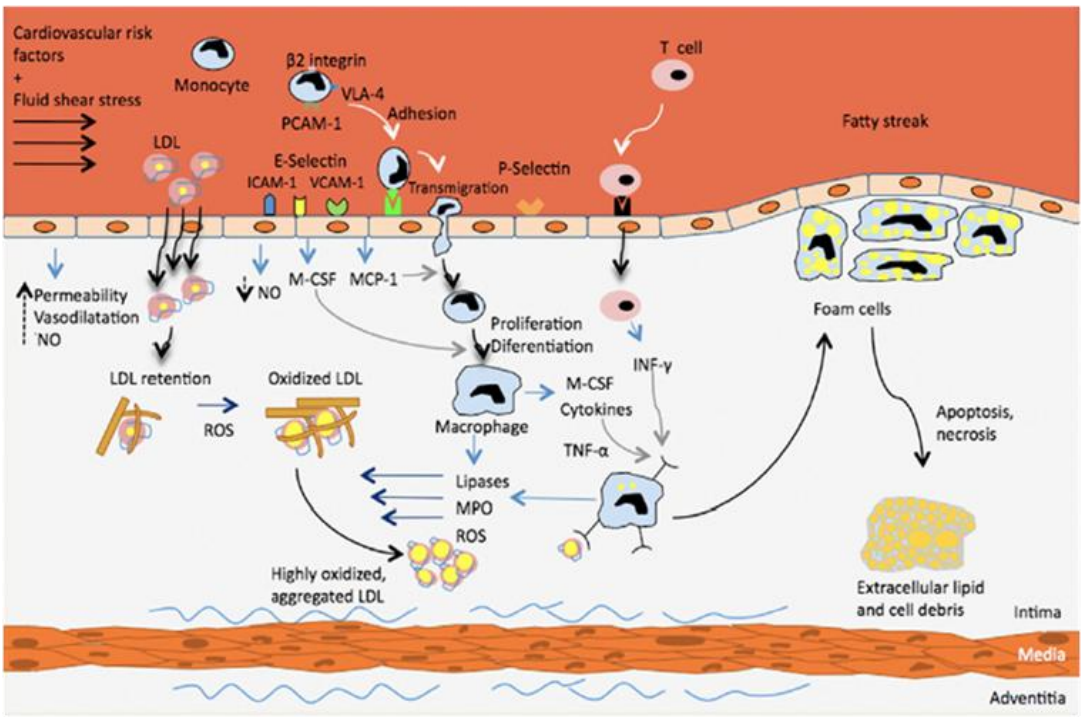


Figure 8 - Atherosclerotic process from the lesion formation to the foam cell formation. **Legend: MCSF**| Macrophage colony-stimulating factor; **MCP-1**| Monocytes chemotactic protein-1; **VLA4 and β2**| Integrins; **TNF-α**| Tumour necrosis factor-α; **INF-ϒ**| interferon-ϒ; **MPO** myeloperoxidase. Image obtained from Ribeiro *et al* [31].

Macrophages use scavenger receptors like CD36 and scavenger receptor type A (SR-A) to recognize and phagocyte modified and aggregated LDL. Oxidized LDL consist of free cholesterol as well as cholesterol esters that are hydrolyzed in lysossomes. Free cholesterol has a number of potential metabolic fates, including esterification by acylCoA: cholesterol transferase A1 (ACAT-1) and storage in the lipid droplets that characterize foam cells. Macrophages are normally protected from the accumulation of toxic cholesterol loads by multiple mechanisms, notably the down-regulation of surface LDL receptor and inhibition of proteolytic activation of the sterol regulatory element-binding protein (SREBP) transcription factors required for cholesterol biosynthesis. However, oxidized or otherwise chemically modified LDL can be taken up by alternate "scavenger" or "oxidized LDL" receptors that are not similarly suppressed when the cholesterol load is in excess, leading to a continuous flux of the LDL and an continuous accumulation of it in the cells, turning them, thereby, a foam cell and drive an inflammatory gene expression in macrophages. Once activated, macrophages secrete a number of inflammatory mediators, such as several cytokines such as TNF-α, some interleukins (IL), as IL-12, IL-6, IL-1β, leukotrienes and several chemokines such as MCP-1, IL-8 and macrophage inflammatory protein 3-α (MIP-3α) that amplify inflammation in the vessel wall and contribute to additional recruitment of monocytes, neutrophils and other inflammatory cells. In addition to the production of inflammatory mediators, macrophages

activation results on the induction of several bactericidal systems such as the Nicotinamide Adenine dinucleotide (NADPH) oxidase enzymes that can damage host tissue due the capacity of superoxide anion and other free radicals produced, to cause the deoxyribonucleic acid (DNA) degradation and inactivation of metabolic enzymes; the release NO which combined with superoxide, generate peroxynitrite that causes cell injury; the generation of reactive nitrogen species (RNS) from MPO that contributes to the conversion of LDL to an atherogenic form; expression of nonspecific esterase, lysossomal hydrolases, ectoenzymes; the secretion of an array of cathepsins and matrix metalloproteinases (MMPs) [30, 32].

Besides the macrophage activation in the atherosclerotic plaques, there is the impairment of the macrophage functions, which is critical for the control and resolution of the inflammation. An important function of macrophages under both resting and inflammatory condition is the rapid uptake of apoptotic cells from tissues, termed efferocytosis, whose is mediated by a range of receptors such as CD36 and cell membrane bound tyrosine Kinase. Nevertheless, in an atherosclerotic lesion a chronic apoptosis of lipid-filled foam cells combined with defective efferocytosis contributes for the formation of the necrotic core and the progression of the atherosclerotic lesion [32].

The transition from a relatively simple fatty streak to a more complex lesion (mature plaque) is characterized by the migration of smooth muscle cells from the media layer (*tunica media)* of the artery wall into the *intima* layer. Intimal smooth muscle cells proliferate and takes modified lipoproteins, contributing to the foam cell formation, and the synthesis of ECM that lead to the development of a fibrous cap [10].

The development of a fibrous cap can be observed in the Figure 9. According with the same figure, the interactions between macrophages foam cells with lymphocytes (Th1 and Th2) establish a chronic inflammatory process. Cytokines secreted by lymphocytes and macrophages exert both pro- and anti-atherogenic effects on each of the cellular elements of the vessel wall. Smooth muscle cells migrate from the media portion of the arterial wall, proliferate and secrete extracellular matrix proteins that form a fibrous plaque [10].

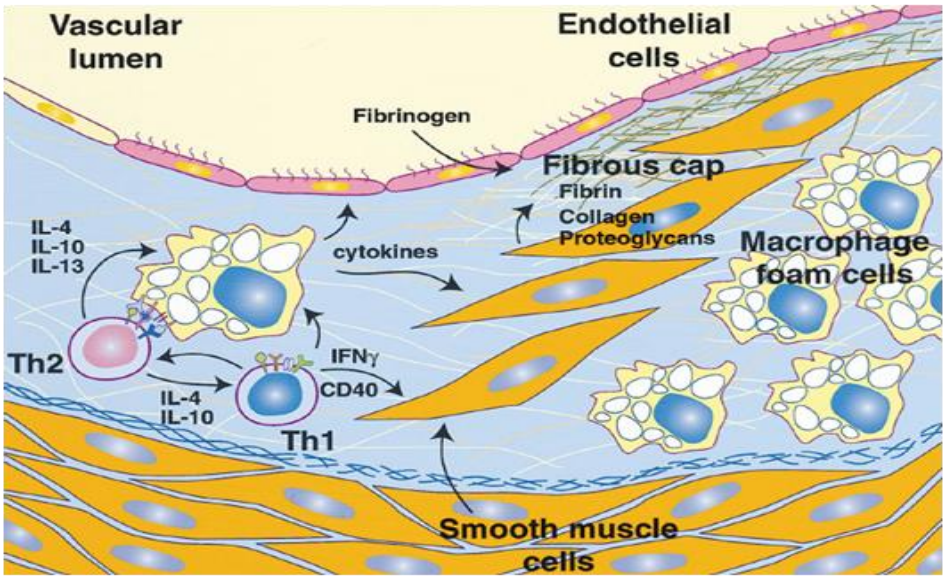


Figure 9 - Progression of an atherosclerotic lesion and the formation of a fibrous cap. Legend: IL| Interleukin; **Th1 and Th2|**Lymphocytes; **CD40|** Scavenger receptor. Image obtained from Glass *et al* [10].

Mature plaques, also known as *atheromas*, have a more complex structure than fatty streaks (Figure 10). In the centre of a plaque, foam cells and extracellular lipid droplets form a core region that is surrounded by a cap of smooth muscle cells and a collagen rich matrix. Other cell types present include dendritic cells (DCs), mast cells, a few B cells and natural killer T (NKT) cells. The shoulder region of the plaque, which is where it grows, and the interface between the cap and the core, has a particularly abundant accumulation of T cells and macrophages. Many of these immune cells show signs of activation and produce pro-inflammatory molecules. With time, the plaque can progress into an even more complex lesion (unstable plaque), which the thinning of the plaque followed a disruption of the cap can lead to a thrombosis event and many of the adverse clinical outcomes associated with atherosclerosis [33].

According with the Figure 10, the atherosclerotic plaque has a core containing lipids (esterified cholesterol and cholesterol crystals) and debris from dead cells. Surrounding it, a fibrous cap containing smooth muscle cells and collagen fibres stabilizes the plaque. Immune cells including macrophages, T cells and mast cells populate the plaque, and are frequently on an activated state. They produce cytokines, proteases, pro-thrombotic molecules and vasoactive substances, which all can affect the plaque inflammation and vascular function. Until complication occurs, an intact endothelium covers the plaque [33].

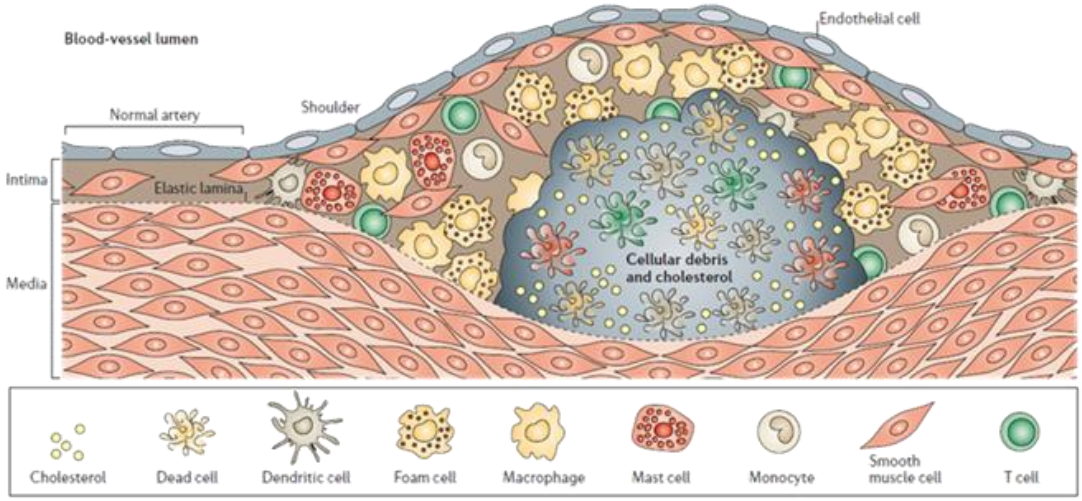


Figure 10 - Cellular composition of an atherosclerotic plaque. Image obtained from Hansson *et al* [33].

1.2.4. Plaque rupture and formation of an occlusive thrombus

As discussed previously, although atherosclerotic lesions can lead to ischemic symptoms due the progressive narrowing of the vessel, acute cardiovascular events that result in myocardial infarction and stroke generally result from a plaque rupture and a thrombosis event [10]. According with some studies, the plaque rupture occurs in approximately 75% of fatal cardiac events [34].

The transition from a stable plaque to an unstable plaque involves the rupture. The vulnerable atherosclerotic plaque has also been called a "high-risk" or "thrombosis-prone" plaque. These plaques typically are composed by a thin inflamed fibrous cap covering a lipid-rich necrotic core and a highly thrombogenic material rich in tissue factor, one of the most potent pro-coagulants. Once this fibrous cap is the only barrier separating the circulation, the thickness and the integrity of the fibrous cap overlying the lipid-rich core is a principal factor in the stability of the plaque. Plaques prone to rupture can be characterized as having thinner fibrous caps, especially in the shoulder region, increased number of inflammatory cells, and a relative small number of smooth muscle cells. Plaque instability, erosion and consequent rupture occur due a degradation rate higher than a regeneration rate, which it owes to an insufficient production of ECM proteins due to the paucity of smooth muscles cells in the *atheroma* and an overproduction of MMPs through the activated cells. Plaque rupture exposes plaque lipids and tissue to the blood, initiating the coagulation cascade, platelets adherence, and thrombosis (Figure 11) [34, 35].

According with the Figure 11, necrosis of some macrophages and smooth muscle cells-derived foam cells leads to the formation of a necrotic core and an accumulation of extracellular cholesterol. Immune cells including macrophages, T cells and mast cells when activated can release pro-inflammatory cytokines, which reduce collagen formation and induce the expression of tissue factor. Proteases that attack the collagenous cap are also released by activated immune cells. The weakened plaque might fissure when subjected to the forces of arterial blood pressure (hemodynamic forces). Exposure of sub-endothelial structures and pro-coagulants such as tissue factor, promotes platelets aggregation and thrombosis. A thrombus forms and might occlude the lumen of the artery, leading to the acute ischemia [33].

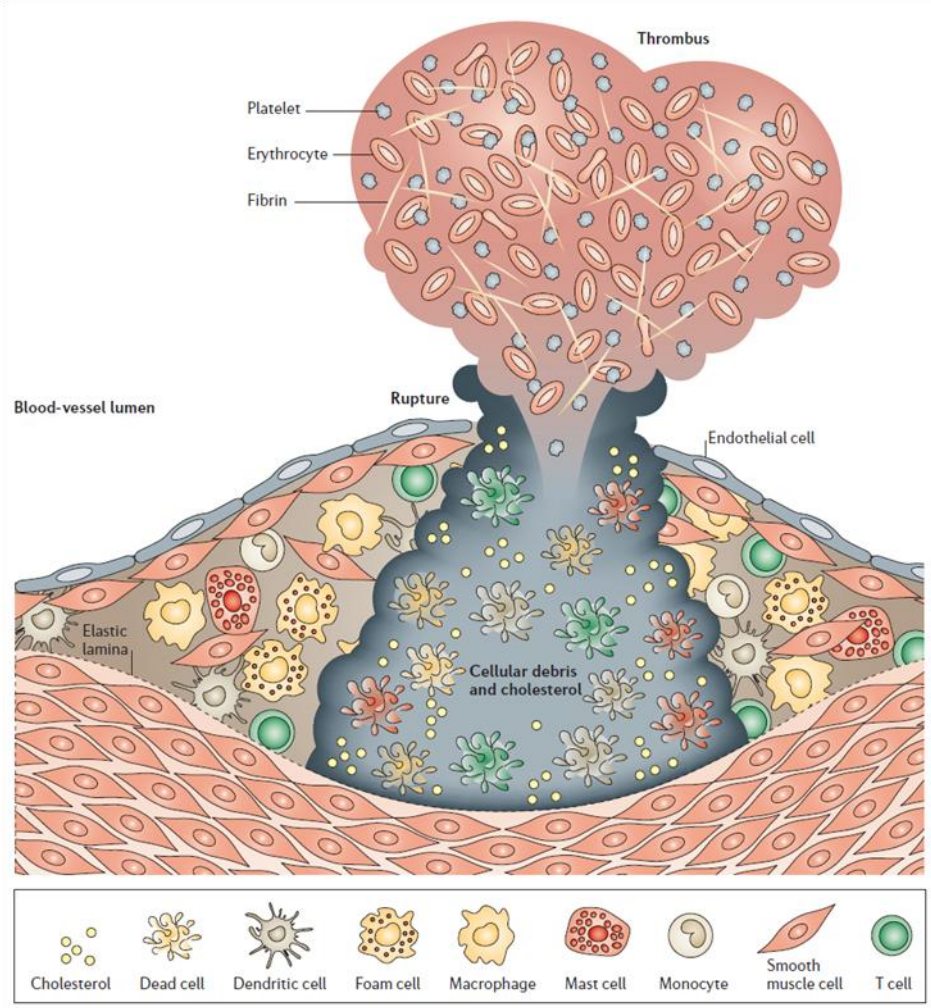


Figure 11 - Plaque rupture and thrombosis. Image obtained from Hansson *et al* [33].

1.3. Homocysteine

Homocysteine (Hcys) is strongly correlated with the onset of some diseases, namely atherosclerosis. The first abnormality in the Hcys metabolism was described in 1962 in mentally retard children and associated to frequent thromboembolic events. A few years later, McCully (1969) described the main feature of this vascular pathology, denoted homocystinuria. Later, Wilken and Wilcken mentioned, for the first time in 1976, the frequent association between abnormal plasma Hcys levels and CHD [36].

It is estimated that up to 40% of the population at risk for CVDs has elevated levels of Hcys. Elevated plasma levels of Hcys lead to atherosclerosis either by directly affecting lipid metabolism and transport or via oxidative damage. Hcys is a non- dietary sulphur-containing amino acid (Figure 12), which is not incorporated into proteins, but exclusively formed as an intermediary product of the methionine (Met) metabolism. It is produced during the conversion of the methionine, an essential dietary amino acid, to glutathione, a powerful antioxidant used by our organism to cope with oxidative stress [37-41].

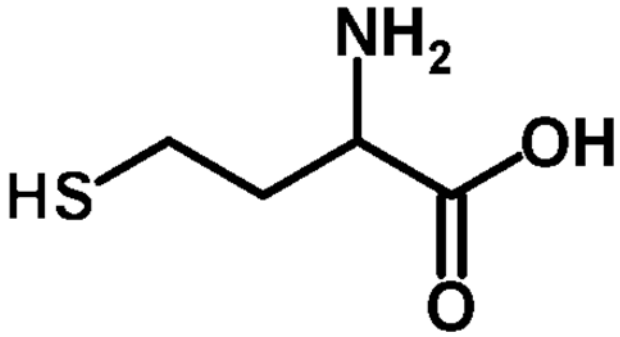


Figure 12 – **Chemical structure of Hcys.** Image obtained from Royal Society of Chemistry [41].

Through the action of methionine adenosyltransferase, methionine is converted to S-adenosylmethionine (SAM) that is the major biological methyl donor required for numerous cellular processes, including the methylation of nucleic acids, proteins (formation of creatinine), polysaccharides and phospholipids. These reactions are catalyzed by various methyltransferases that demethylate SAM to S-adenosylhomocysteine (SAH), which is the immediate precursor of Hcys [40, 42]. The hydrolysis of the SAH by the SAH hydrolase leads to the formation of homocysteine and adenosine [43]. Once Hcys is generated, it may be recovered to Met by methylation, or degraded to cysteine by transsulphuration. Remethylation to Met is catalysed in most tissues by the ubiquitous enzyme, methionine synthase (MetS), which uses vitamin B_{12}, as cofactor, and 5-methyltetrahydrofolate, a derivate compound of folate (vitamin B_9), as a methyl

donor. Met can also be formed from Hcys by betaine-homocysteine methyltransferase and using betaine as a methyl donor. However, this reaction is probably confined to the liver and possibly to the kidney. About the transsulphuration, two vitamin B_6-dependent enzymes are involved. The enzyme cysthathionine β-synthase (CBS) first condenses Hcys with serine to form cystathionine, which is then cleaved into cysteine and α-ketobutyrate by cystathionine ϒ-lyase (CL). Cysteine may be utilized in the synthesis of proteins or as a precursor of the antioxidant glutathione [40]. Changes in the concentration of Met in the body, particularly as a result of its dietary intake, affect the rate of SAM synthesis, as well as the metabolism of Hcys [43]. A simplified overview of this complex metabolic pathway can be observed at the Figure 13. According to it, Hcys is remethylated into methionine using 5-methylhydrofolate (5meTHF) as a methyl donor and vitamin B12 as a cofactor. Met is transformed into SAM, a key intermediate in one-carbon metabolism. Hcys is removed from this cycle in a B6-dependent process leading to production of cysteine. Cysteine can be catabolised or incorporated into proteins or used to the formation of glutathione [42].

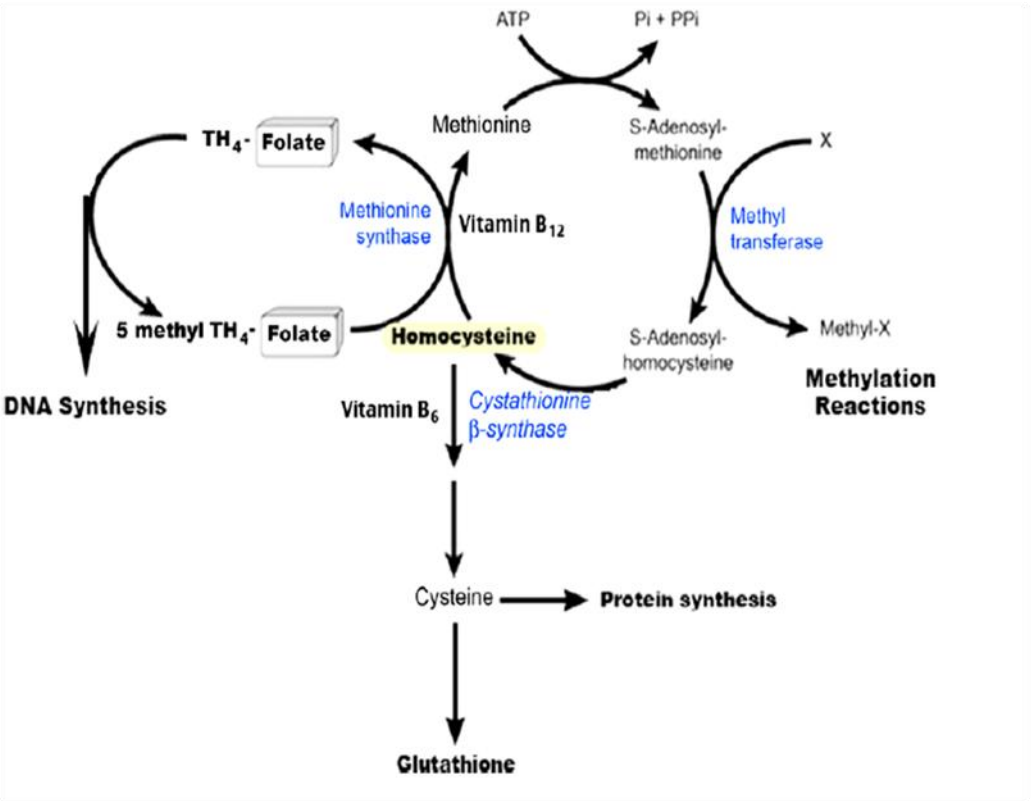


Figure 13 – Homocysteine metabolism pathways. Image obtained from Hoffamn [42].

Under normal metabolic circumstances, there is a strict balance between Hcys formation and elimination. Usually about 50% of the Hcys formed is remethylated to Met. When protein or Met intake is in excess, a large proportion is catabolised by the transsulphuration pathway. If

there is an increased formation of Hcys relative to its consumption, Hcys is excreted from the cells, and this can be detected as an increased level of Hcys in plasma/serum or, even in the urine [40].

Hcys concentration, in plasma or serum, normally, is about 5-10 μmol/L, because only a little amount (~1%) is in reduced form whereas the most part is oxidized and exists as various disulphides (Figure 14). In these dimmers, about 20% appears to be in its free form while the most part (80%) is bound to plasmatic proteins, mainly albumin. However, under abnormal situations, the Hcys metabolism becomes deregulated, total Hcys levels starts to rise and the reduced form starts to be predominant [40].

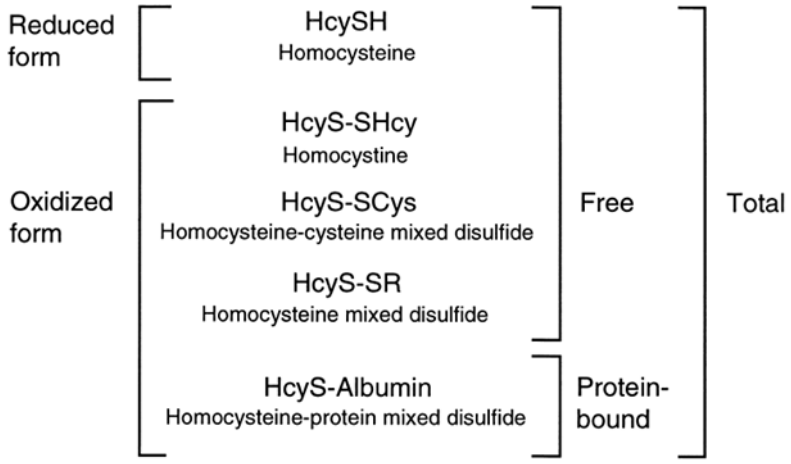


Figure 14 – Various forms of Homocysteine in blood. Image obtained from Nygard *et al* [40].

An elevated level of blood Hcys concentration, or hyperhomocysteinemia (HHcys), is divided into three categories: mild (15 - 30 μmol/L), intermediate (30 - 100 μmol/L) and severe (> 100 μmol/L) [36].

1.3.1. Causes of hyperhomocysteinemia

There are many determinants causes for an elevated blood level of Hcys in humans:

A. Genetic determinants:

Although recently more than fifteen different genes are under investigation for their relationship to plasma total Hcys levels, there are three genes whose alterations or deficiencies

have being already associated with HHcys. These genes code for the enzymes CBS, MetS and Methylenetetrahydrolate reductase (MTHFR) [37].

CBS deficiency, an autosomal recessive disorder, is the most common cause of homocystinuria. Individuals heterozygous for CBS deficiency (between 1 in 200 in the general population) have normal Hcys levels in 30 to 50% of cases [37]. Remain patients with CBS deficiency have extremely high levels of Hcys in the plasma (300-400 μmol/L) and in the urine. Their clinical condition can involve the displacement or malposition of the crystalline lens of the eye *(ectopia lentis*), osteoporosis, skeletal anomalies, mental retardation and a high incidence of premature vascular episodes (arterial and venous thrombosis and premature atherosclerosis) [40, 44].

The most common form of *genetic* HHcys results from the production of a thermolabile variant of MTHFR with an enzymatic activity around 50% (C677T mutation). Homozygosity for this mutant enzyme is present in 9% to 17% of the population, and heterozygosity can be detected in 30% to 41% of the general population. However, there is a substantial interethnic variation. In the population of African descent, Asian Indians or Canadian Inuit, the prevalence for homozygous is 0-2%, whereas it may be about 20% in Asians and even higher in Northern Italy. This difference may partly explain by the variable Hcys levels according to ethnicity. Remarkably, in homozygous individuals, the autosomal recessive mutation in MTHFR provokes a moderate HHcys because the mutated enzyme has a reduced binding to their substrate folate. Folate deficiency could be partially responsible for the expression of the MTHFR thermolabile genotype, as it favours the undesired incorporation of uracil instead thymine into the human DNA [45]. On the other hand , if there is a *genetic* MTHRF suppression, severe HHcys occurs and all the problems associated with that, such as thromboembolic vascular disease even in childhood [37, 40].

Five known mutations affect methylcobalamin synthesis, an essential cofactor of MetS. These mutations induce a functional deficiency in MetS that leads to intermediate HHcys and hypomethioninemia (low levels of methionine). The functional deficiency depends on the mutations in the MetS gene or in the gene encoding MetS reductase, an enzyme involved in the reductive activation of MetS. This gene shows a common polymorphic form (276G) that changes the crucial binding site of the coenzyme (Vitamin B_{12}) and therefore might influence in the secondary structure with a possible reduced functional activity. This mutation is rare and is associated with intermediate and severe HHcys [37].

B. Physiological determinants:

HHcys levels are influenced by gender, being higher in men than in women. The difference between the genders becomes apparent in the puberty and is believed to be related to hormonal factors, but also to the lifestyle, diet and vitamin status. It is also believed that Hcys is higher is men than woman due muscular physiology, as creatine/creatinine synthesis is obtained from SAM methylation with the formation of Hcys [40]. Nygard *et al* (1998) demonstrated that Hcys levels are about 1.65 times higher in men than in women [46].

The total Hcys concentration decreases in normal pregnancies but higher levels are observed in pregnancies complicated by recurrent spontaneous abortions or placental coating separation from the uterus [40].

The levels of Hcys are strongly related to the renal function. The renal function plays a central role for the clearance of Hcys, and because the urinary excretion of Hcys is low, Guldener (2006) suggested that an extensive metabolism of Hcys, probably the transsulfuration, takes place in the kidney [47]. The normal physiological decline in the renal function by age and may to some extent explain the increase in Hcys with age [40].

C. Nutritional determinants:

Dietary intakes and plasma levels of folate and vitamin B_{12} are inversely related to the total Hcys concentration. Deficiencies of these vitamins may cause a moderate or even severe HHcys. Therefore, an increased total Hcys level is a sensitive marker of the disturbed function of both folate and vitamin B_{12} metabolism. The association between vitamin B_6 deficiency and HHcys is, however, less clear, pointing to an inverse correlation.

D. Lifestyle:

Some studies analyzed the influence of various aspects of lifestyle on the Hcys levels, as smoking, coffee and alcohol consumption. It was observed that smoking and heavy coffee consumption are associated with elevated Hcys levels, whereas physical activity is associated with low Hcys [39, 48]. It was also detected that moderate alcohol consumption may be associated with reduced Hcys levels, whereas a chronic high alcohol consumption is linked to elevated Hcys, possible via impaired folate or vitamin B_6 function [39]. According with Dwivedi, *et al* (2011), Hcys levels are about 1.91 times higher in heavy smokers (>20 cigarettes per day) than in non-smokers. Likewise, Hcys levels were also elevated two-fold in high alcohol consumers (>1.5g/Kg/day) compared to non-consumers [37].

E. Drugs:

A number of drugs influence Hcys levels by interfering with its metabolism. Folate antagonists (as methotrexate) and vitamin B_{12} antagonists (as NO), may lead to a noticeable Hcys elevation. The utilization of some immunosuppressive drugs (as cyclosporine), causes a moderate increase in Hcys levels. Antidiabetic drugs (as metformin), may elevate Hcys by affecting folate as well as vitamin B_{12} levels. Anticonvulsant drugs used in the epileptic treatment, cause elevated Hcys levels by interfering with folate polyglutamation and retention. Hypolipidemic drugs, such as colestipol niacin and cholestyramine, may elevate Hcys by a folate-antagonistic effect. Theofylin and azaribine also increase Hcys levels through the inhibition of vitamin B_6 function. Markedly, azaribine, a drug previously used in the psoriasis treatment, was related to an increased incidence of thromboembolism, being prohibited by the Food and Drug Administration (FDA) in 1976. Some anti-Parkinson drugs, such as L-dopa, becomes methylated by SAM and may therefore to increase the levels of Hcys by enhancement of its production. Several hormones-related drugs may influence the Hcys levels. In women, Hcys levels are lowered by some anti-hormones (tamoxifen), by hormone replacement therapy and some oral contraceptives, and increased by the androgen administration. In men, oestrogen plus antiandrogen administration has a substantial effect on Hcys lowering. Aminothiols such as penicillamin, acetylcysteine, and ifosfamide (associated with the use of mesna, which is a drug that is used with to prevent an adverse reaction of ifosfamide called haemorrhagic cystitis or hemorrhagic inflammation of the bladder) reduce Hcys levels probably by increasing renal clearance or by displacing Hcys from the protein binding sites. Such drugs have been suggested for the treatment of homocystinuria [40].

F. Diseases:

Some pathological conditions are associated with elevated Hcys levels. This can usually be explained by low vitamin status, impaired enzyme function and/or renal failure [40]. Hyperproliferative disorders, such as acute lymphoblastic leukemia and severe psoriasis are associated with elevated Hcys levels, probably explained by an increased Hcys export from the proliferating cells [40, 49, 50] . Hypothyroidism is also associated with an increased prevalence of HHcys due the decrease of hepatic levels of enzymes involved in the remethylation [51]. Acute hyperinsulinemia is associated with a decrease in Hcys concentration in normal individuals [52]. In diabetes mellitus, elevated Hcys levels is observed simultaneous with the onset of nephropathy, once the reduced metabolic capacity of the kidney tissue might be rate limiting for renal Hcys clearance [53].

1.3.2. Hcys and atherosclerosis

Apart from the conventional risk factors for CVDs, elevated levels of plasmatic Hcys is now considered to be an independent risk factor for atherosclerosis and thrombosis. Some studies have suggested that HHcys can promote atherosclerosis via oxidative stress by the generation of ROS, and via the cellular impairment through the DNA hypomethylation and the N-homocysteinylation.

1.3.2.1. Hcys and oxidative damage

Oxidative stress induced by HHcys may occur as a result of the decreased expression and/or the activity of key antioxidants enzymes as well as an increased enzymatic generation of superoxide anion (the precursor for multiple ROS and RNS) [54]. Hcys contains a reactive sulfhydryl group (RS-H) and, like most thiols, can undergo oxidation to a disulphide (RS-SR) at physiological pH in the presence of oxygen (O_2) [55]. The general reaction is catalyzed by transition metals, and a variety of ROS can be produced, including superoxide anion radical and hydrogen peroxide (Figure 15) [56, 57].

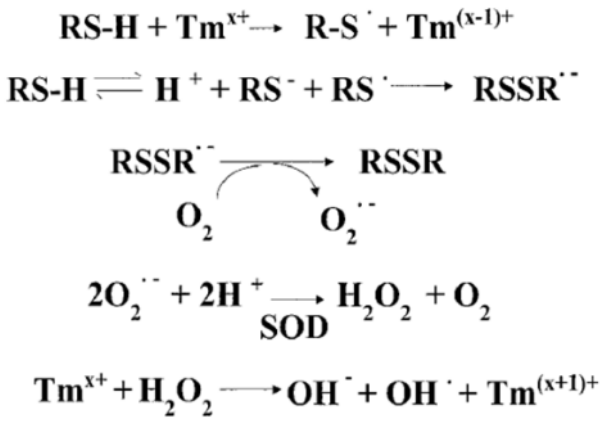


Figure 15 - Hcys oxidation. Legend: RS-H| Hcys; **Tm^{x+}|** transition metal; **RSSR|** Disulphide; **$O_2^{\cdot-}$|** Superoxide anion radical; **SOD|** Superoxide dismutase; **H_2O_2|**Hydrogen peroxide. Image obtained from McDowell *et al* [57].

Once the superoxide is produced, superoxide can enter the cells and the organism tries to deal with it by the action of an enzyme endothelial superoxide dismutase (eSOD). However, superoxide can readily react with NO to form the oxidant peroxynitrate ($OONO^{\cdot}$). This reaction is five-fold faster than superoxide radical degradation by SOD, leading to a decreased bioavailability of NO at the endothelial cells [57]. This occurrence, associated with some others deleterious actions of the oxidative damage of the Hcys, illustrated in the Figure 16, starts a vicious cycle of ROS generation, which leads the cell to a dead end characterized by an inflammatory activation and for last an apoptosis process [37, 58]. According with this figure, HHcys also provokes the decrease secretion and expression of SOD, the formation of S-

nitroshomocysteine, the inhibition of gluthation peroxidase (GPX-1), the depletion of intracellular glutathione and the accumulation of asymmetric dimethylargigine (ADMA) or even inhibiting tetrahydrobiopterin (a cofactor of eNOS) [39].

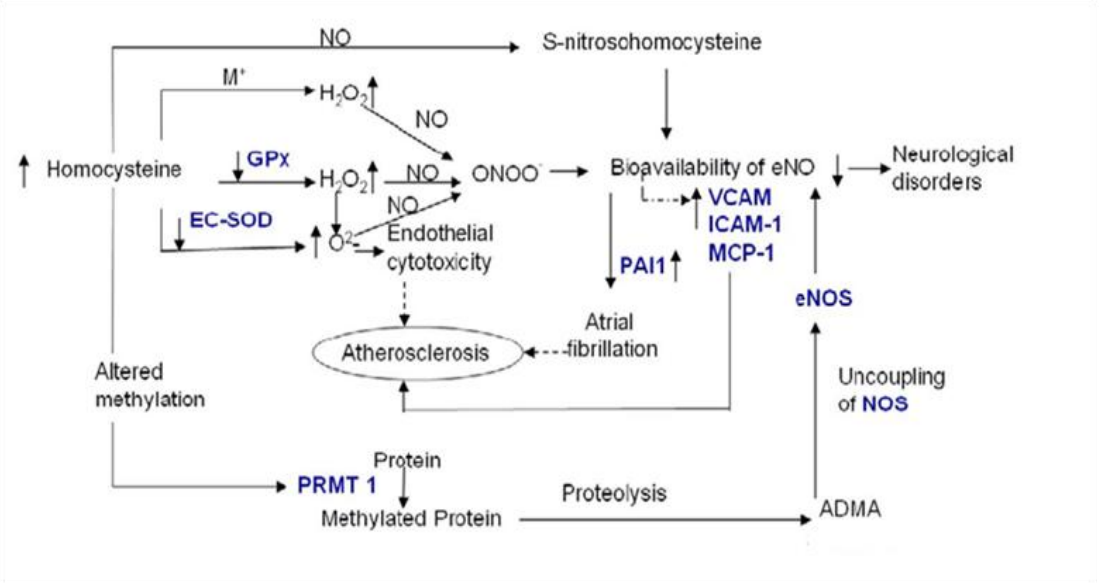


Figure 16 - Hyperhomocysteinemia and oxidative stress. Hcys might directly or indirectly (altered DNA methylation) lead to oxidative stress via the pathways shown in the figure. **Legend: NO|** Nitric oxide; **M^+|**Transition metal; **GPX|** Gluthatione peroxidase; **EC-SOD|** Extracellular superoxide dismutase; **PRMT 1|** Protein Arginine N-Methyltransferase 1; **H_2O_2|** Hydrogen peroxide; **O_2^-|** Superoxide; **ONNO$^-$|** Peroxynitrate; **PAI 1|** Plasminogen Activator Inhibitor 1; **VCAM|** Vascular Cell Adhesion Molecule; **ICAM-1|** Intercellular adhesion molecule-1 **MCP1|** Monocytes chemoattractant protein-1; **eNOS|** Endothelial NO synthase**; NOS|** NO Synthase; **ADMA|** Asymmetric dimethylargigine. Image adapted from Sharma *et al* [39].

Besides the resulting effects of the oxidant stress of Hcys into the cells, ROS generated by the increased concentration of auto-oxidized Hcys, can incite the LDL peroxidation into the vessels walls by the reduction of the copper (Cu) in the Ceruloplasmim (Cp). Cp is an abundant plasma protein (300 μg/mL) that contains seven copper atoms per molecule and accounts for 95% of the total circulating copper in healthy adults [59]. The physiological function of Cp is uncertain but it is believed that this protein has an important role in copper transport, coagulation, angiogenesis, antioxidant action, and iron homeostasis. Reduction of Cu^{2+} to Cu^{+} of the Cp by superoxide radicals produced by the Hcys auto-oxidation may trigger the LDL oxidation activity of this protein [37, 60].

Thus, these reactive species generated cause an oxidative stress inside the cells, which can cause some cellular deregulations that contribute for the endothelium dysfunction, inflammation and oxidant injury [61]. Au-Yeung *et al* (2004) showed that Hcys damages endothelial cells by

increasing ROS production, affecting antioxidant defence systems, promoting lipid peroxidation, as well as triggering apoptosis via mitochondrial oxidant production [62]. Hcys also promotes production of ROS by isolated monocytes and platelets [42]. Likewise, it is believed that Hcys can also deregulate the biosynthesis of cholesterol by hepatic cells due to homocysteine-induced endoplasmic reticulum stress. However, the exact mechanisms involved in all these harmful events still remain poorly understood [58, 63].

1.3.2.2. Hcys and hypomethylation

Associated with the oxidant effect of Hcys, some effects at the genetic level are also provoked by an elevated level of Hcys and they may have a lethal role on cell. DNA methylation is a critical component of epigenetic regulation of the gene expression. DNA hypomethylation is induced by increased Hcys levels and decreased level of Met. Thus, global or selective DNA methylation alteration may contribute to alteration in gene expression and vascular changes during HHcys. Hcys at high concentration also increase the transcription and activity of tissue factor. Hcys elicits a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxins. DNA strands breaks and associated activation of PARP [poly (ADP-ribose) polymerase] and NAD (nicotinamide adenine dinucleotide) depletion occur rapidly after exposure to Hcys and precede mitochondrial dysfunction, oxidative stress and caspase activation [64].

1.3.2.3. Hcys, Hcys-thiolactone and N-homocysteinylation

Recent studies found that the most reactive form of Hcys, which modifies haemostasis, including biological proprieties of blood platelets, may be Hcys- thiolactone (HTL), which represent 0.29% of the total Hcys plasma levels. In all organisms, Hcys is metabolized to the thioester-Hcys-thiolactone in an error-editing in protein biosynthesis when Hcys is selected in place of methionine by methionyl-tRNA synthetase and paraoxonase 1. HTL levels ($\geq$ 15.3 μmol/L) are elevated in HHcys. HTL, known to be cytotoxic in experimental animals and cell cultures, is harmful mostly because of its ability to form isopeptide bonds with protein lysine residues. N-Hcys-proteins, including - fibrinogen may be linked to human pathology such as atherosclerosis (Figure 17). N-homocysteinylation exist in endothelial cells proteins, involves acylation of lysine amino group by the activated carboxyl group of HTL, and depends on the concentration of Hcys [65, 66].

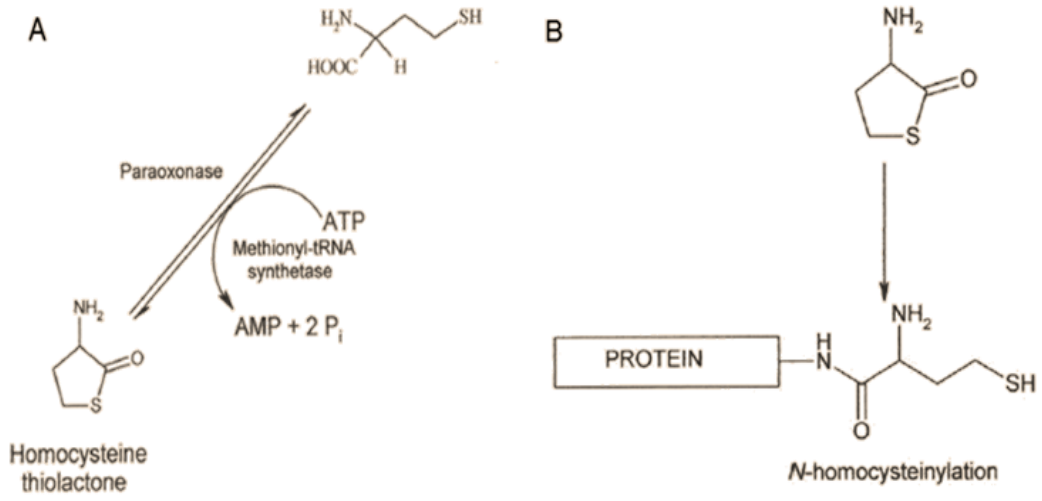


Figure 17 - Hcys-thiolactone metabolism. A| Hcys-thiolactone (HTL) formation; **B|** N-homocysteinylation. Image adapted from Manolescu *et al* [67].

Generally, N-homocysteinylation alters the function of the proteins through the introduction of new free thiol group, and the inactivation of free amino groups, affecting the overall redox potential of the proteins. Moreover, it has been found that proteins modified through this mechanism can act as neo-antigens, triggering the endothelium injury and the activation of the inflammatory response, a key component in atherogenesis, atherothrombosis and stroke. Furthermore, HTL impairs the ability of the vascular endothelium to regenerate itself through the direct inhibition of lysyl oxidase that is responsible for the correct cross-linking/reticulation of the collagen and the elastin into the artery wall [67].

Malinowska *et al* (2012) showed that HTL modulates the platelet adhesion to the collagen and the fibrinogen, promoting the adhesion of the thrombin-activated platelets to the collagen, when platelets were treated with a high concentration of Hcys (50 and 100 μmol/L). This study also demonstrated that HTL is more effective modulator of the platelet adhesion than Hcys. Therefore, the authors conclude that HTL, even at lower concentration than Hcys may be an additional factor which leads to the significant increase of cardiovascular risk. Likewise, they have shown that a combination of Hcys and HTL had even greater modulation action on the platelet adhesion than any of tested thiol compound alone [66].

1.3.3. Hyperhomocysteinemia, vitamin B-cofactor and lowering therapies

As already referred, vitamins B-cofactors (folate, vitamin B_6 and vitamin B_{12}) play an essential role in Hcys metabolism; particularly the catabolic enzymes CBS and CL require vitamin B_6 and the remethylation enzyme (MetS) require vitamin B_{12} and folate for its catalytic activity. It is therefore evident that there is an inverse correlation between Hcys levels and with folate and vitamin B levels (Fig. 13). This strong metabolic relationship is particularly relevant in the quantification of Hcys levels in HHcys conditions and a strong incentive to investigate folate, vitamin B_6 and B_{12} levels in Hcys lowering therapies [40, 68]. This is not, however, a consensual issue as the role of folic acid, vitamin B_6 and B_{12} supplementations in the prevention of CVDs events is not yet fully understood. Although some studies have shown a relationship between the use of vitamin B-cofactors supplements for lowering Hcys level [69-71] most of them have suggested that the existing evidence indicates that the Hcys lowering therapies of the folic acid, vitamin B_6 and B_{12} do not lower the risk of CVD. This context is clearly illustrated in the review from Clarke, *et al* (2010) in which they performed a meta-analysis of 8 randomized trials involving 37485 individuals with increased risk of CVD and concluded that doses of folic acid (ranging from 0.8 to 40 mg/day) can reduce Hcys levels in 25%. However, in the same review, the authors also concluded that dietary supplementation with folic acid to lower the Hcys levels had no significant effects in cardiovascular events or on overall cancer or mortality in the population studied within a 5 years period [72]. Regarding this, the American Heart Association guidelines for CVD prevention do not recommend the use of folic acid supplementation to prevent CVDs. Some potential causes responsible for the lack of consensus between these trials could be the difference between the study groups. Hcys concentrations, inclusion of patients from countries that do not have regulations about the food enrichment with the folic acid, trial period, composition of the vitamin formulation, gender distribution, other patients' medication and patient's disease, among others, lie behind this lack of consensus [67, 73].

1.4. Vitamin B-cofactors

Vitamin B-cofactors play an important role on the strict balance of the Hcys metabolism. Their deficiencies can promote an accumulation of Hcys in the organism and contribute for the development of hyperhomocysteinemic conditions and associated pathological situations.

1.4.1. Vitamin B_6

Vitamin B_6 is a water-soluble vitamin, and occurs in three closely related compounds with similar physiological actions, pyridoxine, pyridoxal and pyridoxamine. The first is found in

large quantities in plant sources and the other two in animal tissues. Liver, grain cereals, peanuts and bananas are rich in vitamin B_6. In humans, vitamin B_6 is also obtained from several intestinal microorganisms able to synthesize this vitamin. Adult requirements are about 2mg/day. The biological active form of Vitamin B_6, pyridoxal 5'-phoshate (Figure 18), participates in an important coenzyme system in the protein synthesis and is involved in the lipids and the carbohydrate metabolism. This vitamin deficiency (< 30 nmol/L or 7.4 ng/mL) has a serious effect on brain function, as the vitamin plays a crucial role in the synthesis of several neurotransmitters (serotonin, dopamine, norepinephrine, among others). The vitamin B_6 deficit contributes to the development of several neuropsychological disorders such as Parkinson disease, delayed dykinesia and depression. The lack of this vitamin also affects the immune function, resulting in impaired cell-mediated immunity and reduced antibody responses. Its deficiency also contributes for the development of CVDs, as the B_6 active form plays a key role as a coenzyme of two enzymes (CBS and CL) required for the metabolism of the Hcys to cysteine [74-76].

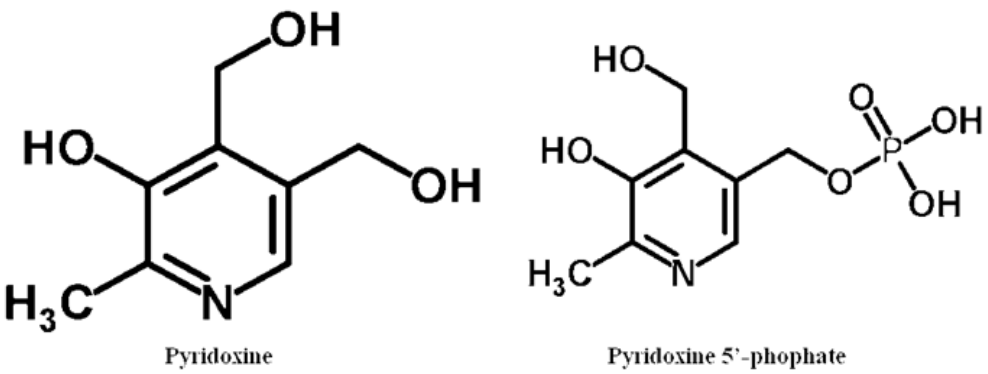


Figure 18 - Pyridoxine and Pyridoxine 5'-phospate structures. Image adapted from Royal Society of Chemistry [75, 76].

1.4.2. Vitamin B_9

Folate or vitamin B_9, is a water-soluble B vitamin present in cells as a family of structurally related and metabolically interconvertable enzyme cofactors that plays an important role in one-carbon metabolism. This pathway is necessary for the synthesis of purine and thymidine nucleotides and for the synthesis of methionine from Hcys. Inadequate folate intake has been linked to the risk of anaemia, neuropsychiatric disorders, neural tube defects and CVD, by the elevation of Hcys concentration. Moreover, folate deficiency leads to the incorporation of uracil instead of thymine into human DNA and to an increased frequency of chromosomal breaks, causing disruption of DNA synthesis, repair and methylation, which may increase the

risk to develop some cancers. Important food sources of folate include vegetables, especially green leafy vegetables, cereals, fruits, nuts, seed, liver and its derived products [45]. Dietary folates predominantly exist as polyglutamates (Figure 19), which have to be hydrolyzed to folate monoglutamates by the folate reductase in the jejunal mucosa in order to be absorbed. Folate polyglutamates comprises a chemically reduced pteridine moiety that is likened to p-aminobenzolglutamate through a methylene group [77].

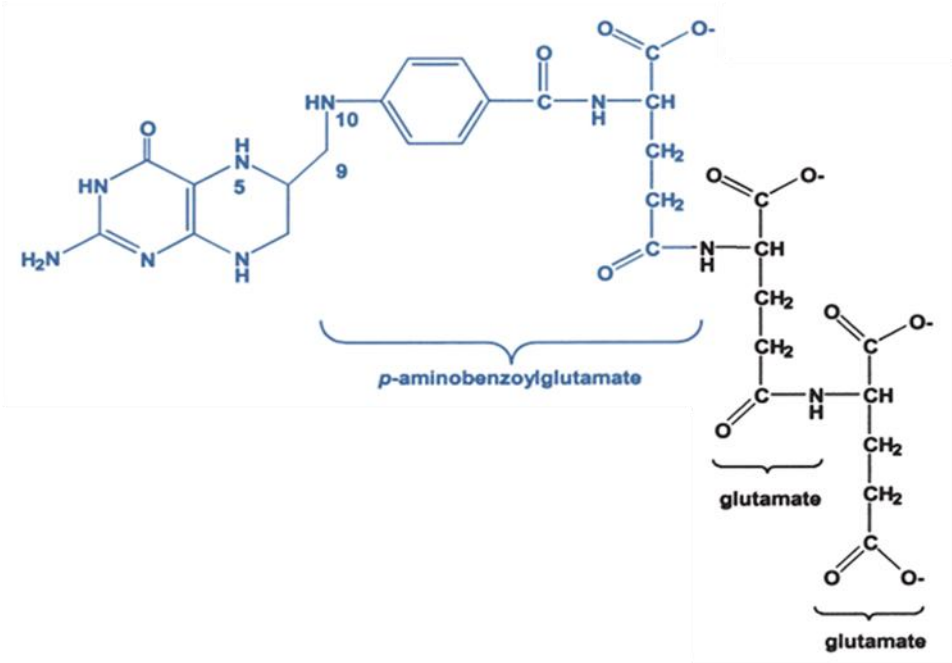


Figure 19 - Structure of the Tetrahydrofolate triglutamate. Legend: The blue fraction of the structure corresponds to the folate monoglutamate. Image adapted from Park *et al* [77].

Once absorbed, inside the enterocytes cells, folate monoglutamates are transported into mitochondria and are processed into functional metabolic cofactors by the addition of ϒ-linked polyglutamate peptide that ranges from 2 to 9 glutamate residues in length. Tetrahydrofolate (THF) polyglutamates chemically activate and carry one-carbon units at the oxidation levels of 5-formyltetrahydrofolate, 10-formyltetrahydrofolate, methylenetetrahydrofolate and 5-methyltetrahydrofolate (5-methylTHF). Each one-carbon form of folate is a required cofactor for one or more biosynthetic pathways (Figure 20), with the exception of 5-formyltetrahydrofolate, which does not serve as a metabolic cofactor and may be a storage form of folate. These metabolites have different roles, being 10-formyltetrahydrofolate required for the synthesis of the purine ring; methylenetetrahydrofolate is required for the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate (dTMP), which in turn is used in DNA building; and 5-methyltetrahydrofolate is required for the remethylation of Hcys to methionine [77].

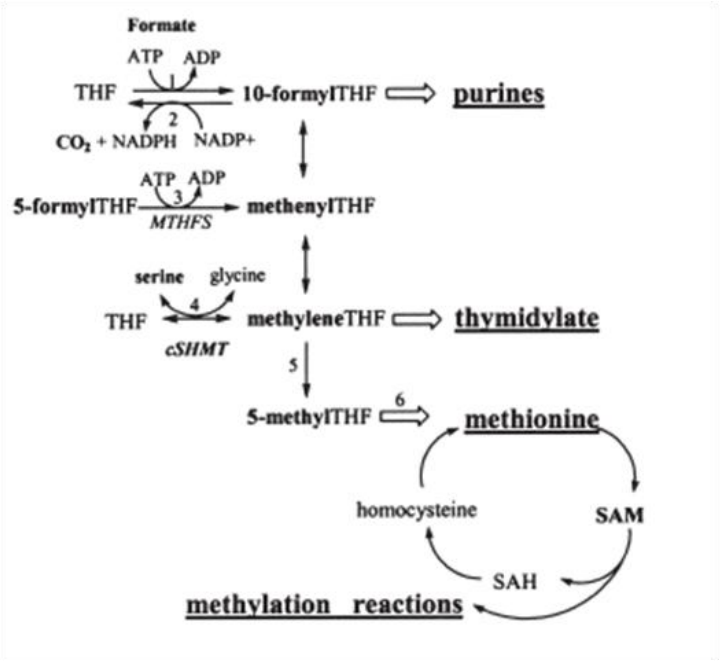


Figure 20 - Folate metabolism. Folate metabolism is required for purine, thymidylate and methionine biosynthesis. **Legend: 1|** 10-formylTHF synthetase; **2|** 10-formyTHF dehydrogenase; **3|** MethenylTHF synthetase (MTHFS); **4|** Serine hydroxymethyltransferase (cSHMT); **5|** MethyleneTHF dehydrogenase (MTHFR); **6|** Methionine synthase (MS) Image obtained from Park *et al* [77].

Following the formation process of THF polyglutamates inside the enterocytes, 5-methyTHF is released into the portal circulation, being the main folate form in the plasma. Much of this folate is taken by the liver, although some is released into the bile where it is recirculated by the enterohepatic cycle. Deficiency of 5-methylTHF is considered when plasma levels are lower than 10 nmol/L (4 ng/mL) [78, 79].

Folic acid, a folate monoglutamate, is a stable, synthetic analog, which is merely the parent structure of this large family of vitamin coenzymes. Due the only single glutamate in its structure, folic acid is absorbed more efficiently, exhibiting greater bioavailability than naturally occurring folate, which assumes, as already referred, the polyglutamate form [77]. The relative bioavailability of dietary folate is estimated to be only 50% when compared with the synthetic folic acid [80].

1.4.3. Vitamin B_{12}

Vitamin B_{12} or cobalamin (Figure 21) plays an important role in the DNA synthesis and neurological function. Vitamin B_{12} is a cobamide (cobalt-containing corrins), which is a presumed archetype of tetrapyrrole (family of compounds which has evolved to exploit the special properties of the ionic forms of cobalt, iron, magnesium and nickel) [81, 82]. The usual dietary sources of vitamin B_{12} are mainly animal food (chicken, mutton and liver), eggs, fish and shellfish [83]. This vitamin is also produced by the most species of enteric bacterias, as *Salmonella*, *Escherichia* and *Enterobacter* species [84].

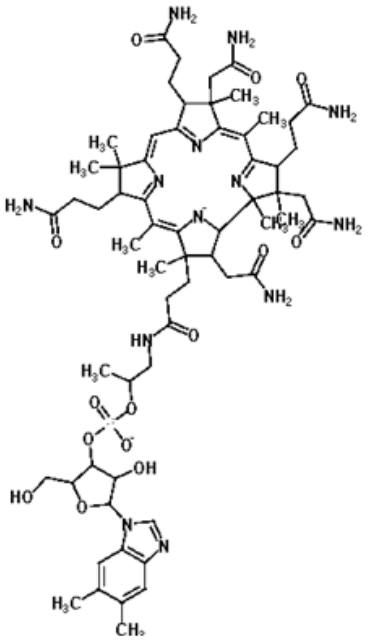


Figure 21 - Cobalamin structure. Image obtained from Royal Society of Chemistry [82].

Cobalamin deficiency (low serum level, usually, less than 74 pmol/L or 100 pg/mL) is associated with hematologic, neurologic, and psychiatric manifestations. It is a common cause of macrocytic (megaloblastic) anemia and, in advanced cases, pancytopenia (leukopenia or reduction of leukocytes number and thrombocytopenia or reduction of platelets number). Neurologic sequels from cobalamin deficiency include paresthesias (skin feelings of tingling, tickling, stinging and burning), peripheral neuropathy, and demyelization of the corticospinal tract and dorsal column. This vitamin deficit has been also linked to psychiatric disorders, including impaired memory, irritability, depression, dementia and, rarely, psychosis. In addition to these hematologic and neuropsychiatry manifestations, vitamin B_{12} deficiency may exert, like folate deficit, indirect cardiovascular effects by increasing the Hcys levels [85].

Vitamin B12 can be absorbed by two pathways: intrinsic factor (active diffusion) and passive diffusion [83]. The first one is used for the absorption of large amounts of vitamin that normally enter the organism by ingested food [83]. Once ingested, the acidic environment of the stomach facilitates the breakdown of cobalamin that is bound to food. An intrinsic factor (gastric intrinsic factor), a glycoprotein that is released by parietal cells in the stomach, binds to vitamin B_{12} in the duodenum [83, 85] and this complex subsequently aids on the absorption of the vitamin in the terminal ileum (Figure 22). The other pathway (passive diffusion) is normally used for small amounts of vitamins [83] Once absorbed, cobalamin binds to transcobalamin II and is transported throughout the body [85].

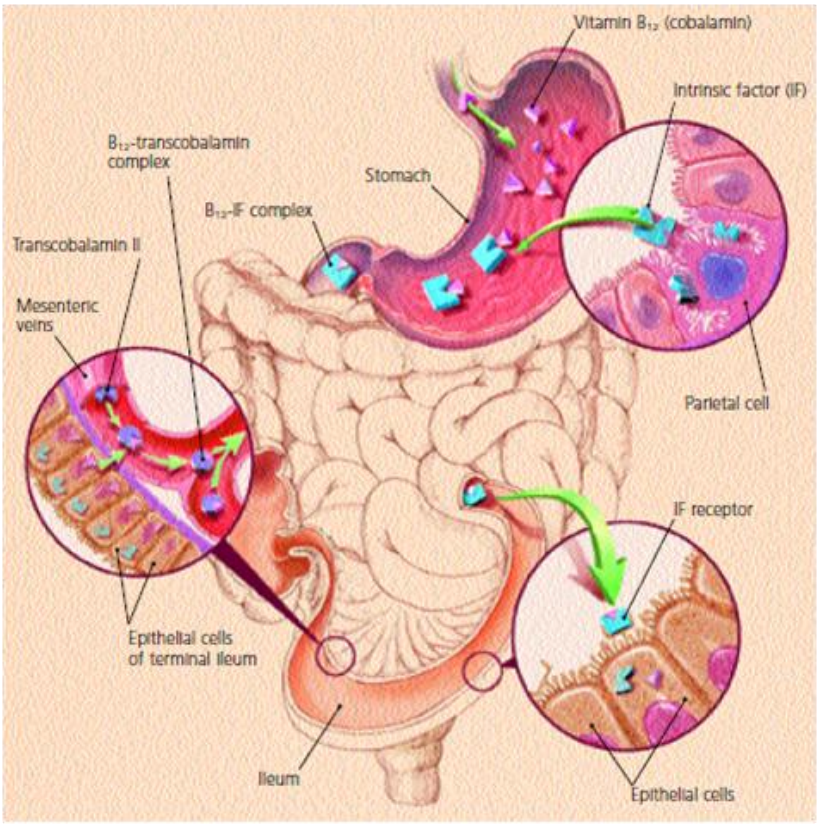


Figure 22 - Cobalamin absorption and transport. Image obtained from C. Robert *et al* [85].

1.5. Hcys and vitamin B-cofactors: analytical determination

The determination of the total Hcys levels has gained high interest within the biomedical community over recent years as the molecule was considered an important biomarker for a wide range of diseases, especially atherosclerosis. Associated to Hcys metabolism, the vitamin B-cofactors are also interesting molecules to quantify, allowing obtaining a better overview of Hcys levels and possible causes of their abnormal levels. Regarding this, only very recently a study using HPLC for the simultaneous quantification of vitamin B-cofactors (B_6, B_9 and B_{12}) and Hcys in human serum has been reported [86]. In other previous study, simultaneous quantification of folate and Hcys has been performed using LC coupled to tandem mass spectrometry (LC/MS/MS) detection [87, 88]. Beyond these reports, vitamins B-cofactors and Hcys are usually quantified individually most commonly using radioimmunoassay [89, 90] and competitive chemioluminescent enzyme immunoassays [91] (for folate and vitamin B_{12}), and HPLC (for vitamin B_6) [89, 91, 92] .

Regarding the analytical measure of Hcys, several types of methods are actually available for its determination in the plasma. Among them, some are nearly obsolete like radioenzymatic determination, whereas chromatographic methods are still used and immunoassays become broadly employed due to their easy full automation possibilities. Table 1 summarizes the main features of current analytical methods to quantify Hcys (reviewed in [36]). According to this table, it is possible to note different methodologies used for Hcys analysis as well as their advantages and drawbacks. From several methodologies presented, capillary electrophoresis with laser-induced fluorescence (CE-LIF) and LC/MS/MS detection are the techniques that allow the cheaper analysis of Hcys with high number of samples analysis performed (throughput). Nevertheless, these two methods present some crucial drawbacks that are essential for the development of the technique that aims of this study. Unlike the methodology developed here, CE-LIF uses derivatisation to increase sensibility and precision in Hcys analysis, requiring more time of analysis, LC-MS/MS requires equipment that is not available monetarily for the most laboratories, and both methods require usually a laborious process (high workload) for processing the sample.

Table 1 - The main characteristics of the current analytical methods. Adapted from Drucros, *et al* [36].

Method [a]	Sample pre-treatment	Plasma volume (µL)	Upper limit of linearity (µM)	Coefficient of variation for mean total Hcys level (%)	Throughput (test/hour or day)	Reagent cost estimation by Euros [b]
GC-ID-MS	High workload (laborious pre-treatment process) +derivatization	100	30-300	2.6 -5.7	96-160/day	2.3
LC-MS-MS	High workload	100	60	5.9	40/h	1.0
HPLC-FD	High workload +derivatization	60-150	50-300	3.2 - 4.8	90-150/day	5.4
HPLC-ED	High workload	60	100	5.6	60/day	
IEC	Medium workload	599	100-1000	7.8	25-50/day	1.73
FPIA	None	50	45-50	3.1	20-60/h	11.5
ICL	None	15	50	3.9	150/h	11.5
EIA	Low workload	25	50	6.2	96/2.5h	12 (test in duplicate)
Enzymatic method	None/Low workload	5-100	80-100	2.8-3.7	45/h	
CE-LIF	High workload +derivatization	100	200	7.8	100/day	0.76

[a] **GC-ID-MS|** Gas chromatography-mass spectrometry with isotopic dilution; **LC-MS/MS|** Liquid chromatography with tandem mass spectrometry; **HPLC-FD|** High-pressure liquid chromatography with fluorescence detection; **HPLC-ED|** High-pressure liquid chromatography with electrochemical detection; **IEC|** Ion-exchange chromatography; **FPIA|** Fluorescence polarization immunoassay; **ICL|** Chemioluminescence immunoassay; **EIA|** Enzyme-linked immunoassay; **CE-LIF|** Capillary electrophoresis with laser-induced fluorescence.

[b] Taking into account neither the equipment cost, nor the technician salary.

Immunoassays, such as fluorescence polarization, chemioluminescent microparticle and enzyme-linked, are the more convenient especially for large screening programs of HHcys detections. But knowing their limitations, high values of Hcys needed to be checked by using reference methods in a specialized laboratory. Moreover, this kind of analysis also shows some limitations about of the reagent costs and the price of the equipment[36].

Chromatographic methods have been extensively used for the determination of total Hcys until the introduction of the immunoassays. Therefore, there is an abundance of chromatographic techniques available, using HPLC or ion-exchange liquid chromatography with fluorescence, Ultraviolet/visible (UV/Vis) and electrochemical detection and also hyphenated techniques using Gas Chromatography (GC) or LC coupled to MS detection. All these techniques have shown to produce various advantages in terms of sensitivity depending on the conditions required and they can allow the co-determination of the other amino acids used for diagnosis. However, the lack of selectivity present in many of the procedures forces the utilization of a separation technique (pre-treatment) previously to the chromatographic separation. Despite the advantage of increase both selectivity and sensitivity, this often add time and money to the methodology, which can compromise its suitability to routine and high throughput clinical analysis. LC coupled with MS/MS has been proposed for Hcys determination due the undoubted advantages in terms of the quality of the results owing its specificity, sensitivity and the utilization of labelled internal standards, and because the cost of reagents is definitely lower than that for immunoassays. However, the cost of equipment is the biggest drawback to include LC/MS/MS in the routine clinical quantification [36, 55].

1.5.1. Micro-extraction in package sorbent as a potential tool for Hcys and vitamin B-cofactors extraction from biological samples

When chromatographic assays are used in the analysis of total Hcys, the most common technique applied for the separation of this compound from biological matrix, is a pre-treatment procedure named derivatisation. This is based on the bond between the derivatisation reagents with the Hcys, forming a complex that can be detected with more accuracy, allowing to differentiate Hcys from others compounds on the sample. Several studies have used some derivatisation reagents, such as monobromimane (mBrB), halogenosulfonylbenzofurazans (SBD-F and ABD-F) and o-phthaldialdehyde (OPA), to increase sensibility and precision in Hcys analysis. This technique allows the direct injection of processed plasma and it is usually used with less sensitive LC detectors, such as fluorescence and UV/Vis, or when gas-chromatography is the selected chromatographic methodology. However, this pre-treatment procedure presents some drawbacks that can compromise its automation. Among them are the lack of specificity of the derivatisation reagents for Hcys molecule, the production of secondary metabolites that can interfere with Hcys determination; the incompatibility of the reduction reagents needed to break the sulfhydryl bonds of the disulphides in the plasma; and complex gradient elution programs for the proper separation the analytes, requiring a laborious method [36, 55]. These drawbacks are not present when accurate LC detectors, such as MS and electrochemical detection (EC), are used as in these cases the critical derivatisation pre-treatment step is not required to quantify Hcys with good accuracy and precision. Recent studies

reported the quantification of Hcys and vitamin B-cofactor (folate) using the extractive technique solid phase extraction (SPE) [87], or through direct injection [88]. As stated by Nelson *et al* [87] the determination of total Hcys is difficult because of the instability of its reduced form and the diversity of its oxidized forms. The determination of folate is also very challenged due to its susceptibility to convert to other forms and to degrade during sample preparation. Moreover, according to the same authors, most LC/MS methods, but not all, depend on the selective extraction of the analytes using reversed-phase, anion exchange or affinity SPE. Likewise, this represents a problem because most SPE protocols are inherently more oriented to the extraction of Hcys or folate, but not both analytes simultaneously due to the differences in analyte stabilization and polarity (Hcys is strongly polar in solution while folates are only moderately polar). Even so, Nelson *et al* [87] managed to get good results in the simultaneous quantification of Hcys and folate by performing a two-step C_{18} SPE previously to a LC/MS/MS analysis. In turn, Persichill, *et al* [88] obtained a good analytical performance in the plasmatic Hcys and folate quantification only using protein precipitation followed by direct injection into the LC/MS/MS equipped with a strong cation exchange column. This methodology is obviously quite more simple and less expensive than the SPE method presented by Nelson *et al* [87]. Nevertheless, according with Abdel-Rehim *et al* [93], high matrix background in the extracted samples with consequent significant signal suppression is often observed when protein precipitation (PP) is used as a sample preparation methodology. The same authors also affirmed that SPE is more efficient recovering drug analytes because of the ability to efficiently retain highly functionalized compounds from aqueous samples and release them into organic solvents in the following elution step.

Regarding this, microextraction by packed sorbent (MEPS) is a new sensitive, selective, accurate and promising sample extraction technique. MEPS is a development of conventional SPE that has miniaturized the sorbent bed so that it can be incorporated into the sample path without voids. MEPS is a logical extension of SPE for handling biological fluids because, unlike SPE, this technique uses small operating volumes that reduce the size of the sample required. MEPS uses the same sorbents as conventional SPE columns and so is suitable for use with most existing methods by reducing the volume of reagents and sample, being in this way more easily automated than SPE. Extraction performance is comparable to conventional SPE because the MEPS sorbent bed retains the same dimensional ratios of the conventional SPE cartridges and adaption of existing methods is achieved by converting all steps in proportion to the bed volumes, typically 1 mL for SPE and 10 μL for MEPS. MEPS can handle both small and large volumes (from 10 μL up to 1000 μL) and is suitable for normal phases, reversed phase, mixed mode and ion exchangers. This approach is much less expensive than SPE because the MEPS syringe can be used several times, up to 100 times or more, while

conventional SPE columns and cartridges are used only once. Moreover, MEPS can be fully automated, taking only about one minute to process each sample. Its utilization prior to LC is an excellent tool for screening the presence of drugs and metabolites in blood, plasma, urine and saliva samples [94]. Therefore, MEPS can easily substitute most existing methods using SPE [93, 95].

Unlike conventional SPE columns, in MEPS the solid packing material that constitutes the sorbent, 1-4 mg, is integrated into a liquid handling syringe (50-1000 µL) barrel. The structure of the BIN can be observed in the Figure 23 [95-97].

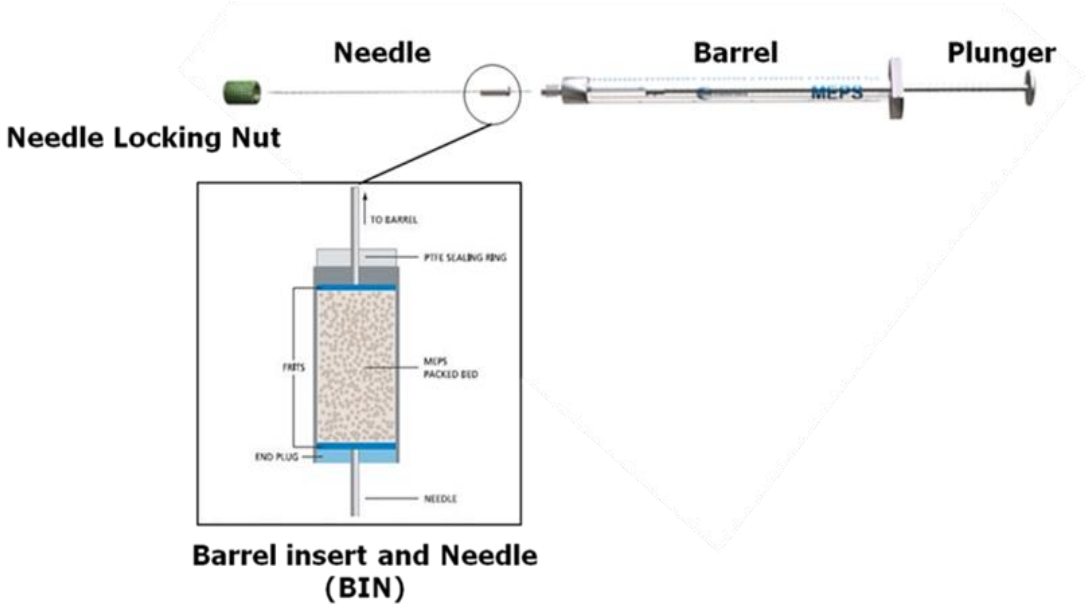


Figure 23 - MEPS structure. Image adapted from SGE Analytical Science and Metrolab [95, 96].

When the sample passes through the solid support, the analytes are adsorbed to the packed solid phase. The cartridge is packed to provide selective and suitable sampling conditions. According with Pereira *et al*, MEPS sorbents are generally based in silica particles or polymeric phases which are then fine-tuned to obtain different chemical properties and a large portfolio of specificities for target analytes [98]. Any sorbent material such silica based (C2, C8, C18); strong anion exchange (SAX); strong cation exchanger (SCX) using sulfonic acid bonded silica, such as M1 (mix of C8 plus SCX); restricted access material (RAM); carbon; polystyrene-divinylbenzene copolymer (PS-DVB) or molecular imprinted polymers (MIPs) can be used [93, 95]. Silica based sorbents have a high retention capability and can cover a wide range of interactions, from hydrophobic to hydrophilic (C2 to C18, SAX, SCX, etc.) by the addition of different polymers (amines, carboxylic acids, etc.) to silica. Carbon sorbents have great absorption capacity and chemical, thermal and mechanical resistance. Polymerics sorbents (PS-DVB) are robust have higher surface contact areas, are suitable for extractions all over the entire

pH range and work at elevated temperatures and posses improved retention for polar compounds as the number of points of interaction between the sorbent and the analyte is significantly increased. However, there are also some drawbacks. Silica based sorbents present a narrow pH stability (2 to 7.5), a poor surface contact with aqueous matrices that result in low recoveries in extracting polar compounds and the presence of some residual silanol groups. Carbon sorbents have a low specific surface area while polymerics sorbents have a lower selectivity than the other sorbents [98].

MEPS technique can be performed manually or can be semi or fully automated by loading the Xchange® MEPS syringes in the digital analytical syringe (eVol®) [99, 100]. The Figure 24 shows the two types of equipments for MEPS technique. This automation brings some advantages, such as: less glassware usage; less solvent required; reduces waste fluid; significant time saving; improved accuracy and reproducibility; improver operator safety (lower spill and splash risk); and complete workflow simplification [99].

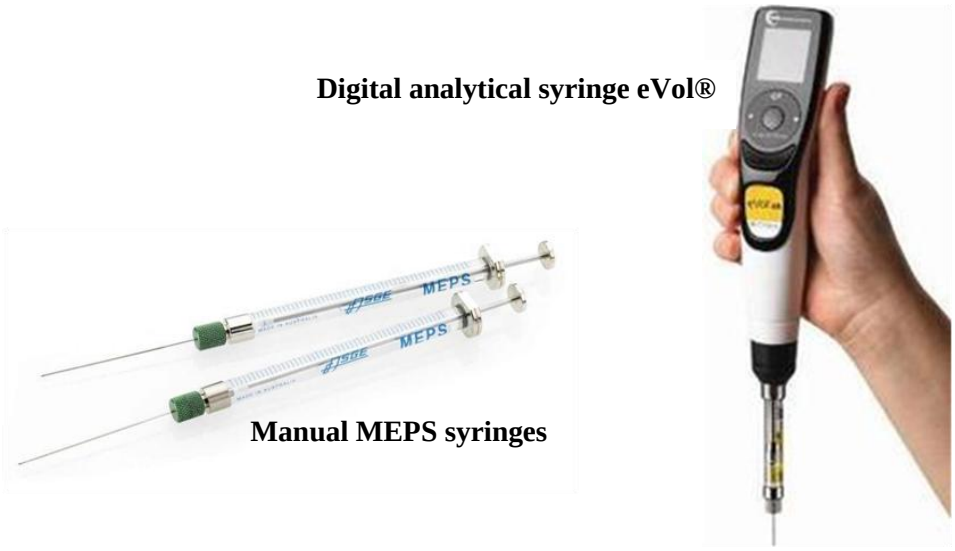


Figure 24 – The equipments for MEPS technique. Image adapted from SGE Analytical [98, 99].

The MEPS extraction procedure is normally realized by essential steps: **1) MEPS cartridge condition**: wherein there is a passage of an organic solvent followed by; the passage of water; **2) Sample loading**: corresponds to the passage of the sample a desired number of times to retain analytes; **3) Sorbent washing:** normally it is used acidified water to remove interfering compounds ; **4) Elution:** corresponding to the phase where the captured analytes are released in a particular solvent, [93, 101].

Actually there are countless studies reported using MEPS in different areas of application, however, there are not any published articles known about the utilization of MEPS for the analytical quantification both Hcys as well as vitamin B cofactors (B6, B9 and B12).

1.5.2. Ultra-performance liquid chromatography

Over the past 40 years the practice of chromatography has witnessed a continuous growth in almost every respect, since the number of chromatographers, the amount of published work, the variety and complexity of samples being separated, separation speed and convenience. Actually chromatography is the most versatile and widespread technique employed in modern analytical chemistry [102].

HPLC is a technique that has arisen from the application to liquid chromatography of theories and instrumentation that were originally developed for GC. HPLC has proven to be the predominant technology used in laboratories worldwide during the last 30 years, contributing for the actually position of chromatography in analytical chemistry world. One of the primary drivers for the growth of this technique has been the evolution of packing materials (stationary phase) used to effect separation [103]. In the Figure 25 is possible to look how HPLC works [104].

By the same Figure, HPLC works by the detection of several analytes according with their retention time. The sample is injected into a mobile phase that crosses a column (stationary phase) made out to interact and trap, for few time, the analytes present in the sample. After the column, there is a detector that will identify the compounds according with certain parameters (depending on which type of detector is being used), namely the time that the analytes are retained since the injection point time (retention time). If an UV-Vis detector is being used, a specific wavelength, ideally corresponding to the maximum absorbance of the target analyte(s), is used to obtain a chromatogram. Then, the target analytes concentration can be inferred by comparison of the respective peak areas in the chromatograms of pure standards and samples.

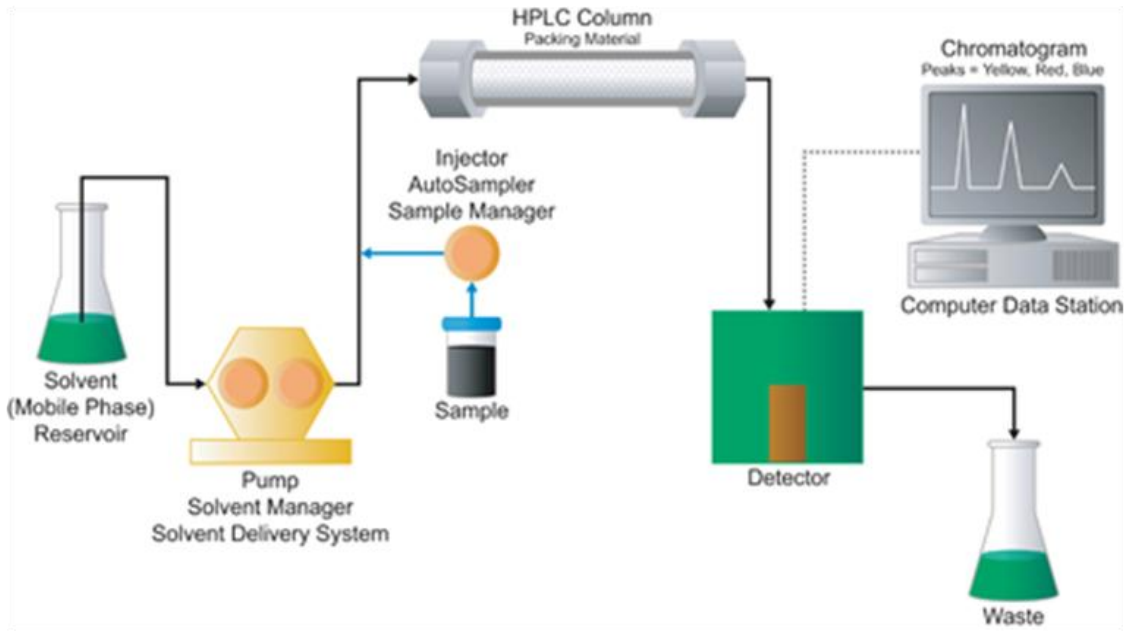


Figure 25 – Schematic diagram of HPLC technique. Image obtained from Waters [102].

For many years, researchers have looked for HPLC as a way to speed up analyses. The need for speed has been driven by the sheer numbers of samples in some laboratories and the availability of affordable, easy to use mass spectrometers. Smaller columns and faster flow rates have been used. Elevated temperatures, having the dual advantages of lowering viscosity, and increasing mass transfer by increasing the diffusivity of the analytes, has also been investigated. However, using the conventional particles size and pressures, limitations are soon reached and compromises must be made, sacrificing resolution for time [105].

In this way, in early 2004, the first commercially available Ultra Performance Liquid Chromatography (UPLC) system that personified these requirements was described for the separation of various pharmaceutical related small organic molecules, proteins, and peptides. Using UPLC it is now possible to take full advantage of chromatographic principles to run separation using shorter columns, and small flow rates for increased speed, with superior resolution and sensibility. Faster separation and higher resolution UPLC separation can lead to higher throughput and time saving when running multiples samples and can cut method development time from days, to hours or even minutes [105].

Chapter II: The experimental

Throughout this chapter it will be described all information concerning to the experimental layout of this in this study.

2.1. Experimental

2.1.1. Material and reagents

During this work, all the reagents used were from an analytical purity greater than 95%, not being necessary, therefore, any further purification. The Table 2 summarizes all the materials and chemicals used for this trial.

Table 2 – List of chemicals and equipments used throughout this experiment.

Chemicals	Supplier
Formic Acid (FA) Sodium Hydroxide (NaOH)	Panreac Química (Barcelona, Spain)
Acetonitrile (ACN) HPLC degree	Fisher Scientific (Lisbon, Portugal)
Methanol (MetOH) HPLC degree	
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) Folic acid Pyridoxine DL-Homocysteine Vitamin B_{12}	Sigma-Aldrich (St. Louis, MO, USA)
Equipments	
Integral water purification system (18 MΩ cm, 23 °C)	Milli-Q Millipore Corporation (Billerica, MA, USA)
Polytetrafluoroethylene (PTFE) membrane filters	Millipore (Billerica, MA, USA)
Semi-automated digital analytical syringe eVol® MEPS needle and BIN	SGE Analytical Science (Melbourne, VIC, Australia)
Ultra Performance Liquid Chromatography (UPLC) Analytical Column Waters Acquity UPLC HSS T3 (100 mm x 2.1 mm, 1.8 µm particle size) Acquity UPLC™ HSS T3 Van Guard™ Pre-column	Waters (Milford, MA, USA)
Expresso Personal Microcentrifuge	Thermo Fisher Scientific (Waltham, MA, USA)

2.1.2. Preparation of the standards stock solutions

A stock solution of DL-Homocysteine (7397 µmol/L or 1 mg/mL) was prepared according to Huang *et al* (2007). Briefly, 10 mg of Hcys powder were dissolved in 10 mL of acidified water (0.4% Formic acid) and stored at -20 ºC in 2 mL of aliquots [106]. As described by Valente *et al* (2012), a stock solution of TCEP (390 mmol/L or 100 mg/mL) was prepared in water and stored at -20 ºC [107]. It was also prepared stock solutions of vitamin B_6 (5910 µmol/L or 1 mg/mL) and vitamin B_{12} (737.8 µmol/L or 1 mg/mL) in water, and vitamin B_9 (2265 µmol/L or 1 mg/mL) in 0.1 mol/L of Sodium hydroxide (NaOH), based on Chatzimichalakis *et al* (2004) [108]. These last stock solutions (vitamin B_6, vitamin B_9 and vitamin B_{12}) were subdivided into 2 mL of aliquots, protected from light and storage at -20ºC.

2.1.3. Plasma samples

Validation assays, were performed with five plasma samples from patients with CVD. All samples were collected in the laboratory of clinical pathological of the Hospital Dr. Nélio Mendonça and stored at -80 ºC until analysis. This study was approved by the ethics committee of the Hospital Dr. Nélio Mendonça and all subjects of the study were selected on a voluntary way, consenting to participate in the study. The Table 3 summarizes the information (gender and age) of the CVD patients group.

Table 3 – Patients that participate in the study.

		Gender	Age
Patients	CVD 1	Male	74
	CVD 2	Female	75
	CVD 3	Male	63
	CVD 4	Male	67
	CVD 5	Male	66

2.1.4. Chromatographic conditions

The method for the simultaneous quantification of Hcys and vitamins B was carried out on a Waters Ultra Performance Liquid Chromatographic Acquity system (UPLC, Acquity H-Class)

equipped with a quaternary solvent manager (QSM), a sample manager (SM), a strength silica HSS T3 analytical column (100 mm x 2.1 mm, 1.8 μm particle size) protected with a HSS T3 Van Guard™ Pre-column Acquity, a column heater and a PDA detector. The whole configuration was driven by Empower software v2.0 from Waters Corporation.

Several gradients with different mobile phases combinations were performed (Annex I) in an attempt to combine all analytes in a same chromatographic methodology.

From all the set of gradients mobile phase programs tested (Annex I), two gradients, one for Hcys and other for vitamins, provided an optimal separation. All sorbents and samples were filtered through 0.22 μm PTFE filters, before use.

The separation for Hcys and vitamins cofactors occurred with a constant flow rate of 200 μL min^{-1}, giving a maximum back pressure of 6000 psi, which is within the capabilities of the UPLC™. The column temperature was thermostated at 30ºC and the samples were kept at 23 ºC in the sample manager. The analysis was performed during 5 min followed by a re-equilibration time of 2 minutes. Beyond the difference in the gradients, different volumes of injection were used 2μL and 5 μL for Hcys and vitamins assays, respectively.

For quantification purposes the PDA detection was set with four distinct channels corresponding to the maximum absorbance wavelength of each analyte (Hcys: 280 nm; Vitamin B6: 290 nm; Vitamin B9: 284 nm; Vitamin B12: 360 nm). The analytes were identified by comparing the retention time and spectral characteristics of their peaks.

2.1.5. Method for the extraction of Hcys and vitamins B

2.1.5.1. MEPS extraction and optimization

For a proper extraction prior to the chromatographic separation of Hcys and vitamins, two different MEPS procedures were performed. The Table 4 summarizes this two extraction techniques performed along with the optimization conditions also performed in this study. The method A corresponds to the MEPS extraction of Hcys and the method B corresponds to the MEPS extraction of vitamins.

Table 4 – MEPS extraction procedures for Hcys and vitamins.

	Optimization conditions	A (Hcys)	B(Vitamins)
MEPS steps	Sorbents	C2, C8, C18, SIL, M1, DVB, HLB DVB, R-AX, R-CX, PGC, PEP and C18(Thermo)	
Cartridge condition		250 µL of ACN 250 µL of 0.01%F A	250 µL of MetOH 250 µL of 0.05% FA
Sample loading	Number of sample cycles	Standards/plasma containing 20 µL of TCEP (390 mmol/L) **C1:** 200 µL **C2:** 2 x 100 µL **C3**: 4 x 50 µL	Standard/plasma **C1:** 200 µL **C2:** 2 x 100 µL **C3:** 4 x 50 µL
Sorbent washing	Washing volume	0.01% FA **C4:** 250 µL **C5:** 100µL	0.05 % FA **C4:** 250 µL **C5:** 100 µL
Elution	Solvent organic percentage	ACN/0.01% FA **C6:** 60% **C7:** 80% **C8:** 90%	MetOH/0.05% FA **C6:** 60% **C7:** 80% **C8:** 90%

The standard solutions used for the experiments were prepared from the initial stock solutions refereed at the subtopic 2.1.2, by diluting in acidified water (0.05% FA). However, for the method A the dilution was performed in 0.05%AF containing 10% of TCEP (7.8 mmol/L). It was used for the method A, 25 ng/µL (184 µmol/L) of Hcys as standard solution, and for the method B, 0.4 ng/µL of each vitamin (vitamin B6: 1.6 µmol/L; B9: 100 nmol/L and B12: 296 nmol/L).

The plasma samples were previously filtrated with 0.2µm PTFE. For the method A, before the plasma filtration it was added to a certain volume of plasma 10 % of TCEP. To optimize the

two techniques, several recovery points (after sample loading; after washing, and elution) were collected to evaluate the best conditions for the extraction. Through the collecting of these recovery points it is possible to evaluate if there is retention of the analyte by the sorbent and/or undesired loss of analyte between the steps until the elution. The Figure 26 shows this recovery points.

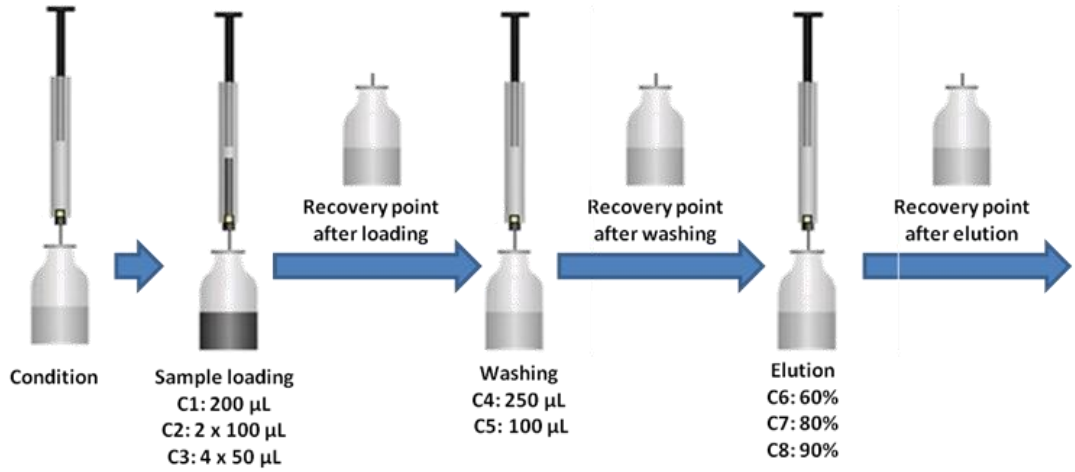


Figure 26 – MEPS recovery points for the optimization.

The optimization of the best fibre, best number of sample cycles (C1 C2 and C3), the volume of washing (C4 and C5) and the elution (C6, C7 and C8) were evaluated by collecting the recovery points referred previously. For the best sorbent, the best number of sample cycles and for the best volume of washing, it was used the elution condition C7 (80% ACN/0.01% FA for Hcys and 80% MetOH/0.05% FA for Vitamins B). The evaluation of the best condition was based on the comparison of the average peak area of each analyte. For the method B, however, as it was used for the simultaneous extraction of vitamins, the best condition was chosen among the better condition for the three vitamins.

2.1.5.2. Protein precipitation for Hcys extraction

The evaluation of the extraction of Hcys by protein precipitation (PP) was performed as described by Persichilli *et al* (2010) [88]. First, 200 µL of plasma were submitted to the reduction with 20 µL of TCEP (390 mmol/L), to break the sulphur bonds of Hcys; then 200 µL of MetOH were added to precipitate the proteins. This mixture centrifuged at 10.000 during 5 minutes. Finally, 200 µL of supernatant was collected and injected (2 µL of the 200 µL) into the UPLC system. This same procedure was also performed with the calibrations solutions used for the validation of the method.

2.2. Validation of the analytical methods

For the validation of the methods, several calibration solutions were prepared The range of concentrations used for each biomarker, were selected according with sensibility of UPLC-PDA and the normal plasma range levels of each compound. The Table 5 summarizes all range of concentrations used as calibration solutions of Hcys, vitamin B_6, vitamin B_9 and vitamin B_{12}.

Table 5 – Calibration solutions used for the validation of Hcys and vitamins B.

Compound	Concentration (µmol/L)	Amount (ng)
Hcys	4	0.5
	7	1
	30	4
	184	25
	370	50
	555	75
Vitamin B_6	0.030	7.4×10^{-3}
	0.300	7.4×10^{-2}
	1.6	0.4
	6.5	1.6
	300	74
	3000	740
Vitamin B_9	0.010	4×10^{-3}
	0.100	0.4
	0.400	1.6
	1	4
	10	40
	200	800
Vitamin B_{12}	7.4×10^{-5}	1×10^{-4}
	7.4×10^{-4}	1×10^{-3}
	0.296	0.4
	1	1.6
	7.40	10
	74	100

The methods performed were submitted to the same validation parameters. Selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision inter- and intra-day, recovery (accuracy) and matrix effect were therefore assessed. These parameters were calculated for each analyte using concentrations usually found in human plasma.

The selectivity was evaluated by the absence of interfering compounds on the retention time of the analytes, in plasma samples analysis by UPLC-PDA.

The linearity was estimated through the determination of the correlation coefficient. Three calibration curves for each analyte were performed, with six different concentrations prepared in acidified water (0.05% FA). Each concentration was injected thrice. The calibration curve was calculated by the graphical representation of a set of different media of peak areas corresponding to different concentrations of the analytes [109].

The sensibility of the methods was evaluated by the LOD and the LOQ for each compound. The LOD and LOQ were calculated through the lowest concentration level of the analyte in the linearity evaluation. LOD was obtained by 3 x standard deviation (SD) and LOQ was calculated by 10 x SD [109].

The precision, expressed by the percentage of relative standard deviation (RSD), was evaluated by the injection of three concentration levels of the analytes, corresponding to low level (LL), medium level (ML) and high level (HL) from the calibration curve. It were analysed six replicas (n=6) at the same day, in order to obtain repeatability (precision intra-day). For the reproducibility (precision inter-day), it were analysed six replicas for each level of concentration during three non-consecutive days (n=18) [109].

The recovery of the method, performed to both methods, was estimated by the recovery percentage of the different analytes through the extraction procedures previous discussed. This evaluation was performed using three levels of concentrations, LL, ML and HL. Recovery was calculated according with the following equation: recovery percentage = 100 x (concentration of the fortified standard – concentration of the standard)/ added analyte concentration [109].

The matrix effect was evaluated by the percentage of the quotient between the slopes of the standards in blank matrix (acidified water for Hcys method and 80% or 90% of acidified methanol for vitamins method) and those obtained by fortified plasma (standard addition method) [110].

Chapter III: Results and Discussion

Throughout this chapter, the data obtained in this work will be presented, interpreted and discussed. The steps used in the developing of the methodology for the determination of the Hcys and vitamins B, the optimization of the processes and the validation of the procedures, will be discussed and compared with published work whenever possible.

3.1. A unique methodology for Hcys and vitamins B chromatographic separation

After optimizing the extraction technique for plasma samples, several gradients were used in an attempt to combine all the analytes in a single chromatographic methodology. In Figure 27A and 27B, are shown the gradient that allowed the best separation and the respective chromatogram. The remaining gradients tested in this work are indicated in the Annex I.

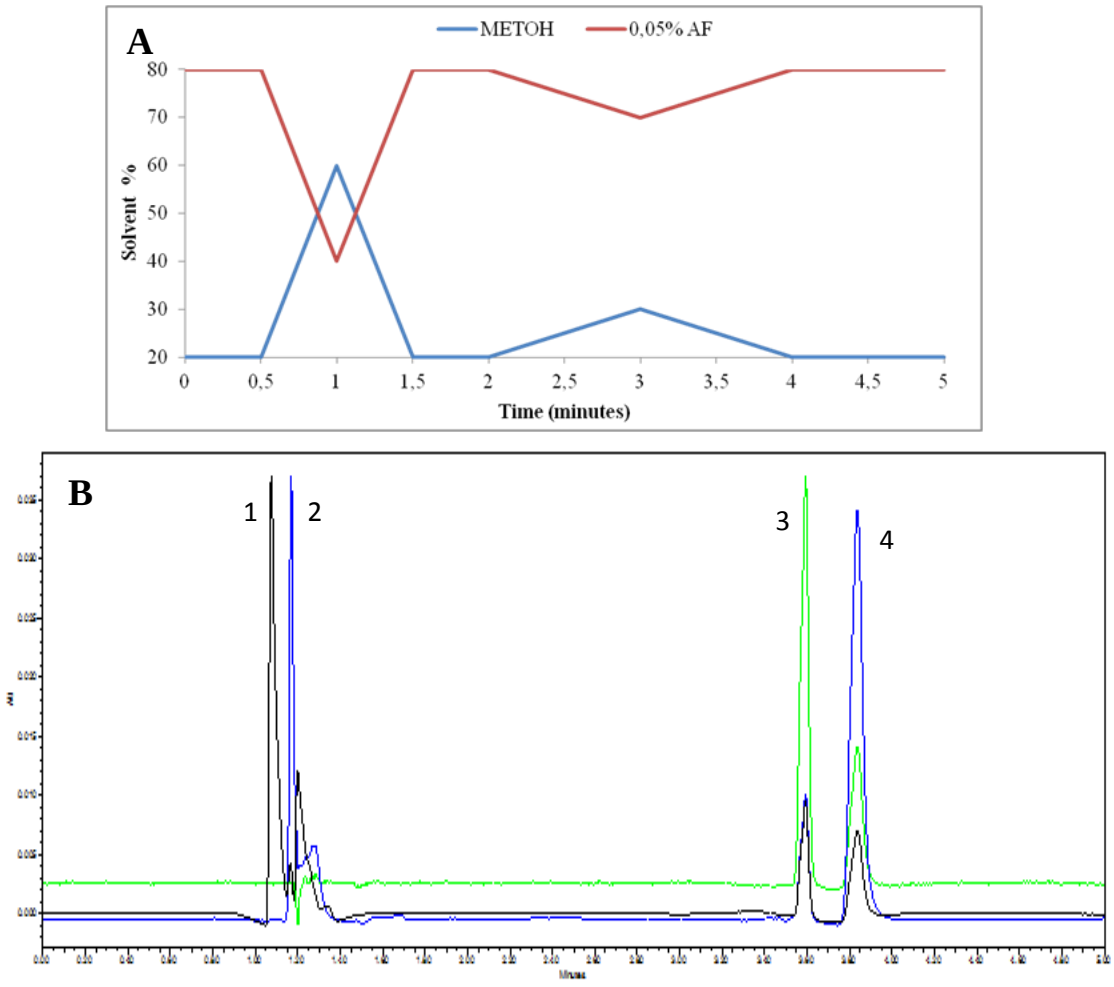


Figure 27 – Chromatographic separation of Hcys and vitamins B. Legend: A| Gradient mobile phase scheme. B| Chromatograms. The black chromatogram correspond to the max wavelength of 238 nm; the blue to the max wavelength of 290 nm; the green to the max wavelength of 260 nm; **1|** peak of Hcys + interfering compound; **2|** peak of vitamin B_6; **3|** peak of vitamin B_{12}; **4|** peak of vitamin B_9.

As Figure 27B shows, the gradients tested did not allowed the simultaneously quantification of the four analytes on the available columns as Hcys and vitamin B_6 separation was not fully achieved and moreover, there is a non-identified interferents coeluting with Hcys. The difficulty to find optimum conditions for target compounds involves a number of reasons. The selected analytes have acid dissociation constant (pKa) values very different (2.46, 9.41 and 10.28 to Hcys [111]; 5.58, 9.40, 14.53 and 15.20 to vitamin B_6 [112]; 3.37, 4.17, 7.61, 7.61, and 18.09 to vitamin B_9 [113]; and 8 successive pKa for B_{12}, ranging from 0.53 to 12.59 [114], as indicated in Annex II), our column has a limited range of pH (2-8), and the use of TCEP (which is acidic) was necessary to maintain the Hcys stable (at physiological pH (pH=7) it tends to form dimmers [55]). Associated to this, compounds with lower pKa values elute faster, whereas compounds with higher pKa values tend to elute slower at lower pH because higher ionization tends to occur at lower pH [86]. Therefore, there were too many factors playing a crucial role for an optimal separation of the selected analytes and it was not possible to optimize them in order to be possible the chromatographic separation in the UPLC system and conditions available.

Although my best efforts by the use of several of gradients with different profiles, solvents constitutions, percentages and pH, in order to solve the problems refereed above, I was not succeeded in obtain Hcys and vitamins B-cofactors simultaneous quantification. In this sense, particularly Hcys quantification followed a different methodology than what was the initial objective of the study. Nevertheless, this new method sought accomplishes with the aim of the study in getting a simple, fast, cheaper and reliable method for the clinical quantification of Hcys. To achieve this, Hcys and vitamins B quantification and MEPS extraction were assayed using the same conditions previously reported at the experimental section.

3.2. Identification and quantification of Hcys

3.2.1. Chromatographic separation

As referred previously, the impossibility to resolve Hcys and vitamins B in a single chromatographic run, lead us to developing separated method for Hcys and vitamins B (B_6, B_9 and B_{12}) that accomplishes with the aims of the study.

From the set of different gradients previously tested (Fig. 28 and Annex I), the best gradient was selected and further optimized for the optimal separation of Hcys. This optimization involved column temperature and flow rate adjustments. Table 6 shows the optimal chromatographic conditions for Hcys separation.

Table 6 – Optimal chromatographic conditions for quantify the Hcys.

Chromatographic parameters	Optimal Chromatographic conditions		
Column	Analytical Column Waters Acquity UPLC HSS T3 (100 mm x 2.1 mm, 1.8 µm particle size)		
Gradient mobile phase program	**Time (min)**	**0.1% FA**	**ACN/ 0.1% FA**
	0	95	5
	0.5-1.5	100	0
	2	95	5
	3	80	20
	4-5	95	5
Flow rate	200 mL min^{-1}		
Column temperature	30 ºC		
Injected volume	2 µL		
UV detection wavelenght	280 nm		
Time of retention	1.25 min		

3.2.2. Methods for extracting Hcys

As referred in the experimental section (subtopic 2.5 of the chapter II), two extraction procedures were performed: 1) extraction by MEPS and 2) extraction by PP.

3.2.2.1. Extraction by MEPS

In order to improve the efficiency of Hcys MEPS extraction from plasma samples, the sorbents were optimized in the first place. For this evaluation, volumes in the crucial steps of the MEPS methodology identified in the experimental section as recovery points (Figure 26) were collected. The conditions assayed, C1, C4, and C7, are described in the Table 4. The Figure 28 shows the average peak area of Hcys obtained from each recovery point per sorbent and the direct injection of Hcys (25 ng per volume injected, corresponding to the same amount of Hcys in volume of standard solution loaded).

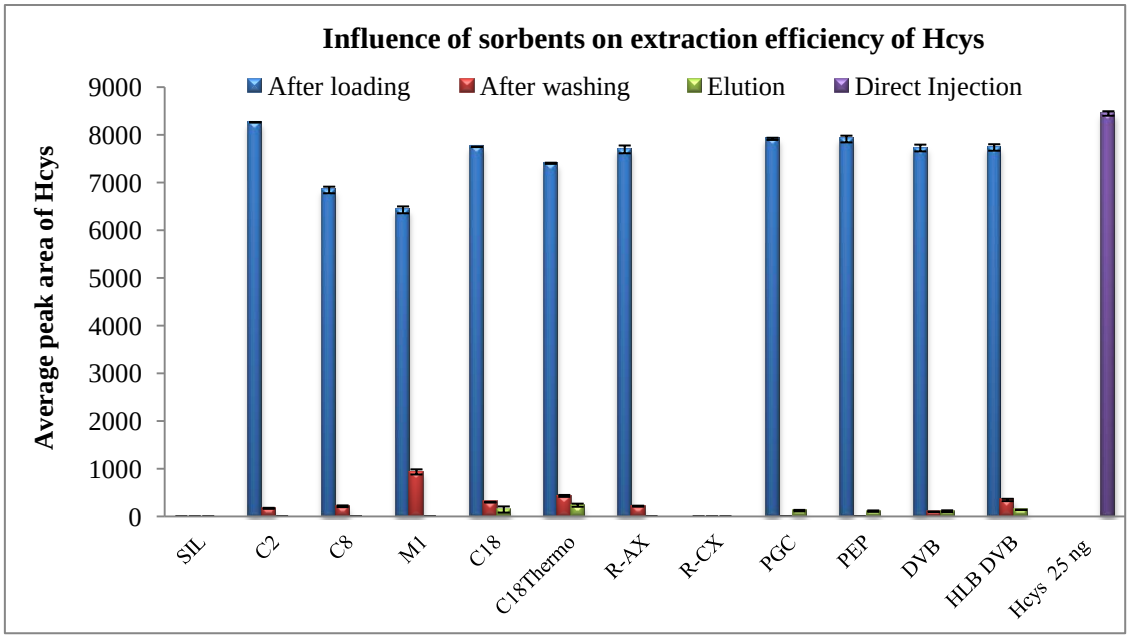


Figure 28 – Influence of sorbents on extraction efficiency of Hcys.

According to this figure, it is possible to note that all the sorbents had low levels of retention at the sample loading step. The values of recovery of all the sorbents ranged from 2 up to 10 % (Table 7). Even the very low amounts retained are mostly washed away in the washing step. This situation is clearly observed in the M1 fibre, which although presents the higher Hcys retention (10.59%), the analyte is lost during wash and nothing is recovered in the elution step. In opposition, there is only a very residual retention of Hcys in the PGC and PEP fibres (5.72% and 2.45%, respectively; Table 7) that is not lost during the wash step, being eluted in the final MEPS extraction step. Nevertheless, M1 was selected as the best sorbent for the next optimization steps due to its higher retention abilities for Hcys.

Table 7 –Influence of sorbents on the extraction efficiency of Hcys.

Sorbents	Recovery %
SIL	-
C2	2.18
C8	4.23
M1	10.59
C18	6.37
C18 (Thermo)	9.03
R-AX	3.87
R-CX	-
PGC	5.72
PEP	2.45
DVB	3.81
HLB DVB	7.04

The number of sample loading cycles was then performed. To do that, C1 (1x 200 µL), C2 (2 x 100 µL) and C3 (4 x 50 µL) conditions were evaluated and compared. The Figure 29 shows the average peak area of Hcys obtained from each recovery point per each condition.

According with the same figure, it was not possible to obtain a better extraction efficiency of Hcys. By contrast, it was noted that with the increase of number of cycles less Hcys were retained. This was also confirmed by the recovery rates (10.59 % for C1, 5.13% for C2 and 2.38% for C3).

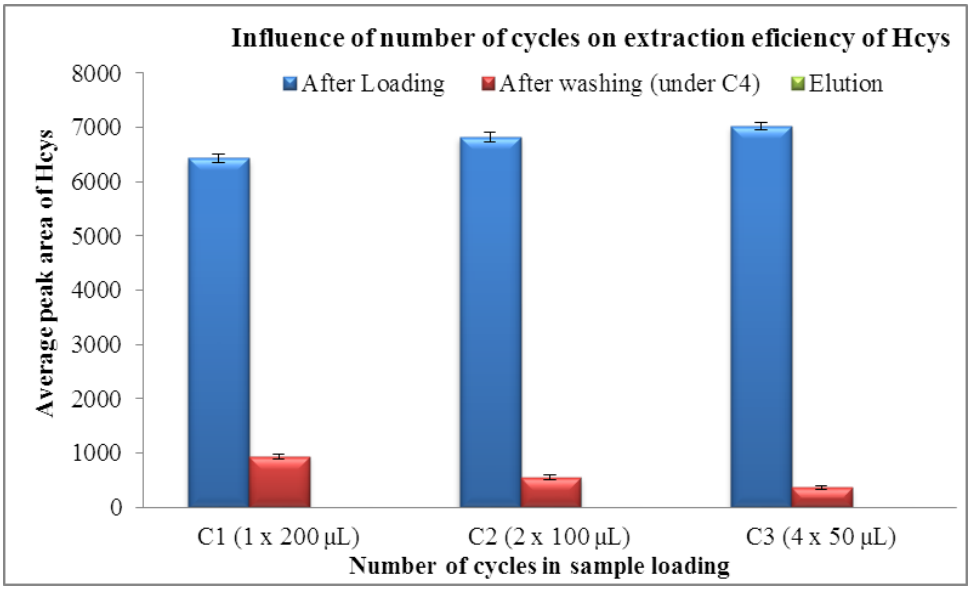


Figure 29 – Influence of number of cycles on extraction efficiency of Hcys.

The imperative of using a lower pH (3-4) to maintain the Hcys stabilized, results in a lower intermolecular interaction between the analytes and the compounds that constitute the sorbent, favouring the sorption of them [115]. This is most likely the main reason for the low recovery rates of Hcys and for the difficulty in improving its efficiency of extraction. In the annex II it is possible to see the most probably form of Hcys at the pH of 3-4.

Several others methods were adapted from SPE descriptions (Annex III) and used in an attempt to retain the Hcys on MEPS sorbents, at least with the same efficiency than the method here described. Nevertheless, attempts provide better results than the ones described.

3.2.2.2. Extraction by PP

Plasma PP was performed as described in the experimental section as an alternative to MEPS extraction. The Figure 30 shows a chromatogram of Hcys obtained from the use of protein precipitation in plasma samples.

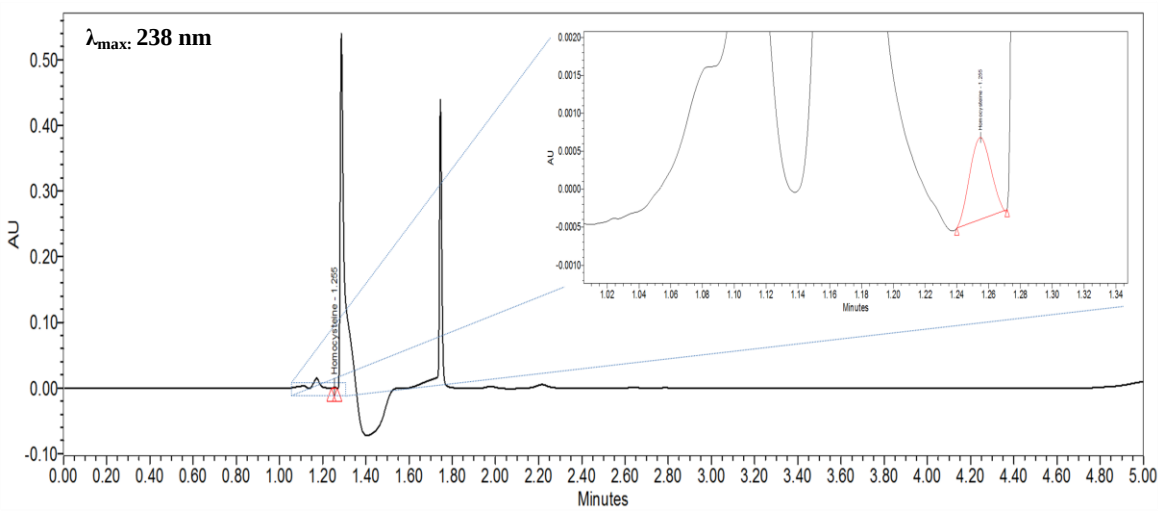


Figure 30 – Chromatogram of Hcys extraction by protein precipitation of plasma. Legend: 1| peak area of **12±2.4 µmol/L of Hcys.** The Hcys concentration was calculated by the equation of the line of the calibration curve.

To evaluate the extraction efficiency of Hcys by this method, it was performed a simulation of plasma PP. For this, it was substituted plasma for 200 µL of acidified water (0.05% FA) containing a known concentration of Hcys (25 ng/µL) and compared the peak area of Hcys obtained of from it with other peak are of Hcys obtained from a direct injection without PP. It was also performed a blank assay without Hcys.

The extraction efficiency of Hcys through PP (88.6%) was much higher than the obtained by MEPS.

3.2.2.3. Using MEPS and PP to improve Hcys quantification

According with results reported previously, PP is most efficiency to extract Hcys than MEPS. However, as it is possible to see at the Figure 30, there are some interfering compounds near the peak of Hcys that it would be interesting to improve.

In this way, MEPS was used following PP to try to cleanup samples from the interfering compound. In this way, after performing the PP of a fortified plasma sample (25 ng of Hcys), the supernatant (200 µL) was submitted to MEPS. C2 was the select sorbent, as it was the sorbent with less recovery rate (Table 7). The recovery point after sample loading was collected to compare with a sample from the same aliquot submitted only to PP.

The Figure 31 shows the average peak area of Hcys from a sample submitted only to PP and other that was performed protein precipitation and MEPS as cleanup (PP+MEPS).

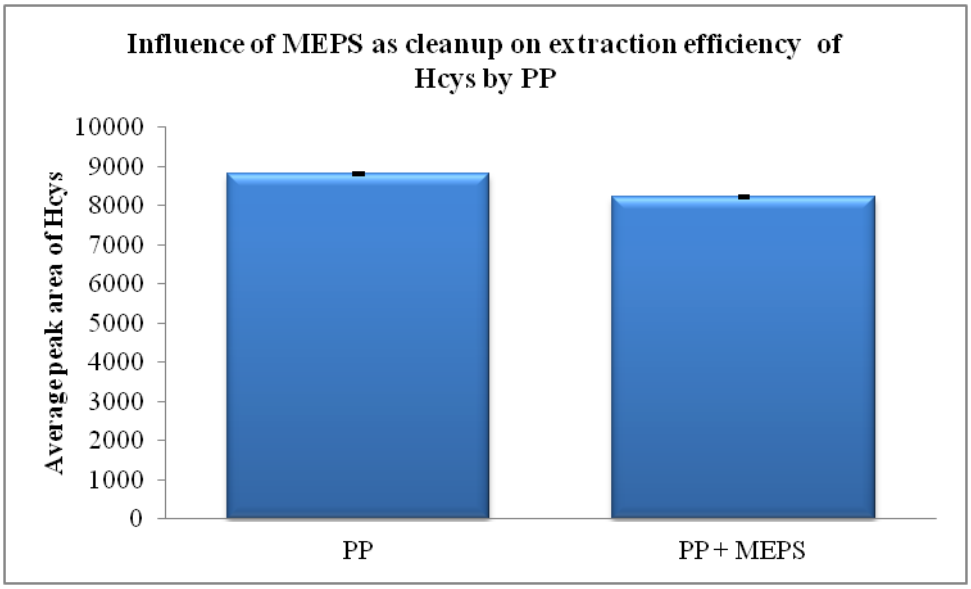


Figure 31 – Influence of MEPS on the extraction efficiency of Hcys by PP. Legend: PP| Plasma sample submitted only to protein precipitation; **PP+MEPS|** Plasma samples submitted to protein precipitation and MEPS.

According with the Figure 31, there is practically no differences regarding average peak area of Hcys of a sample submitted to PP and other submitted to PP and MEPS.

Concerning to interfering compounds near the peak of Hcys, as Figure 32 shows, there are almost any difference between the two samples. Therefore, MEPS, as cleanup technique, does not improve the chromatographic separation of Hcys and its utilization in Hcys extraction was completely discarded both as extraction or cleanup methodologies.

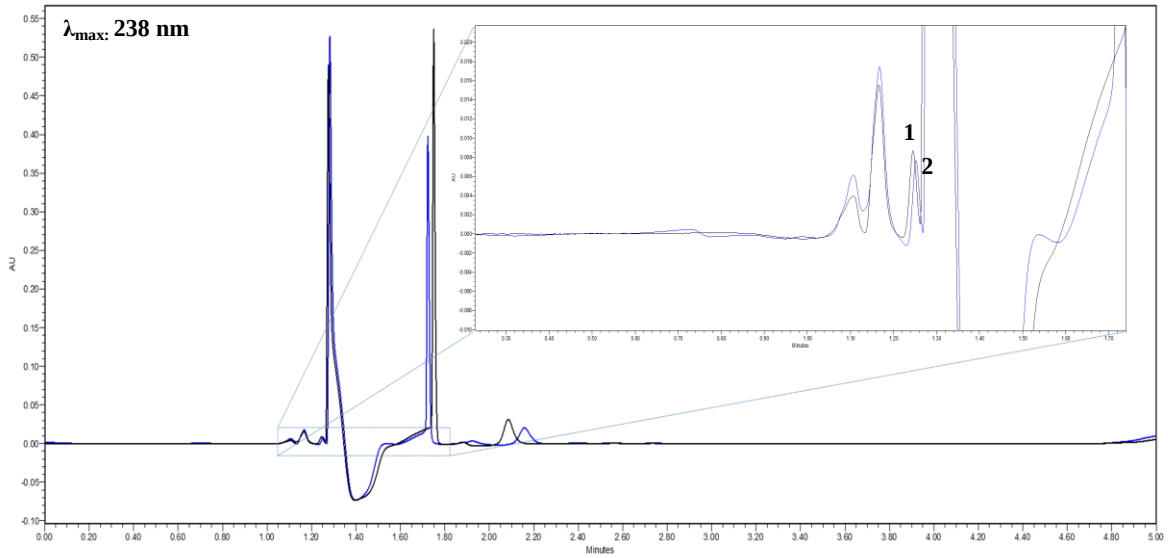


Figure 32 – Chromatogram of the influence of MEPS on efficiency of Hcys by protein precipitation. The black chromatogram corresponds to the plasma sample submitted only to protein precipitation, and the blue one corresponds to plasma samples submitted to protein precipitation and MEPS. **Legend: 1|** peak area of Hcys obtained from PP; **2|** peak area of Hcys obtained from PP followed by MEPS.

3.2.4. Validation the best method for Hcys assay

As reported before, PP allowed an extraction of Hcys more efficiently than MEPS and was therefore validated for the according with the following parameters:

a. Selectivity

The selectivity is an important analytical parameter to ensure that the quantification of the analytes of interest are not afected by the presence of interfering compounds in the matrix. For the evaluation of this parameter, the chromatogram obtained by a direct injection of the standards solution was compared with the chromatogram obtained by the direct injection of the plasma from a patient, verifying the absence of the interferents on the same time of retention of the Hcys. The selectivity was also checked by the resolution of the peak of Hcys in the plasma, that revealed the absence of co-eluting interferents.

b. Linearity

Another parameter that was evaluted was the linearity of the method, which can be defined as the ability of the method to obtain test results proportional to the concentration of the analyte within a given range of concentration [109]. The linearity for Hcys was estimated according to a range of concentrations which contains LL, ML and HL of Hcys according with what was expected to find in plasma. It was perfomed a calibration curve built by the three replicas of the

six calibration solutions (ranging from 4 to 555 µmol/L, as indicated in Table 5) prepared in acidified water (0.05% FA). The Figure 33 shows the calibration curve for Hcys and the Figure 34 shows the overlap of different chromatograms from each concentration used for the evaluation of linearity.

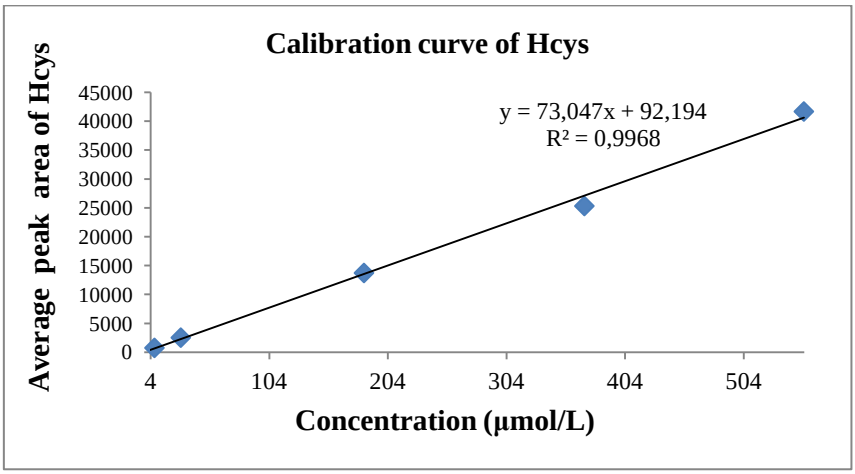


Figure 33 – Calibration curve of Hcys.

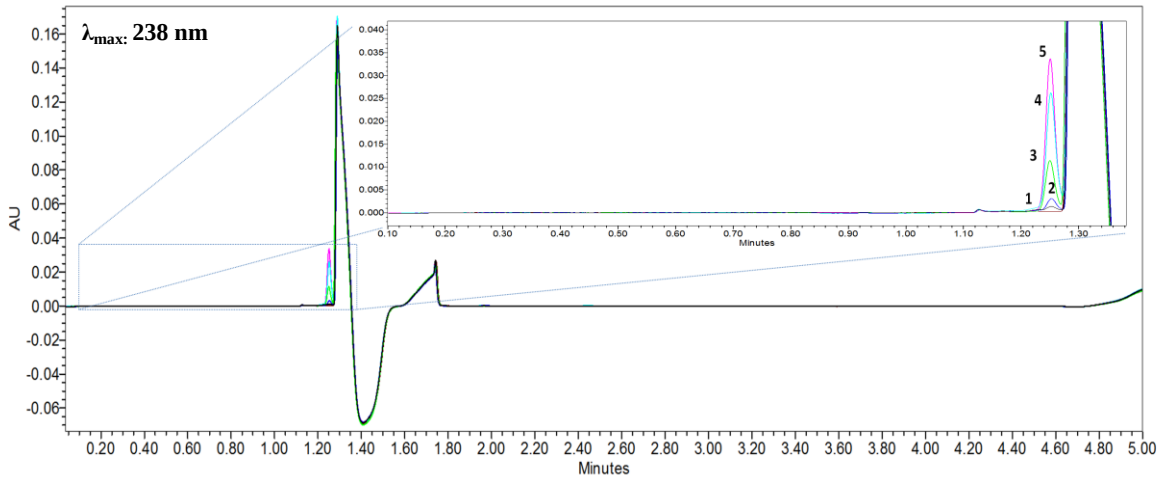


Figure 34 – Overlap of the chromatograms related to the linearity of the Hcys. Legend: Hcys peak corresponding to: 1| 7 µmol/L; **2|** 30 µmol/L; **3|** 184 µmol/L; **4|** 370 µmol/L; **5|** 555 µmol/L.

c. LOD and LOQ

The LOD and the LOQ were calculated by the lowest concentration of the analyte detected by UPLC. For LOD this value should be reliably distinguished from zero or be detected/measured with reasonable statistical certain. LOD should be three times higher than the noise level, being therefore calculated by 3 x SD. For LOQ this value should be determined quantitatively with an acceptable level of repeatability, precision and trueness, and should be ten

times higher than the nose level, being in this way, calculated by 10 x SD of the lowest concentration [109].

For Hcys , LOD and LOQ were calculated based on the lowest concentration used for the linearty, 4 µmol/L. The Table 8 presents the LOD and LOQ obtained for Hcys as well as the values obtained from other similar study that use the same equipment (UPLC and UV detector).

Table 8 – Comparison of the LOD and LOQ obtained with other study.

	Studies	LOD (µmol/L)	LOQ (µmol/L)
Hcys	Our study	**0.07**	**0.21**
	Valente *et al* [107]	0.05	0.15

According with Nekrassova *et al* (2003), the values of LOD and LOQ obtained in this studies are lower than the most of the common chromatographic techniques, used for quantify Hcys which do not use MS, and unlike this study, most of them require laborious samples pre-treatments and complex gradient programs with high column temperatures and longer chromatographic separations [55].

Valente *et al* (2012) obtained a much higher analytical performance than the one here developed, with LODs and LOQs at least five times lower than the reported limits in the literature. However, unlike this study, their method uses derivatizing agentes (SDB-F and ABD-F) that need at least 40 min to react with thiol groups, a mobile-phase composition based on phosphate buffer (30-100mM) and a chromatographic run of 10 min (Hcys retention time of 8.5 min) [107].

d. Precision

The precision for the method here presented, expressed by the RSD, was evaluated by the injection of three concentration levels corresponding to 7µmol/L to LL, 184 µmol/L to ML and 555 µmol/L HL. To obtain the repeatability (precision intra-day), it was injected six replicas of each concentration (n=6). For the evaluation of reproducibility (precision inter-day), it were injected six replicas of each concentration during three non-consecutive days (n=18). The Table 9 presents the RSD values of each concentration for the precision intra-days and inter-days.

Table 9 –Intra-day and inter-day precision.

	Levels of concentration	RSD (%)
Intra-day (n=6)	LL	3.32
	ML	1.23
	HL	1.31
Inter-day (n=18)	LL	6.15
	ML	6.99
	HL	5.43

According with United Nations Office on Drugs and Crime, this value should be better than 20% at lower concentrations and better than 15% at higher concentrations [116]. In this sense, the results obtained in this study do not fulfil this criteria for the validation of analytical methodologies, as all the RSD values obtained are bellow than 15%. The precision intra-day ranged from 1.31 to 3.32 %, whereas the precision inter-day ranged from 5.43% to 6.99%.

e. Recovery

This evaluation was performed by the use of three levels of different concentrations, 7 μmol/L (LL), 184 μmol/L (ML) and 555 μmol/L (HL). The values of recovery percentage were calculated according with the following equation: recovery percentage = 100 x (concentration of the fortified standard – concentration of the standard)/ added analyte concentration.

The Table 10 shows the recovery values of the three levels of concentration.

Table 10 – Values of recovery.

Concentration	Recovery (%)
7 µmol/L	93.1
184 µmol/L	88.5
555 µmol/L	82.5

f. Matrix effect

The matrix effect was estimated by the percentage of the quotient between the slopes of the standard in blank and the obtained by fortified plasma, through the use of three Hcys levels of concentration, 7 µmol/L (LL), 184 µmol/L (ML) and 555 µmol/L (HL). The three levels of concentration were added to the plasma by the standard addition method.

According Figure 35, it is possible to observe that there is a matrix effect for Hcys (>120 %). The value of matrix effect was estimated by the percentage of the quotient between the slopes of the standards in blank matrix and those obtained by fortified plasma.

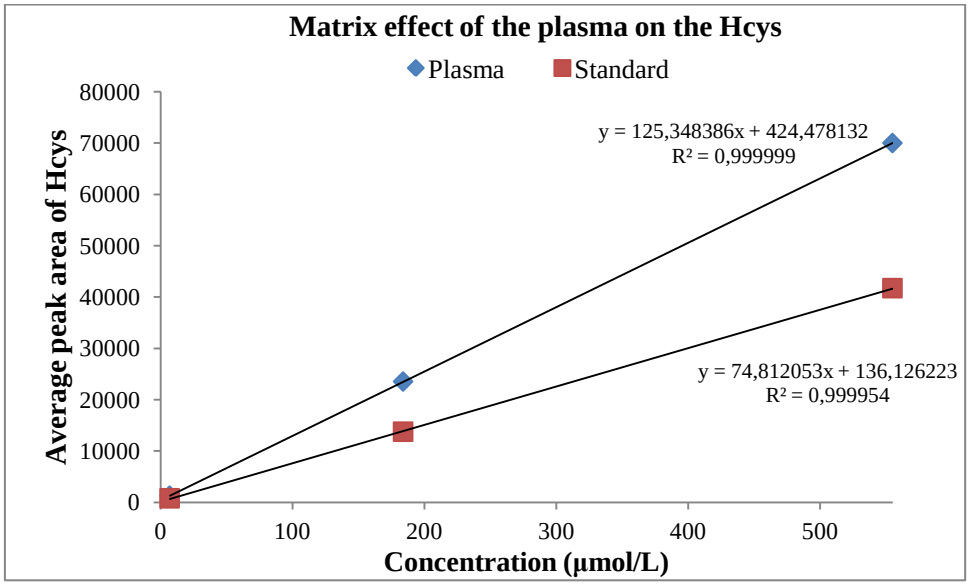


Figure 35 – Matrix effect of the plasma on the Hcys.

3.2.4. Clinical application of the methodology for Hcys quantification

After the validation of the methodology to quantify Hcys, five plasma of patients with CVD were quantified. The table 11 summarizes the results for Hcys concentration in each sample.

Table 11 – Values of the clinical application of the method for quantify the Hcys.

	Hcys	Measure Mean + RSD (µmol/L)
Samples	CVD 1	4.3 ± 1.2
	CVD 2	0.4 ± 0.2
	CVD 3	13.8 ± 4.1
	CVD 4	23 ± 4.6
	CVD 5	4.6 ±0.9

From our results, only two patients had Hcys levels higher than normal (5-10 µmol/L), but only one had a level that is considered as HHcys (23 ± 4.6 µmol/L) However, due to the sample size, and the lack of information of the clinical situtation of the patients it is not possible to make any conclusion in relation to high levels of homocysteine and CVDs. This clinical application of the method serves only to demonstrate the feasibility of this method in the Hcys quantification.

3.3. Identification and quantification of vitamins B

3.3.1. Chromatographic separation

As already discussed, a different methodology from the one used in Hcys, have been adopted to identify and quantify the three vitamins B. After various optimizations of gradient programs (Annex I), columm types (T3 HSS and BEH C18) and column temperatures, it was found an optimal condition to separate all the three vitamins in the same chromatographic method. The table 12 summarizes all the parameters that have contributed for a sucessful chromatographic separation of the standards containing the three vitamins. This search for best chromatographic method involved the direct injection of the standard solutions containing all the vitamins, leaving the optimization of the extractive technique for the following steps.

Table 12 – Optimal chromatographic conditions for quantify the vitamins B-cofactors.

Chromatographic parameters	Optimal Chromatographic conditions		
Column	Analytical Column Waters Acquity UPLC HSS T3 (100 mm x 2.1 mm, 1.8 µm particle size)		
Gradient mobile phase program	**Time (min)**	**0.05% FA**	**Methanol**
	0-0.5	80	20
	1	40	60
	1.5-2	80	20
	3	70	30
	4-5	80	20
Flow rate	200 mL min^{-1}		
Column temperature	30 ºC		
Injected volume	5 µL		
UV detection wavelenght	**Vitamin B_6**	290 nm	
	Vitamin B_9	284 nm	
	Vitamin B_{12}	360 nm	
Time of retention	**Vitamin B_6**	1.2 min	
	Vitamin B_9	3.8 min	
	Vitamin B_{12}	3.5 min	

The Figure 36, shows the chromatogram of the best chromatographic contition obtain from the direct injection of the standard solutions of the three vitamins B.

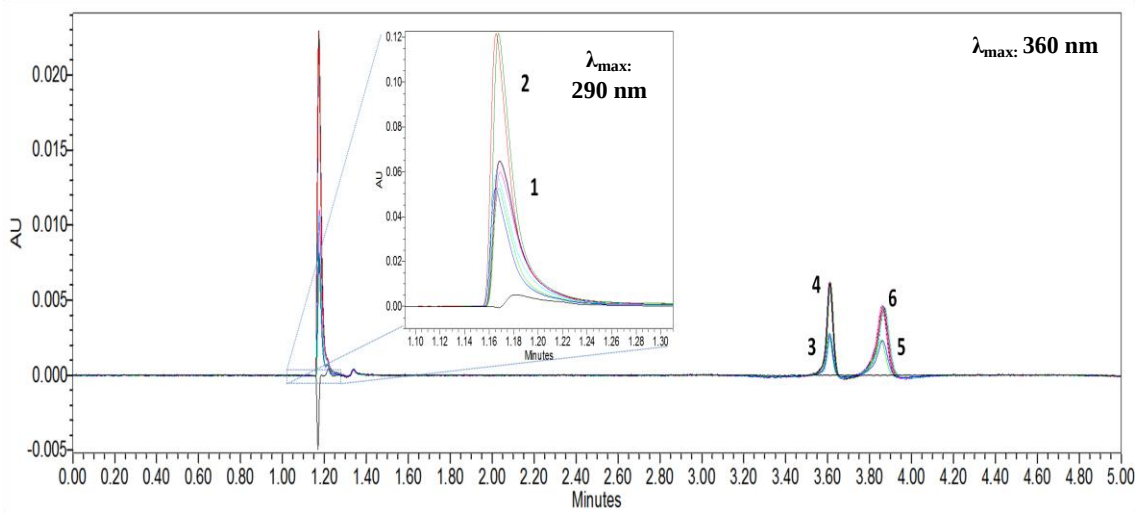


Figure 36 - Chromatograms of vitamins B standards obtained by the optimal chromatographic condition. Legend: Peak identification: **Vitamin B_6: 1|** 6.5 µmol/L (1.6 ng/µL**)** and **2|** 13 µmol/L (3.2 ng/µL); **Vitamin B_{12}: 3|** 1 µmol/L (1.6 ng/µL) and **4|** 2 µmol/L (3.2 ng/µL); **Vitamin B_9: 5|** 0.400 µmol/L (1.6 ng/µL) and **6|** 0.800 µmol/L (3.2 ng/µL).

Vitamins exist in vestigial levels in biological matrix, so it was necessary an extraction technique to separate and concentrate them, bellow the sensitivity levels of the equipment.

3.3.2. Optimization the MEPS for extracting the vitamins B_6, B_9 and B_{12}

The optimization of the vitamins extraction using MEPS followed the layout defined in the Table 4. Accordingly, the optimization began with the selection of the best sorbent, followed by optimization of the number of sample loading cycles, the volume of washing, and the percentage of organic solvent of the elution solution. This optimization involved the collection of the recovery points after loading sample, after washing and elution, as previously defined and illustrated in the Figure 26.

For all the optimization procedures, the sorbent was loaded with 320 ng from each vitamin B in 200 µL of acidified water (0.05% FA). In the literature is reported the fortification of 40 µL of plasma for SPE extraction [108]. In this work, we preferred to use a higher sample volume (200 µL) in order to obtain higher detection sensitivity, as vitamins B are at vestigial levels in plasma.

a. Selection of the best fibre

For selecting the best sorbent, which has more analytes retained, it was evaluated twelve sorbents, SIL, C2, C8, M1, C18, C18 Thermo, R-AX, R-CX, PGC, PEP, DVB and HLB DVB, using, the C4, C7 conditions described in Table 4 (briefly, 200 µL of sample loading, 250 µL 0.05% FA as the washing volume and elution with 80% MetOH/0.05%FA). The elution recovery point of each sorbent was used for the analysis of the peak area of vitamins, in order to compare all the sorbents, as shown in Figure 37.

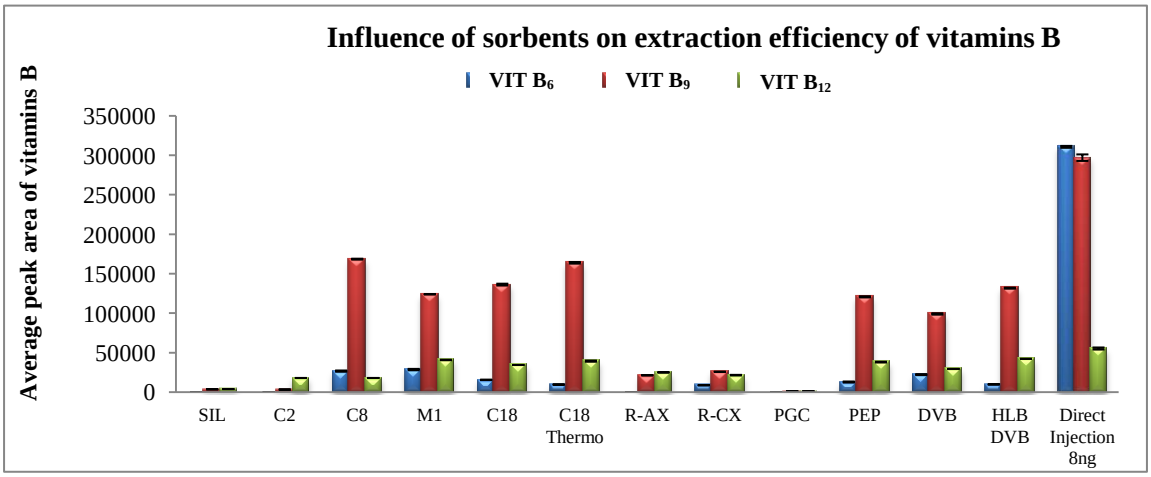


Figure 37 – Comparison of the influence of MEPS sorbents and direct injection on the extraction efficiency of vitamins B.

According with Figure 37, it is possible to note that M1 is the best sorbent for the simultaneous extraction of the three vitamins. A direct injection of a standard solution containing 8 ng of each vitamin was used as control (equivalent to a value of 100% of recovery of the analytes upon the MEPS extraction).

b. Optimization of the number of loading cycles

After the selection of the best fibre, M1, it was evaluated the optimal number of sample loading cycles in this sorbent. In this way, it was performed the evaluation the conditions of number of loading cycles in order to select the best cycle for a better retention of all analytes: C1 (1 X 200 µL); C2 (2 x 100 µL) and C3 (4 x 50 µL).

For this evaluation, it was used a standard solution containing 320 ng/ 200µL, and it was collected the recovery points, after loading and elution (80%MetOH/0.05%FA) of each sample loading condition. C1, C2 and C3 indicated in Table 4 (respectively 1 x 200 µL, 2 x 100 µL and 4 x 50 µL), using 320 ng of each vitamin / assay, and it were collected the recovery points, after loading and elution (condition C7 for elution: 80%MetOH/0.05%FA) of each sample loading condition. The washing condition used for this evaluation was the condition C4 (250 µL of 0.05% FA).

The Figure 38 shows the average peak area of vitamins B in each recovery point.

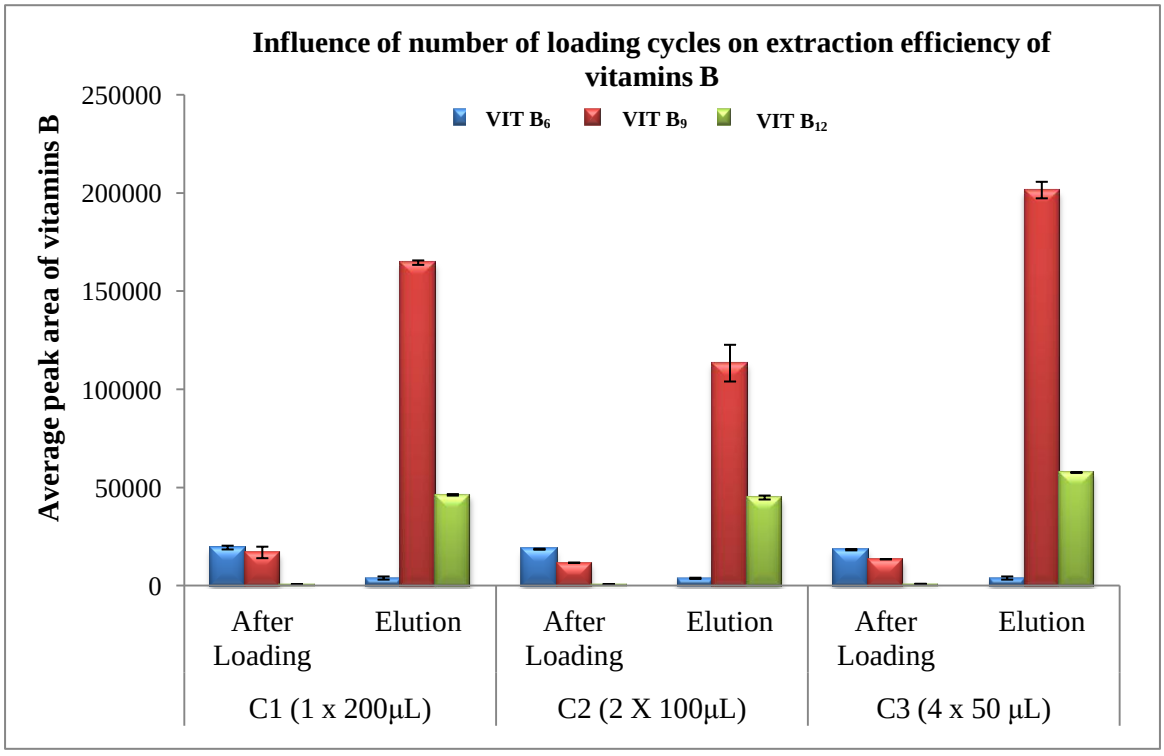


Figure 38 – Influence of number of loading cycles on extraction efficiency of vitamins B.

According with this figure it is possible to note that the best cycle corresponds to four sample loading cycles of 50 µL each one (C3 condition), being the condition that presents cumulatively the higher extraction efficiency for the three vitamins analysed.

c. Optimization of the washing volume

The next step of the MEPS optimization was the washing volume (conditions C4 and C5, 100µL or 250 µL of acidified water, respectively, see Table 4 for details). Both the rejected volume after each washing step and the correspondent elution phase (C7 condition: 80% MetOH/0.05%FA) were collected for comparison. For the evaluation of this procedure it was used the number of cycles previously optimized and a standard solution with the same concentration of vitamins B. Figure 39 shows the washing effect in the vitamins retention. As can be depicted from the figure, a higher washing volume corresponds to a higher loss of the target analytes (condition C4).

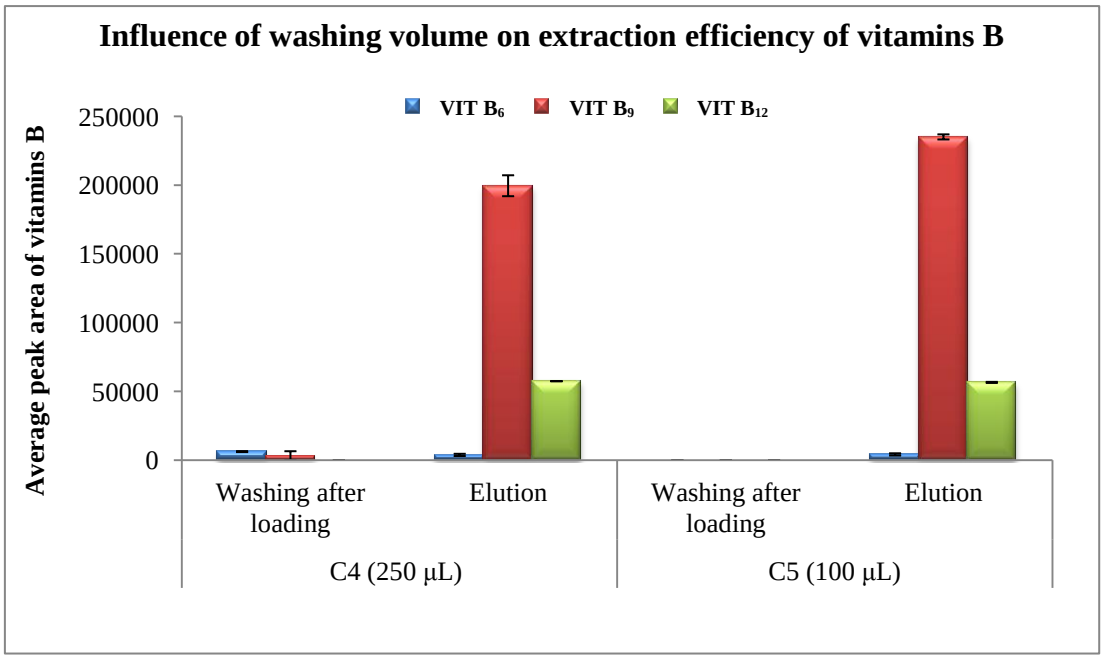


Figure 39 – Influence of washing volume on the extraction efficiency of vitamins B.

According the previous graphic, it is possible to note that the best washing volume was 100 µL (C5), as it presents less loss of vitamins at the washing step and more vitamins at the correspondent elution phase.

.

d. Optimization of the elution system

At last, once the best washing volume was chosen, the percentage of organic solvent at the elution phase was evaluated. Thus, three elution conditions corresponding to 60% (C6), 80% (C7) and 90% (C8) of acidified methanol (0.05% FA), were assayed in order to observe which one favours a higher recovery of the target analytes retained in the MEPS fibre. The Figure 42 shows the different elution phases and amount of analytes eluted.

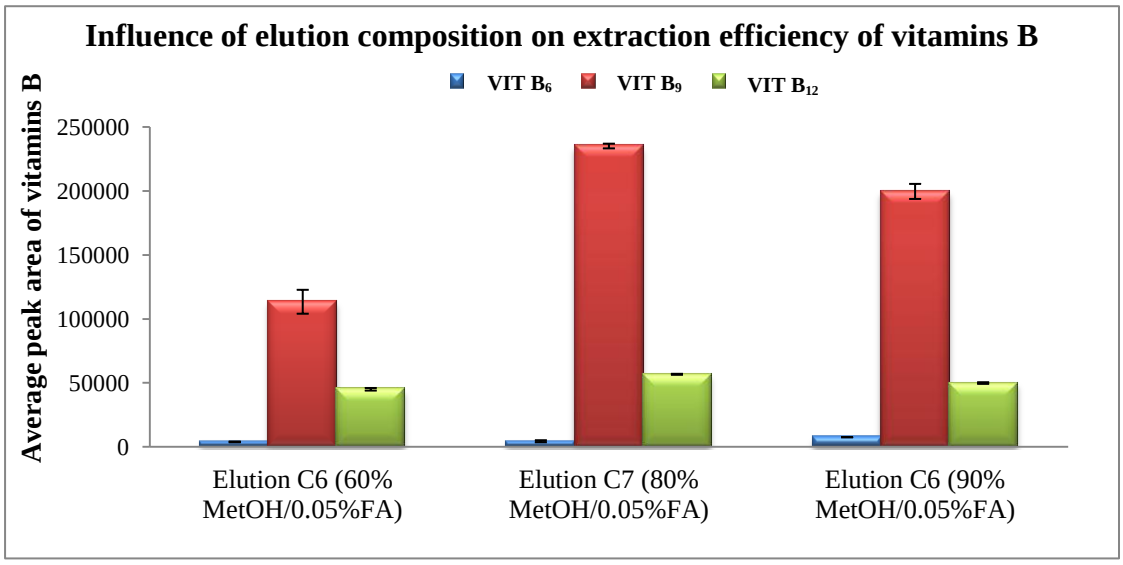


Figure 40 – Influence of elution composition system on the extraction efficiency of vitamins B.

According to Figure 40, it is possible to observe that the best percentage of methanol in the elution solution is 80% for all three vitamins B (condition 7).

3.3.3. Validation of the method for vitamins B

After the optimization of the MEPS procedure for vitamins extraction, the validation of whole analytical methodology using calibrations curves was performed with the vitamins solutions indicated in the experimental section (Table 5).

The selectivity parameter was performed in first place to ensure that the quantification of the vitamins B were not afected by the presence of interfering compounds of the matrix. For the evaluation of this parameter, the chromatogram obtained in the standards solution MEPS extraction was compared with the one corresponding to a plasma sample and verified the inexistence of inferents at the same retention time of each vitamin. . As we can observe in Figure 41, there are no interfering compounds at the retention times for the three vitamins B analysed. In the same figure, however, we can also depicted that the sensibility of the analytical method is very low and it is not yet suitable to detected the vestigial levels of vitamins usually present in plasma samples. In the upper painel of Figure 41, we can observe that the 320 ng of

each vitamin B that were used to fortify the 200 µL plasma sample are hardly distinguished from the basal line.

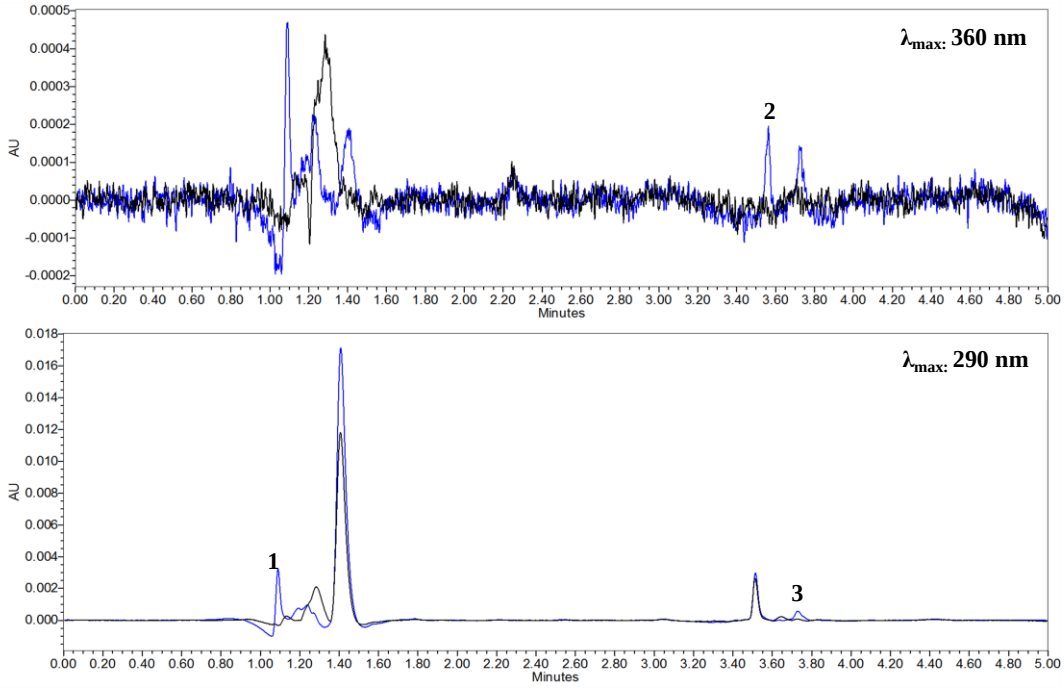


Figure 41 – Chromatograms of the detection of vitamins B in plasma samples. Legend: The black chromatogram corresponds to the plasma sample without fortification and the blue corresponds to the fortified plasma (320 ng/200 µL). **1|** peak area of 6.5 µmol/L of vitamin B_6; **2|** peak area of 1 µmol/L of vitamin B_{12}; **3|** peak are of 0.4 µmol/L of vitamin B_9.

In the next step, linearity was evaluated for the higher concentration of vitamins solutions (Table 5). This parameter was performed to evaluate the viability of this method to extract and quantify efficiency vitamins B from other biological samples, once it has not yet the sensibility necessary for plasma samples. However, it was not possible to obtain the necessary calibration curves as it were obtained uncoherent values due to a non-identified experimental error. Unfortunatelly, the time contraints did not allowed to go further in the validation of vitamins B quantification using MEPS extraction followed by LC-UV analysis. This approach has potentiality to be suitable for this purpose as Chatzimichalakis *et al* (2004) succeeded in developing a method for quantify vitamins in biological fluids, using SPE as the extraction technique, followed by HPLC –UV analysis [108]. Nevertheless, their study worked with higher levels of vitamins concentration. Despite their study used only 40 µL of plasma for extraction, they spiked the aliquots with 100 µL of water soluble vitamin solutions at concentration levels of 1, 2, 5, 10 and 15 ng/µL, injecting 20 µL of the 100 µL of the elution phase.

4. Conclusion

The experimental work here described allowed to reach part of the objectives initially proposed, while other require more time to be achieved. We had developed a simple, fast and cheaper methodology that allows the proper quantification of Hcys in biological samples using only 7 min, including the sample process and the chromatographic separation. This method is easy and suitable for the clinical environment, just involving a protein precipitation of the plasma samples followed by direct injection in the UPLC system and UV detection. This methodology is also even faster than initial anticipated because MEPS extraction was shown not to be efficient enough in comparison with the protein extraction. Previously, Persichilli *et al* [88] also reported the plasma protein precipitation as extraction procedure for the Hcys quantification. Our methodology is, however, simpler, faster and cheaper as it is performed in an UPLC and does not involve the expensive MS detection. Unfortunately, it was not possible to conjugate Hcys and vitamins B_6, B_9 and B_{12} in a single methodology. This was previously described [86], but using chromatographic conditions not recommended for the UPLC system available, namely buffered gradients. Moreover, we also only have two different chromatographic columns available (C18 BEH and HSS – high strenght silica), which clearly limit our options to separate such different molecules.

A separated methodology to quantify vitamins B_6, B_9 and $B_{12,}$ was therefore developed, although not fully validated due to the limited time available to perform this work. Different parameters were already optimized, including the MEPS extraction conditions as the best fibre, sample volume loading, washing volume and elution solution composition.

For future perspectives, we suggest the optimization of the method to the clinical environment, preferentially combining the four analytes used in this study in a single methodology by using new gradients conditions and other chromatographic columns.

5. References

1. Celermajer D, Chow C, Marijon E, Anstey N, Woo K. Cardiovascular disease in the developing world: prevalences, patterns, and the potential of early disease detection. Journal of the American College of Cardiology 2012 Oct 2;60(14):1207-16.
2. WHO. Global status report: on noncommunicable diseases. World Health Organization 2011.
3. WHO. Global Atlas on cardiovascular disease prevention and control. World Health Organization 2011.
4. Mathers C, Loncar D. Projections of Global Mortality and Burden of Disease from 2002 to 2030. Plos Medicine 2006;3(11):2011-30.
5. WHF. Cardiovascular disease: Risk factors. Available from: http://www.world-heart-federation.org/fileadmin/user_upload/documents/Fact_sheets/2012/PressBackgrounderApril2012RiskFactors.pdf.
6. Rader D, Daugherty A. Translating molecular discoveries into new therapies for atherosclerosis. Nature 2008 Feb 21;451(7181):904-13.
7. Born G, Medina R, Shafi S, Cardona-Sanclemente L. Endothelial factors in the flux of atherogenic plasma proteins into artery walls. Clinical Hemorheology and Microcirculation 2002;26:107-16.
8. Pugsley M, Tabrizchi R. The vascular system: An overview of structure and function. Journal of Pharmacological and Toxicological Methods 2000;44:333-40.
9. Hansson G. Inflammation, Atherosclerosis, and Coronary Artery Disease. The New England Journal of Medicine 2005;352(16):1685-95.
10. Glass C, Witztum J. Atherosclerosis: The Road Ahead. Cell 2001 Feb 23;104:503-16.
11. Vogel R. Coronary Risk Factors, Endothelial Function, and Atherosclerosis: A Review. Clinical Cardiology 1997;20:426-32.
12. Gallino A, Stuber M, Crea F, Falk E, Corti R, Lekakis J, et al. "In vivo" imaging of atherosclerosis. Atherosclerosis 2012 Sep;224(1):25-36.
13. Sitia S, Tomasoni L, Atzeni F, Ambrosio G, Cordiano C, Catapano A, *et al*. From endothelial dysfunction to atherosclerosis. Autoimmunity Reviews 2010 Oct;9(12):830-4.
14. Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. Circulation 2004 Jun 15;109(23 Suppl 1):III27-32.
15. Brevetti G, Schiano V, Chiariello M. Endothelial dysfunction: a key to the pathophysiology and natural history of peripheral arterial disease? Atherosclerosis 2008 Mar;197(1):1-11.
16. Andor M. The role of inflammation in endothelial dysfunction and progression of atherosclerosis in metabolic syndrome. Timisoara Medical Journal 2005;55(4):330-4.
17. Celermajer D. Endothelial dysfunction: Does it matter? Is it reversible? Journal of the American College of Cardiology 1997;30(2):325-33.
18. Schiffrin E. Remodeling of resistance arteries in essential hypertension and effects of antihypertensive treatment. American Journal of Hypertension 2004;17(12):1192-200.
19. Belin de Chantemele E, Stepp D. Influence of obesity and metabolic dysfunction on the endothelial control in the coronary circulation. Journal of Molecular and Cellular Cardiology 2012 Apr;52(4):840-7.
20. Hegele R. Plasma lipoproteins: genetic influences and clinical implications. Nature Reviews Genetics 2009 Feb;10(2):109-21.
21. Saba A, Oridup O. Lipoproteins and Cardiovascular Diseases. In: InTech, editor. Lipoproteins - role in health and diseases 2012. p. 197-222.
22. Wasan K, Brocks D, Lee S, Sachsbarrable K, Thornton S. Impact of lipoproteins on the biological activity and disposition of drugs: implications for drug discovery. Nature Reviews Drug Discovery 2008;7:84-99.
23. Salway J. Compêndio de bioquímica médica 2006.

24. Fogelstrand P, Boren J. Retention of atherogenic lipoproteins in the artery wall and its role in atherogenesis. Nutrition, Metabolism & Cardiovascular Diseases 2012 Jan;22(1):1-7.
25. Camejo G, Hurt-Camejo E, Wiklund O, Bondjers G. Association of apo B lipoproteins with arterial proteoglycans: Pathological significance and molecular basis. Atherosclerosis 1998;139:205-22.
26. Badimon L, Vilahur G, Padro T. Lipoproteins, platelets and atherothrombosis. Revista Española de Cardiología 2009;62(10):1161-78.
27. Niki E. Lipid peroxidation: Physiological levels and dual biological effects. Free Radical Biology & Medicine 2009;47(5):469-84.
28. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. Nature Medicine 2011;17(11):1410-22.
29. Yoshida H, Kisugi R. Mechanisms of LDL oxidation. Clinica Chimica Acta 2010 Dec 14;411(23-24):1875-82.
30. Osterud B, Bjorklid E. Role of Monocystes in Atherogenesis. Physiological Reviews 2003;83:1069-112.
31. Ribeiro F, Alves A, Duarte J, Oliveira J. Is exercise training an effective therapy targeting endothelial dysfunction and vascular wall inflammation? International Journal of Cardiology 2010 Jun 11;141(3):214-21.
32. Maiuri M, Grassia G, Platt A, Carnuccio R, Ialenti A, Maffia P. Macrophage autophagy in atherosclerosis. Mediators of Inflammation 2013;2013:584715.
33. Hansson G, Libby P. The immune response in atherosclerosis: a double-edged sword. Nature Reviews Immunology 2006 Jul;6(7):508-19.
34. Fishbein M. The vulnerable and unstable atherosclerotic plaque. Cardiovascular Pathology 2010 Jan-Feb;19(1):6-11.
35. Plutzky J. Atherosclerotic plaque rupture: Emerging insights and opportunities. The American Journal of Cardiology 1999;84(1A):15J-20J.
36. Ducros V, Demuth K, Sauvant M, Quillard M, Caussé E, Candito M, *et al*. Methods for homocysteine analysis and biological relevance of the results. Journal of Chromatography B 2002;781:207-26.
37. Dwivedi M, Tripathi A, Shukla S, Khan S, Chauhan U. Homocysteine and cardiovascular disease. Biotechnology and Molecular Biology Review 2011;5(5):101-7.
38. Mosharov E, Cranford M, Banerjee R. The Quantitatively important relationship between homocysteine metabolism and glutathione synthesis by the transsulfuration pathway and its regulation by redox changes. Biochemistry 2000;39(42):13005-11.
39. Sharma P, Senthilkumar R, Brahmachari V, Sundaramoorthy E, Mahajan A, Sharma A, *et al*. Mining literature for a comprehensive pathway analysis: a case study for retrieval of homocysteine related genes for genetic and epigenetic studies. Lipids in Health and Disease 2006;5:1.
40. Nygard O, Voliset S, Refusem H, Bratistrom I, Ueland P. Total homocysteine and cardiovascular disease. Journal of Internal Medicine 1999;246:425-54.
41. Royal Society of Chemisty. Chemspider: Homocysteine. 2013; Available from: http://www.chemspider.com/Chemical-Structure.757.html?rid=db0ff207-058b-4f97-8288-50a7f8301394.
42. Hoffman M. Hypothesis: hyperhomocysteinemia is an indicator of oxidant stress. Medical Hypotheses 2011 Dec;77(6):1088-93.
43. Medina M, Urdiales J, Amores-Sánchez M. Roles of homocysteine in cell metabolism old and new functions. European journal of biochemistry 2001;268:3871-82.
44. Stein J, McBride P. Hyperhomocysteinemia and atherosclerotic vascular disease. Archives of Internal Medicine 1998;158:1301-6.
45. Park J, Vollset S, Melse-Boonstra A, Chajes V, Ueland P, Slimani N. Dietary intake and biological measurement of folate: a qualitative review of validation studies. Molecular Nutrition & Food Research 2013 Apr;57(4):562-81.

46. Nygard O, Refsum H, Ueland P, Vollset S. Major lifestyle determinants of plasma total homocysteine distribution: the horland homocysteine study. The American Journal of Clinical Nutrition 1998;67:263-70.
47. Guldener C. Why is homocysteine elevated in renal failure and what can be expected from homocysteine-lowering? Nephrology Dialysis Transplantation 2006 May;21(5):1161-6.
48. Bree A, Verschuren W, Blom H, Kromhout D. Lyfestyle factors and plasma homocysteine concentration in a general population sample. American journal of Epidemiology 2001;154:150-4.
49. Krajinovic M, Lamothe S, Labuda D, Lemieux-Blanchard E, Theoret Y, Moghrabi A, *et al*. Role of MTHFR genetic polymorphisms in the susceptibility to childhood acute lymphoblastic leukemia. Blood 2004 Jan 1;103(1):252-7.
50. Cakmak S, Guil U, Kiliç C, Gonul M, Soylu S, Kiliç A. Homocysteine, vitamin B12, and folic acid levels in psoriasis patients. Journal of the European Academy of Dermatology and Venereology 2009;23(3):300-3.
51. Cartarqi B, Parrot-Roulad F, Cochet C, Doucassous D, Roger P, Tabarin A. Homocysteine, hypothyroidism, and effect of thyroid hormone replacement. Thyroid 1999;9(12):1163-6.
52. Sanchez-Margalet V, Valle M, Ruz F, Gascon F, Mateo J, Goberna R. Elevated plasma total homocysteine levels in hyperinsulinemic obese subjects. The Journal of Nutritional Biochemistry 2002;13(2):75-9.
53. Wollesen F, Bratistrom L, Refsum H, Ueland P, Berglund L, Berne C. Plasma total homocysteine and cysteine in relation to glomerular filtration rate in diabetes mellitus. Kidney International 1999;55:1028-35.
54. Faraci F, Lentz S. Hyperhomocysteinemia, oxidative stress, and cerebral vascular dysfunction. Stroke; A Journal of Cerebral Circulation 2004 Feb;35(2):345-7.
55. Nekrassova O. Analytical determination of homocysteine: a review. Talanta 2003;60(6):1085-95.
56. Jacobsen D. Hyperhomocysteinemia and Oxidative Stress : Time for a Reality Check? Arteriosclerosis, Thrombosis, and Vascular Biology 2000;20(5):1182-4.
57. McDowell I, Derek L. Homocysteine and endothelial dysfunction: A link with cardiovascular disease. The Journal of Nutrition 2000;130:369s-72S.
58. Wang H, Tan H, Yang F. Mechanisms in homocysteine-induced vascular disease. Drug Discovery Today: Disease Mechanisms 2005;2(1):25-31.
59. Fox P, Mazumder B, Ehernwald E, Mukhopadhyay C. Ceruloplasmin and cardiovascular disease. Free Radical Biology & Medicine 2000;28(12):1735-44.
60. Exner M, Hermann M, Hofbauer R, Hartmann B, Kapiotis S, Gmeiner B. Homocysteine promotes the LDL oxidase activity of ceruloplasmin. FEBS Letters 2002;531:402-6.
61. Antoniades C, Antonopoulos A, Tousoulis D, Marinou K, Stefanadis C. Homocysteine and coronary atherosclerosis: from folate fortification to the recent clinical trials. European Heart Journal 2009 Jan;30(1):6-15.
62. Au-Yeung K, Woo C, Sung F, Yip J, Siow Y, Karmin O. Hyperhomocysteinemia activates nuclear factor-kbB in endothelial cells via oxidative stress. Circulation Research 2004;94:28-36.
63. Jia F, Wu C, Chen Z, Lu G. Atorvastatin inhibits homocysteine-induced endoplasmic reticulum stress through activation of AMP-activated protein kinase. Cardiovascular Therapeutics 2012 Dec;30(6):317-25.
64. Tayal D, Goswami B, Koner B, Mallika V. Role of Homocysteine and Lipoprotein (A) in atherosclerosis: An update. Biomedical Research 2011;22(4):391-405.
65. Olas B, Kolodziejczyk J, Kedzierska M, Rywaniak J, Wachowicz B. Modification of human blood platelet proteins induced by homocysteine and its thiolactone in vitro. Thrombosis Research 2009 Dec;124(6):689-94.

66. Malinowska J, Tomczynska M, Olas B. Changes of blood platelet adhesion to collagen and fibrinogen induced by homocysteine and its thiolactone. Clinical Biochemistry 2012 Oct;45(15):1225-8.
67. Manolescu B, Oprea E, Farcasanu I, Berteanu M, Cercasov C. Homocysteine and vitamin therapy in stroke prevention and treatment: a review. Acta Biochimica Polonica 2010;57(4):467-77.
68. Langman L, Cole D. Homocysteine cholesterol of the 90s? Clinica Chimica Acta 1999;286:63-80.
69. Lonn E, Yusuf S, Arnold M, Sheridan P, Pogue J, Micks M, *et al*. Homocysteine lowering with folic acid and B vitamins in vascular disease. . The New England Journal of Medicine 2006;354(15):1567-77.
70. Toole J, Malinow R, Chambless L, Spence J, Pettigrew C, Howard V, *et al*. Lowering homocysteine in patients with ischemic stroke to prevent recurrent stroke, myocardial infaction and death. JAMA 2004;291(5):565-75.
71. Bonna K, Njolstad I, Ueland P, Schirmer H, Tverdal A, Steigne T, *et al*. Homocysteine lowering and cardiovascular events after acute myocardial infarction. The New England Journal of Medicine 2006;354(15):1578-88.
72. Clarke R, Halsey J, Lewington S, Lonn E, Armitage J, Manson JE, *et al*. Effects of lowering homocysteine levels with B vitamins on cardiovascular disease, cancer, and cause-specific mortality. Archives of Internal Medicine 2010;170(18):1622-31.
73. Abraham J, Cho L. The homocysteine hypothesis: still relevant to the prevention and treatment of cardiovascular disease? Cleveland Clinic Journal of Medicine 2010 Dec;77(12):911-8.
74. Malouf R, Grimley E. Vitamin B6 for cognition (Review). Cohrane Database of Systematic Reviews 2003(4):1-32.
75. Royal Society of Chemisty. Chemspider: Pyridoxine 5'-phosphate. 2013; Available from: http://www.chemspider.com/Chemical-Structure.1026.html?rid=ca3e3229-afe0-4cf0-8a26-72fcd4cc07a6.
76. Royal Society of Chemisty. Chemspider: Pyridoxine. 2013; Available from: http://www.chemspider.com/Chemical-Structure.1025.html?rid=3a7f71f0-60d8-4333-ac23-409f9fa2a6cf.
77. Stover P. Physiology of Folate and Vitamin B12 in Health and Disease. Nutrition Reviews 2004;62(6):3-12.
78. Lucock M. Folic acid: nutritional biochemistry, molecular biology, and role in disease processes. Molecular Genetics and Metabolism 2000 Sep-Oct;71(1-2):121-38.
79. WHO. Serum and red blood cell folate concentrations for assessing folate status in population. Vitamin and Mineral Nutrition information System World Health Organization 2012.
80. Melse-Boonstra A, Bree A, Verhoef P, Bjorke-Monsen A, Verschuren W. Dietary monoglutamate and polyglutamate folate are associated with plasma folate concentrations in dutch men and women aged 20-65 years The Journal of Nutrition 2002;132:1307-12.
81. Toohey J. Vitamin B12 and methionine synthesis: A critical review. Is nature most beautiful cofactor misunderstood? BioFactors 2006;26:45-57.
82. Royal Society of Chemisty. Chemspider: Cobalamin. 2013; Available from: http://www.chemspider.com/Chemical-Structure.4942647.html.
83. Baik H, Russel R. Vitamin B12 deficiency in the elderly. Annual Review of Nutrition 1999;19:357-77.
84. Lawrence J, Roth J. Evolution of coenzyme b12 synthesis among enteric bacteria: evidence for loss and reacquisition of a multigene complex. Genetics 1996;142:11-24.
85. Robert C, Brown D. Vitamin B12 Deficiency. American Academy of Family Physicians 2003;67(5):979-86.
86. Shaik M, Gan S. Rapid resolution liquid chromatographty method development and validation for simultaneous determination of homocysteine, vitamins b6, b9 and b12 in human serum. Indian Journal of Pharmacology 2013;45(2):159-67.

87. Nelson B, Satterfield M, Sniegoski L, Welch M. Simultaneous Quantification of Homocysteine and Folate in Human Serum or Plasma Using Liquid Chromatography Tandem Mass Spectrometry on stable isotope. Analytical Chemistry 2005.
88. Persichilli S, Gervasoni J, Iavarone F, Zuppi C, Zappacosta B. A simplified method for the determination of total homocysteine in plasma by electrospray tandem mass spectrometry. Journal of Separation Science 2010 Oct;33(20):3119-24.
89. Lopez-Alarcón M, Chávez-Negrete A, Montalvo-Velarde I, Maldonado-Hernández J, Vital-Reyes V. Homocysteine after methionine load in healthy subjects with adequate B-vitamin status. Cirugía y Cirujanos 2011;79(5):432-8.
90. Sadeghian S, Fallahi F, Salarifar M, Davoodi G, Mahmoodian M, Fallah N, *et al*. Homocysteine, vitamin B12 and folate levels in premature coronary artery disease. BMC Cardiovascular Disorders 2006;6:38.
91. Turqut B, Arlsan S, Demir T, Guler M, Kaya M. Levels of circulating homocysteine, vitamin b6, vitamin b12, and folate in different types of open-angle glaucoma. Journal of Clinical Interventions in Aging 2010;26(5):133-9.
92. Vermaak W, Ubbink J, Barnard H, Potgieter G, Jaarvelds H, Groenewald A. Vitamin B6 nutrition status and cigarette smoking. The American Journal of Clinical Nutrition 1990;51:1058-61.
93. Abdel-Rehim M. Microextraction by packed sorbent (MEPS): a tutorial. Analytica Chimica Acta 2011 Sep 9;701(2):119-28.
94. Mandrioli R, Mercolini L, Lateana D, Boncompagni G, Raggi M. Analysis of risperidone and 9-hydroxyrisperidone in human plasma, urine and saliva by MEPS-LC-UV. Journal of Chromatography B 2011;879(2):167-73.
95. Abdel-Rehim M. New trend in sample preparation: on-line microextraction in packed syringe for liquid and gas chromatography applications. Journal of Chromatography B 2004;801(2):317-21.
96. SGE Analytical Science. Microextraction by Packed Sorbent. 2013; Available from: http://www.sge.com/uploads/2G/_V/2G_VKvgzgew13fyfOcLiNA/MEPS-v3_medium.wmv.
97. Metrolab. Microextraction by Packed Sorbent. 2010; Available from: http://www.metrolab.gr/useful_detail_gr.asp?id=10.
98. Pereira J, Gonçalves J, Alves V, Câmara J. The microextraction by packed sorbent as an effective and high-throughput sample extraction technique. Recent applications and future trends. Sample Preparation 2013;1:38-53.
99. SGE Analytical Science. eVol® XR hand-held automated analytical syringe. 2013; Available from: http://www.sge.com/products/evol.
100. SGE Analytical Science. MEPS syringes 2013; Available from: http://www.sge.com/uploads/Dz/sv/DzsvQoEUFy1ra_qxD3OMyw/IM-1051-S_medium.jpg.
101. Lafontan M. Advances in adipose tissue metabolism. International Journal of Obesity 2008;32 Suppl 7:S39-51.
102. Snyder L, Kirkland J. Introduction to modern liquid chromatography. Second ed 1979.
103. Lindsay S. High Performance Liquid Chromatography 1992.
104. Waters. How Does High Performance Liquid Chromatography work? 2013; Available from: http://www.waters.com/waters/pt_PT/How-Does-High-Performance-Liquid-Chromatography-Work%3F/nav.htm?cid=10049055&locale=pt_PT.
105. Swartz M. UPLC™: An Introduction and Review. Journal of Liquid Chromatography & Related Technologies 2005;28(7-8):1253-63.
106. Huang Y, Lu Z, Brown K, Whitehead A, Blair I. Quantification of intracellular homocysteine by stable isotope dilution liquid chromatography/tandem mass spectrometry. Biomedical Chromatography 2007 Jan;21(1):107-12.
107. Valente A, Bronze M, Bicho M, Duarte R, Costa H. Validation and clinical application of an UHPLC method for simultaneous analysis of total homocysteine and cysteine in human plasma. Journal of Separation Science 2012 Dec;35(24):3427-33.

108. Chatzimichalakis P, Samanidou V, Verpoorte R, Papadoyannis I. Development of a validated HPLC method for the determination of B-complex vitamins in pharmaceuticals and biological fluids after solid phase extraction. Journal of Separation Science 2004 Oct;27(14):1181-8.
109. Taverniers I, De Loose M, Van Bockstaele E. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. Trends in Analytical Chemistry 2004;23(8):535-52.
110. Mendes B, Silva P, Mendonça I, Pereira J, Câmara J. A new and fast methodology to assess oxidative damage in cardiovascular diseases risk development through eVol-MEPS–UHPLC analysis of four urinary biomarkers. Talanta 2013;116:164-72.
111. Chemicalize. ChemAxon. Homocysteine. 2013; Available from: http://www.chemicalize.org/structure/#!mol=C%28CS%29C%28C%28%3DO%29O%29N&source=fp.
112. Chemicalize. ChemAxon. Pyridoxine. 2013; Available from: http://www.chemicalize.org/structure/#!mol=pyridoxine&source=calculate.
113. Chemicalize. ChemAxon. Folate. 2013; Available from: http://www.chemicalize.org/structure/#!mol=folate&source=calculate.
114. Chemicalize. ChemAxon. Vitamin B12. 2013; Available from: http://www.chemicalize.org/structure/#!mol=vitamin+B12&source=calculate.
115. Salami F, Queiroz E. Microextraction in packed sorbent for determination of sulfonamides in egg samples by liquid chromatogrpahy and spectrophotometric detection. Journal of the Brazilian Chemical Society 2011;22(9):1656-61.
116. UNODC. Guidance for the validation of analytical methodology and calibration of equipment used for testing of illict drugs in seized materials and biological specimens. United Nations Office on Drugs and Crime 2009.

6. Annexes

6.1. Annex I : Gradient mobile phase programs

Gradient 1	**Time (min)**	**MetOH (%)**	**0.1% FA (%)**
Flow rate 250 µL min^{-1}	0	50	50
Column temperature : 30 ºC	0,5	80	20
	1	5	95
	2	80	20
	3	10	90
	4	10	90
	0	59	50
Gradient 2	**Time (min)**	**MetOH (%)**	**0.1% FA (%)**
Flow rate 250 µL min^{-1}	0	20	80
Column temperature : 30 ºC	0,5	20	80
	1	95	5
	1,5	20	80
	2	20	80
	3	40	60
	4	20	80
Gradient 3	**Time (min)**	**MetOH (%)**	**0.1% FA (%)**
Flow rate 250 µL min^{-1}	0	10	90
Column temperature : 35 ºC	1	5	95
	2	80	20
	3	0,6	40
	4	10	90
	5	10	90
Gradient 4	**Time (min)**	**MetOH (%)**	**0.1% FA (%)**
Flow rate 250 µL min^{-1}	0	20	80
Column temperature : 35 ºC	0,5	95	5
	1	95	5
	1,5	20	80
	2	20	80
	3	40	60
	4	20	80

Gradient 5	**Time (min)**	**MetOH (%)**	**0.1% FA (%)**
Flow rate 250 µL min^{-1}	0	20	80
Column temperature : 35 ºC	0,5	20	80
	1	40	60
	1,5	20	80
	2	20	80
	3	40	60
	4	20	80
	5	20	80
Gradient 6	**Time (min)**	**MetOH (%)**	**0.1% FA (%)**
Flow rate 250 µL min^{-1}	0	90	10
Column temperature : 30 ºC	0,5	95	5
	1	40	60
	1,5	20	80
	2	20	80
	3	40	60
	4	20	80
	5	10	10
Gradient 7	**Time (min)**	**MetOH (%)**	**0.05% FA (%)**
Flow rate 250 µL min^{-1}	0	10	90
Column temperature : 30 ºC	0,5	20	80
	1	95	5
	1,5	20	80
	2	20	80
	3	40	60
	4	20	80
	5	10	90
Gradient 8	**Time (min)**	**MetOH (%)**	**0.05% FA (%)**
Flow rate 250 µL min^{-1}	0	20	80
Column temperature : 40 ºC	0,25	95	5
	0,5	20	80
	1	95	5
	1,5	20	80
	2	20	80

	3	40	60
	4	20	80
	5	20	80
Gradient 9	**Time (min)**	**MetOH (%)**	**0.05% FA (%)**
Flow rate 250 µL min^{-1}	0	20	80
Column temperature : 30 ºC	0,5	20	80
	1	95	5
	1,5	20	80
	2	20	80
	3	40	60
	4	20	80
	5	20	80
Gradient 9	**Time (min)**	**MetOH (%)**	**0.01% FA (%)**
Flow rate 250 µL min^{-1}	0	50	50
Column temperature : 30 ºC	0,5	80	20
	1	5	95
	2	80	2
	3	10	90
	4	50	50
Gradient 10	**Time (min)**	**MetOH (%)**	**0.01% FA (%)**
Flow rate 250 µL min^{-1}	0	10	90
Column temperature : 30 ºC	0,5	80	20
	1	5	95
	2	80	20
	3	10	90
	4	10	90
Gradient 11	**Time (min)**	**MetOH (%)**	**0.01% FA (%)**
Flow rate 250 µL min^{-1}	0	50	50
Column temperature : 30 ºC	0,5	80	20
	1	5	95
	2	80	20
	3	10	90
	4	50	50
Gradient 12	**Time (min)**	**MetOH (%)**	**0.01% FA (%)**

Flow rate 250 µL min^{-1}	0	50	50
Column temperature : 30 ºC	0,5	20	80
	1	95	5
	1,5	20	80
	2	20	80
	3	40	60
	4	20	80
	5	50	50
Gradient 13	**Time (min)**	**MetOH (%)**	**0.01% FA (%)**
Flow rate 250 µL min^{-1}	**0**	50	50
Column temperature : 30 ºC	**0,3**	5	95
	0,5	80	20
	1	5	95
	1,5	80	20
	2	80	20
	3	60	40
	4	50	50
	5	50	50
Gradient 14	**Time (min)**	**MetOH (%)**	**0.01% FA (%)**
Flow rate 250 µL min^{-1}	0	50	50
Column temperature : 35 ºC	0,5	20	80
	1	95	5
	1,5	20	80
	2	20	80
	3	20	80
	5	50	50
Gradient 15	**Time (min)**	**MetOH (%)**	**0.01% FA (%)**
Flow rate 250 µL min^{-1}	0	20	80
Column temperature : 35 ºC	0,05	95	5
	0,07	20	80
	0,1	70	30
	0,5	20	80
	1	95	5
	1,5	20	80
	2	20	80

	3	20	80	
Gradient 16	**Time (min)**	**MetOH (%)**	**0.01% FA (%)**	
Flow rate 250 µL min^{-1}	0	50	50	
Column temperature : 35 ºC	0,3	5	95	
	0,5	80	20	
	1	5	95	
	1,5	80	20	
	2	80	20	
	3	60	40	
	4	50	50	
	5	50	50	
Gradient 17	**Time (min)**	**MetOH (%)**	**H_20 (%)**	
Flow rate 250 µL min^{-1}	0	50	50	
Column temperature : 35 ºC	0,5	20	80	
	5	50	50	
Gradient 18	**Time (min)**	**MetOH (%)**	**H_20 (%)**	
Flow rate 250 µL min^{-1}	0	20	80	
Column temperature : 35 ºC	1	50	50	
	2	80	20	
	4	20	80	
	5	20	80	
Gradient 19	**Time (min)**	**MetOH (%)**	**H_20 (%)**	
Flow rate 250 µL min^{-1}	0	20	80	
Column temperature : 30 ºC	1	30	70	
	2,5	30	70	
	4	20	80	
	5	20	80	
Gradient 20	**Time (min)**	**MetOH (%)**	**H_20 (%)**	
Flow rate 250 µL min^{-1}	0	50	50	
Column temperature : 30 ºC	0,1	10	90	
	0,5	50	50	
	4	50	50	
	5	50	50	
Gradient 20	**Time (min)**	**MetOH (%)**	**0.1% FA**	**H_20 (%)**

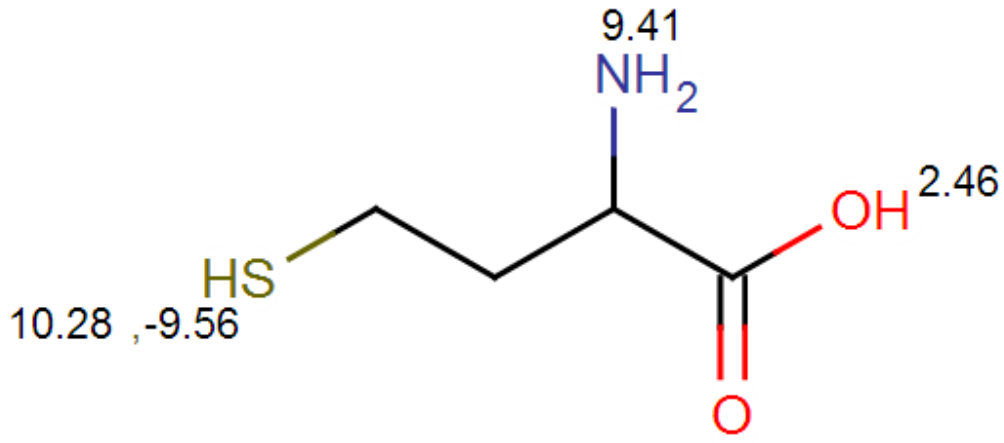
Flow rate 250 µL min^{-1}	0	50	0	50
Column temperature : 30 ºC	1	50	10	40
	2	80	20	0
	4	20	20	60
	5	50	0	50
Gradient 21	**Time (min)**	**MetOH (%)**	**0.1% FA**	**H_20 (%)**
Flow rate 250 µL min^{-1}	0	50	0	50
Column temperature : 30 ºC	0,25	20	0	80
	0,5	80	0	20
	2	80	10	10
	5	50	0	50
Gradient 22	**Time (min)**	**MetOH (%)**	**0.1% FA**	**H_20 (%)**
Flow rate 250 µL min^{-1}	0	50	0	50
Column temperature : 30 ºC	0,25	0	80	20
	0,5	0	80	20
	1	0	5	95
	1,5	0	80	20
	2	0	80	20
	3	0	60	40
	4	0	80	20
	4,75	0	80	20
	5	50	50	50
Gradient 23	**Time (min)**	**MetOH (%)**	**0.1% FA**	**ACN (%)**
Flow rate 250 µL min^{-1}	0	20	50	30
Column temperature : 30 ºC	0,5	20	50	30
	1	50	50	0
	2	30	40	20
	4	20	50	30
	5	20	50	30
Gradient 24	**Time (min)**	**MetOH (%)**	**0.1% FA**	**ACN (%)**
Flow rate 250 µL min^{-1}	0	50	10	40
Column temperature : 30 ºC	0,5	20	50	30
	1	40	50	10
	4	50	10	40

	5	50	10	40
Gradient 24	**Time (min)**	**ACN (%)**	**0.1% FA (%)**	
Flow rate 250 µL min^{-1}	0	20	80	
Column temperature : 30 ºC	1,5	50	50	
	2,5	70	30	
	4	20	80	
	5	20	80	
Gradient 25	**Time (min)**	**ACN (%)**	**0.01% FA (%)**	
Flow rate 200 µL min^{-1}	**0**	80	20	
Column temperature : 30 ºC	0.2	90	10	
	0.5	30	70	
	1	15	85	
	5	80	20	
Gradient 26	**Time (min)**	**ACN (%)**	**0.01% FA (%)**	
Flow rate 200 µL min^{-1}	**0**	95	5	
Column temperature : 30 ºC	0.5	100	0	
	1	100	0	
	2	95	5	
	3	95	5	
	4	80	20	
	5	95	5	

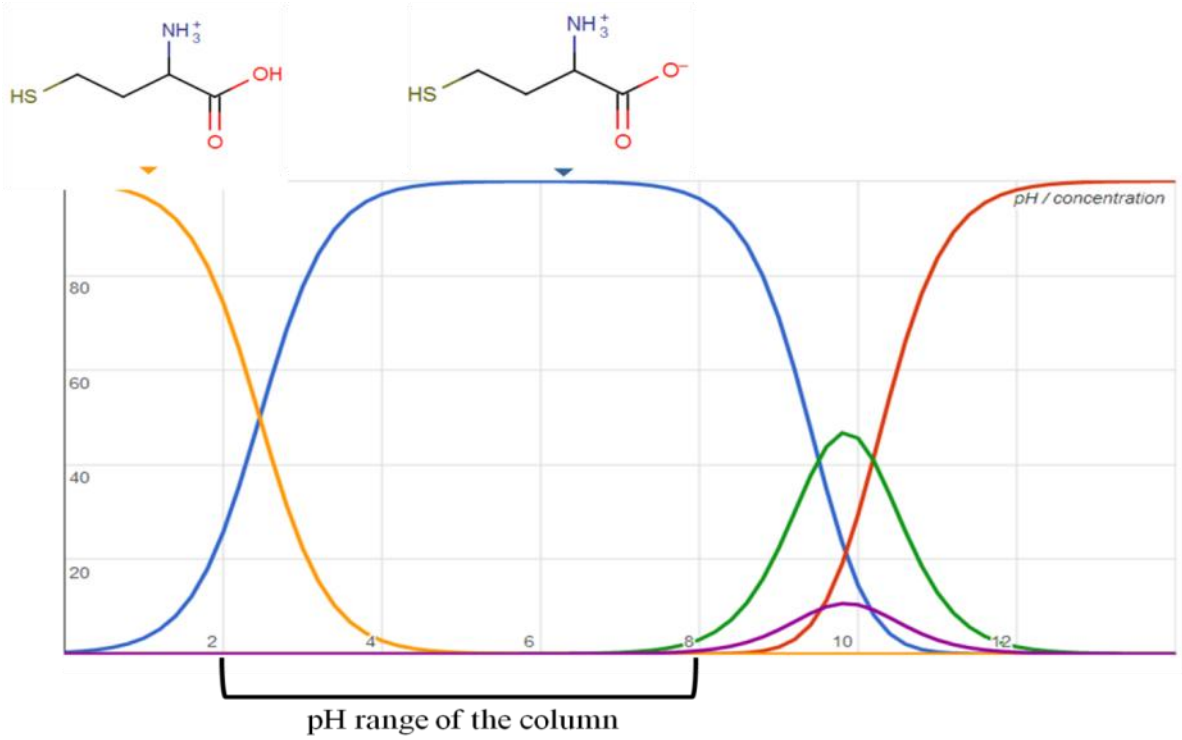
6.2. Annex II : Form of Hcys and vitamins B at pH 2- 8

All images were adapted from chemicalize.org

Hcys

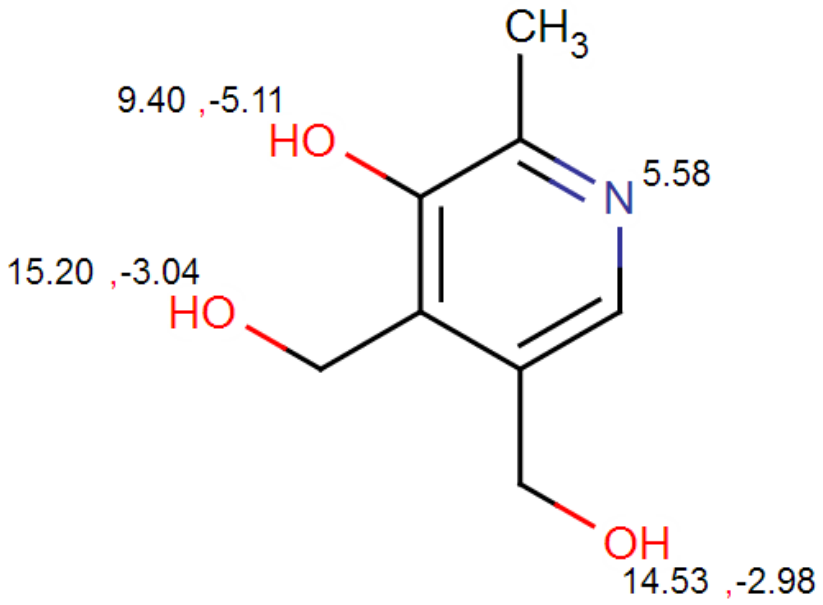


Pka values of Hcys

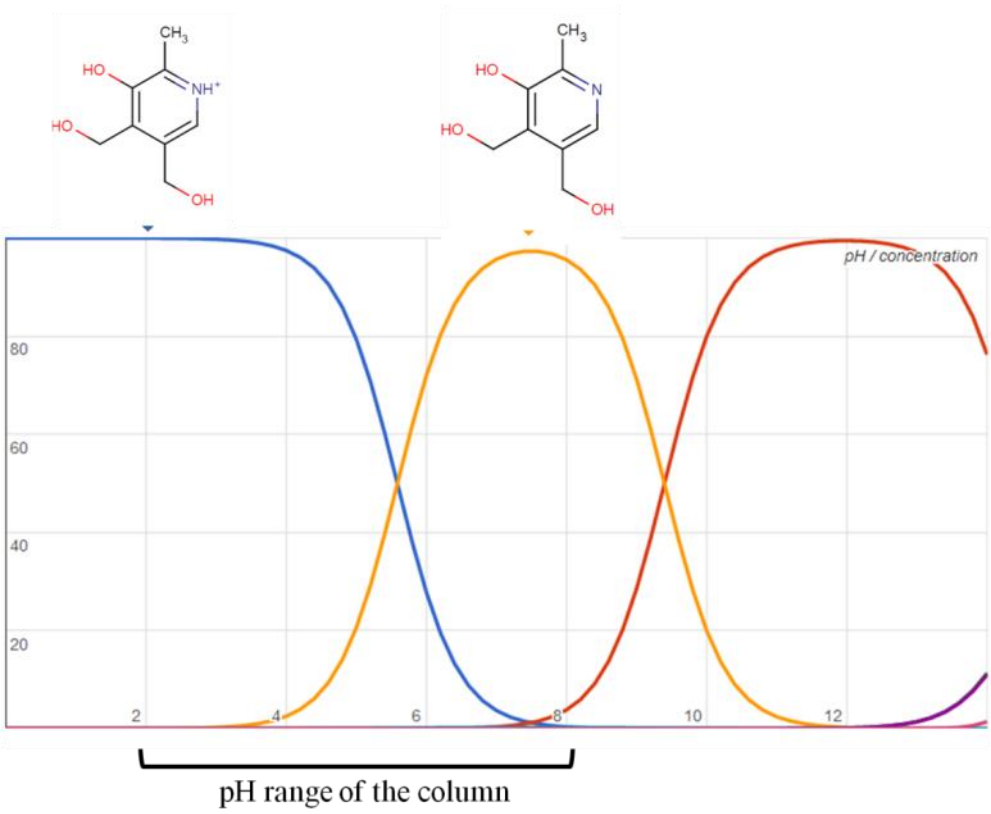


Predominant forms of Hcys within the pH range of the column

Vitamin B_6

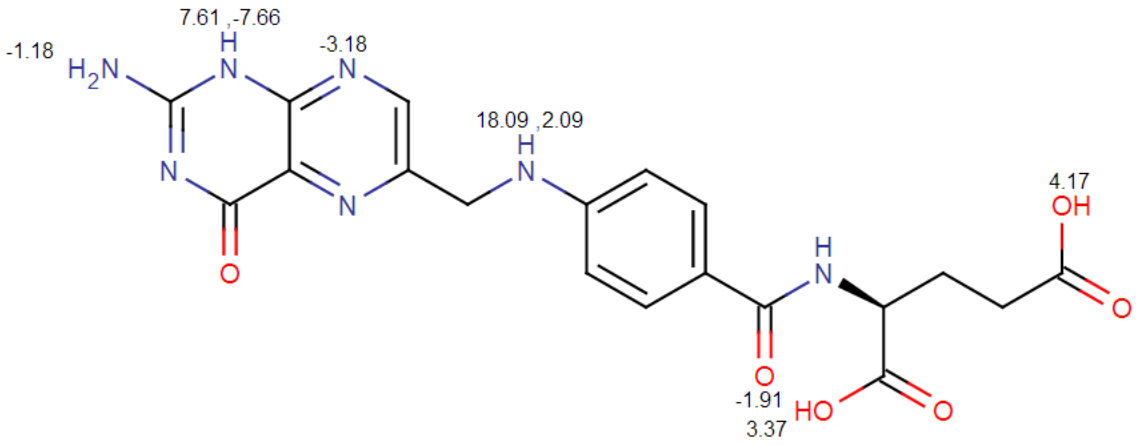


PKA values of Vitamin B_6

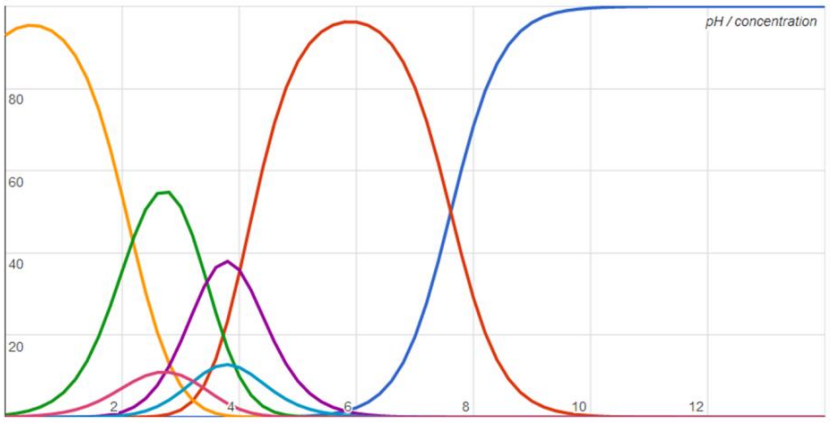


Predominant forms of Vitamin B_6 within the pH range of the column

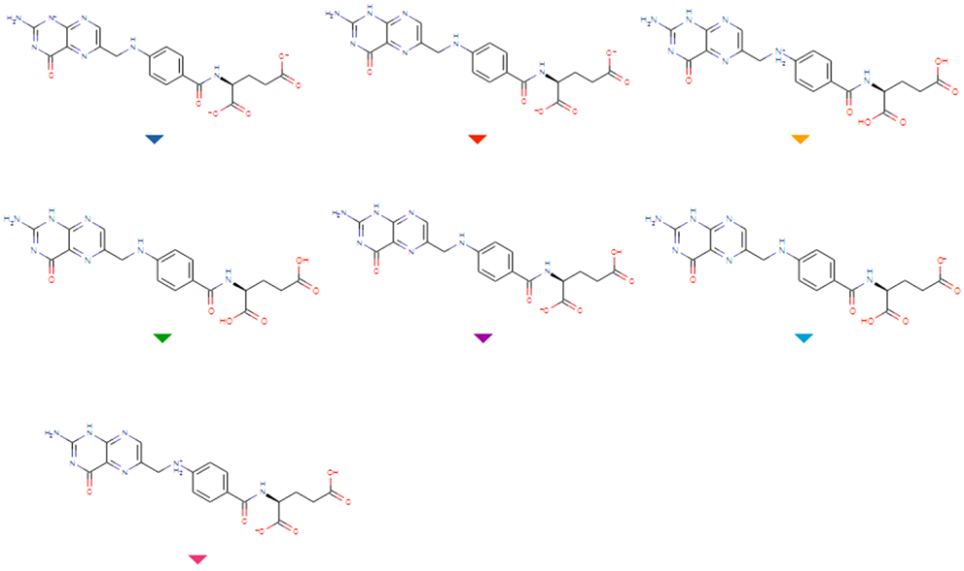
Vitamin B_9



PKA values of Vitamin B_9

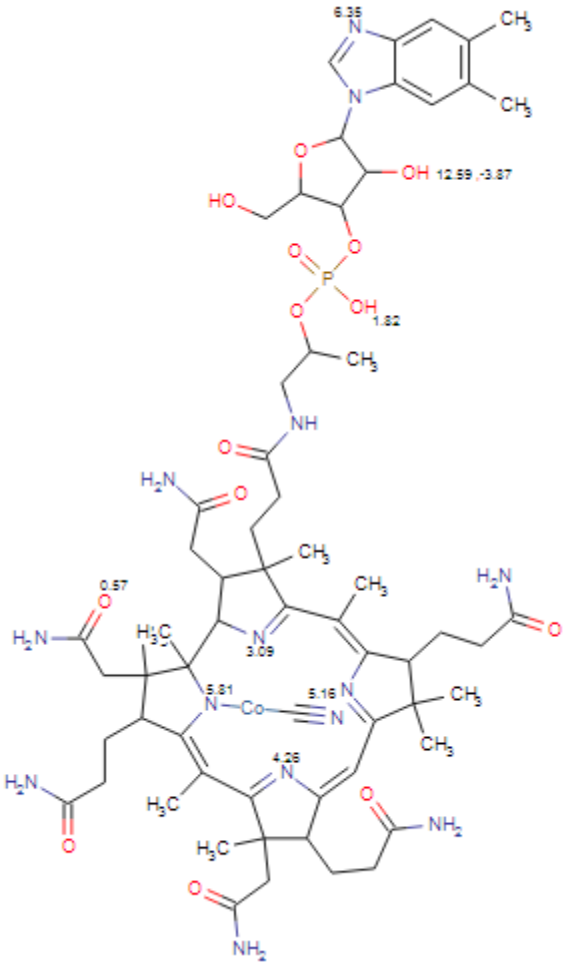


pH range of the column

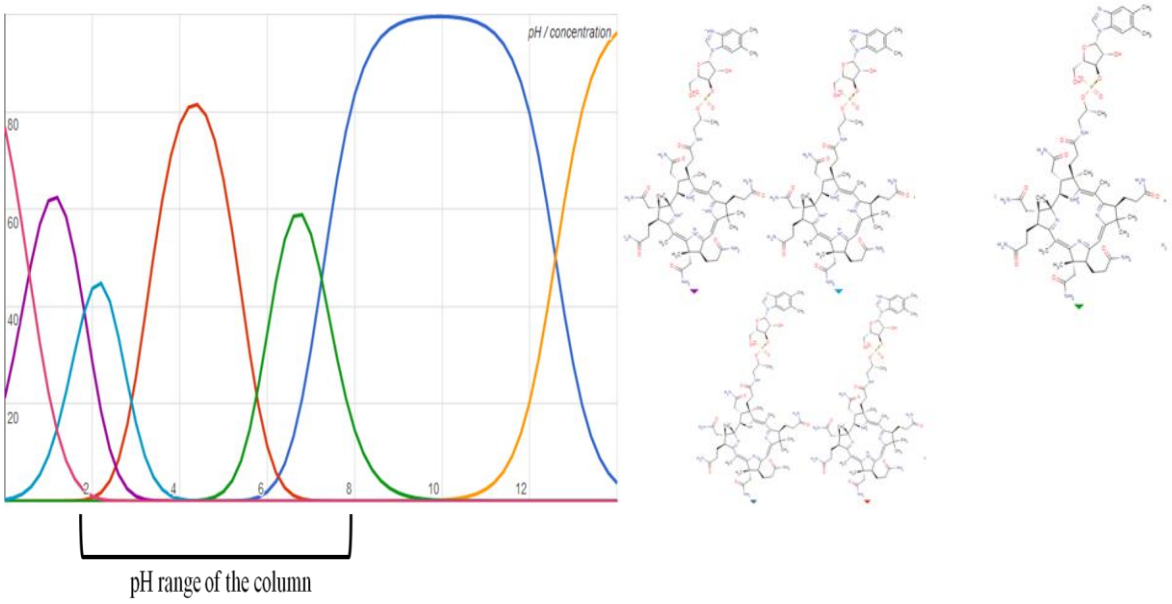


Predominant forms of Vitamin B_9 within the pH range of the column

Vitamin B_{12}



PKA values of Vitamin B_{12}



Predominant forms of Vitamin B_{12} within the pH range of the column

6.3. Annex III : MEPS procedures performed to extract efficiency Hcys

MEPS procedure 1	
Cartridge condition	250 µL of MetOH 250 µL of 0.01%FA
Sample loading	5 x 50 µL of standard containing Hcys and 25 µL of TCEP (390 mmol/L)
Sorbent washing	1 x 50 µL 0.01% FA
Elution	3 x 30 µL 20% MetOH
MEPS procedure 2	
Cartridge condition	100 µL of MetOH 100 µL of H_20
Sample loading	100 µL of standard containing Hcys and 10 µL of TCEP (390 mmol/L)
Sorbent washing	100 µL of H_20
Elution	100 µL of % MetOH A: 20% B: 40% C:80%
MEPS procedure 3	
Cartridge condition	100 µL of MetOH 100 µL of 0.01% FA
Sample loading	2 x100 µL of standard containing Hcys and 20 µL of TCEP (390 mmol/L)
Sorbent washing	100 µL of 0.01% FA
Elution	50 µL of MetOH
MEPS procedure 4	
Cartridge condition	100 µL of MetOH

	100 µL of Sodium Acetate 100 mmol/L
Sample loading	100 µL standard containing Hcys and 20 µL of TCEP (390 mmol/L) prepared in Sodium acetate 100 mmol/L
Sorbent washing	100 µL of Sodium Acetate 100 mmol/L 100µL of MetOH
Drying	5 min of cycles of aspiration of air
Elution	50 µL of 5% Ammonium hydroxide/95% MetOH
MEPS procedure 5	
Cartridge condition	100 µL of MetOH 100 µL of Ammonium Acetate 100 mmol/L
Sample loading	100 µL standard containing Hcys and 20 µL of TCEP (390 mmol/L) prepared in Ammonium acetate 100 mmol/L
Sorbent washing	100 µL of Ammonium Acetate 100 mmol/L 100µL of MetOH
Elution	50 µL of 5% Ammonium hydroxide/95% MetOH
MEPS procedure 6	
Cartridge condition	250 µL of ACN 250 µL of 0.01%F A
Sample loading	200 µL of Standards containing Hcys and 20 µL of TCEP (390 mmol/L)
Sorbent washing	250 µL0.01% FA
Elution	80 µL ACN/0.01% FA