Determination of Lipophilic and Hydrophilic Antioxidants in *Solanum Lycopersicum* L. Tomato From *Gordal* Variety by LL-USAE Combined With UHPLC-PDA/FLR

MASTER DISSERTATION

José Aldónio Oliveira Figueira
MASTER IN APPLIED BIOCHEMISTRY

UNIVERSIDADE da MADEIRA
A Nossa Universidade
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José de Sousa Câmara

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Thesis submitted to UNIVERSIDADE DA MADEIRA
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JOSÉ ALDÓNIO OLIVEIRA FIGUEIRA

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Prof. Doutor José de Sousa Câmara

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“Don't find fault. Find a remedy”

Henry Ford

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RESEARCH WORKS

The development of this work was an excellent opportunity to extend my knowledge in accessing and analyzing scientific information, scheduling and performing research work, as well communicating science. Moreover, it has contributed to the improvement of my knowledge in metabolomics, as well as in my skills in analytical chemistry.

Along the experimental directly involved in my thesis research project, I also had the opportunity to participate in other ongoing research projects in the lab, which resulted in the participation in international and national conferences, through different oral and poster communications.

Published work:

PAPERS IN PEER REVIEWED INTERNATIONAL JOURNALS:

Gonçalves JL, **Figueira JA**, Rodrigues FP, Ornelas LP, Branco RN, Silva CL, et al. A powerful methodological approach combining headspace solid phase microextraction, mass spectrometry and multivariate analysis for profiling the volatile metabolomic pattern of beer starting raw materials. Food Chem 2014;160:266-80.

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ORAL COMMUNICATIONS IN INTERNATIONAL AND NATIONAL CONFERENCES:

Figueira, J.A.; Porto-Figueira P.; Câmara, J.S.; Determination of lipophilic antioxidants α- and δ-tocopherol in tomato from *gordal* variety by ultrasound assisted extraction combined with ultrahigh pressure liquid chromatography; XX Encontro Luso-Galego de Química, Porto, Portugal, November, 2014.

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*POSTER COMMUNICATIONS IN INTERNATIONAL AND NATIONAL CONFERENCES:***:**

Porto-Figueira P; Freitas A; Cruz C; **Figueira JA**; Câmara JS. Differentiation of the volatomic pattern of several *passiflora* L. 12º Encontro de Química dos Alimentos, Lisbon, Portugal, September, 2014

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Porto-Figueira P, **Figueira J**, Câmara JS. Comparison of the effectiveness of different extraction techniques, SPME and QuEChERS, combined with GC-MS for the establishment of the volatomic profile of Eugenia uniflora L. 38th International Symposium on Capillary Chromatography and 9th GCxGC Symposium, Riva del Garda, Italy, May, 2014

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RESUMO

O tomate (*Lycopersicon esculentum* L.) é um dos principais constituintes da dieta mediterrânica. O seu consumo tem sido associado à uma acção eficiente na redução dos riscos cardiovasculares e a certos tipos de cancro. É deste modo um dos vegetais mais populares e amplamente consumidos no mundo. Para avaliar o potencial do *Lycopersicon esculentum* L. como alimento bioactivo, foram desenvolvidos dois métodos analíticos a fim de determinar os níveis dos antioxidantes lipofílicos: α-tocoferol, δ-tocoferol, β-caroteno, licopeno; e hidrofílicos: ácido ascórbico. A quantificação dos carotenóides totais (β-caroteno e licopeno) foi realizada por extração líquido-líquido assistida por ultrassons (LL-USAE) em combinação com a análise de espectroscopia no ultravioleta visível (UV-Vis), segundo o método da média para carotenóides totais, a 450 nm. A cromatografia líquida de ultra eficiência com um sistema de detecção envolvendo o detector fotodíodos e de fluorescência (UHPLC-PDA/FLR) permitiu a caracterização, identificação e quantificação dos antioxidantes lipofílicos e hidrofílicos alvo de estudo. Esta metodologia UHPLC-PDA/FLR, simples e rápida, revelou-se de grande sensibilidade, permitindo obter limites de deteção (LODs) e de quantificação (LOQs) muito inferiores (cerca de 10 vezes) aos encontrados na literatura.

O método LL-USAE/UV-Vis foi validado e aplicado a diferentes amostras de tomate e processados derivados do tomate. Os resultados revelaram um pequeno aumento do conteúdo de carotenóides ao longo da maturação, atingindo o seu teor máximo na maturação. Estes resultados complementam os obtidos pelos ensaios ORAC e TBARS, que evidenciam um aumento da capacidade antioxidante que supera o também aumento da peroxidação lipídica ao longo da maturação. Os LODs e LOQs obtidos para esta metodologia foram significativamente inferiores aos encontrados na literatura.

A determinação do teor de carotenóides em diferentes variedades de tomate, através do método LL-USAE/UV-Vis, indicou uma maior concentração para a variedade *regional*, seguida das variedades *rama* e *gordal*, e por fim a variedade *cacho*. Esta metodologia foi aplicada a diferentes amostras processadas derivadas do tomate, tendo-se verificado que as massas de tomate concentradas apresentaram maior teor de carotenóides.

Palavras-chave: *Lycopersicon esculentum* L.; antioxidantes lipofílicos; antioxidantes hidrofílicos; maturação; UHPLC; LL-USAE: validação do método

ABSTRACT

Tomato (*Lycopersicon esculentum* L.) is one of the main constituents of the Mediterranean diet. Its consumption has been proposed to reduce the risk of cardiovascular diseases and certain types of cancer. It is therefore one of the most popular and extensively consumed vegetable crop worldwide. To gain insights on the potential of *Lycopersicon esculentum* L. as bioactive food, two analytical methodologies were developed to determine the levels of the lipophilic δ-tocopherol, α-tocopherol, β-carotene, lycopene; and hydrophilic antioxidants ascorbic acid. The quantification of total carotenoids (β-carotene and lycopene) was assessed through a liquid–liquid ultrasound assisted extraction (LL-USAE) in combination with ultraviolet-visible spectroscopy (UV-Vis), according to method of mean, for total carotenoids ($\lambda_{máx}$ = 450 nm. The ultra-high performance liquid chromatographic using both photodiode array and fluorescence detection (UHPLC-PDA/FLR), allows the identification and quantification of the target lipophilic and hydrophilic antioxidants. This methodology UHPLC-PDA/FLR is fast, simple and revealed a high sensitivity for the compounds under study. The limits of detection (LODs) and quantification (LOQs) obtained were much lower (about 10 times) than the reported in literature.

The method LL-USAE/UV-Vis was validated and applied to different tomato foodstuffs. The results reveal a small increase of carotenoids content during maturation, reaching the maximum level when ripe. These results complement those obtained by the ORAC and TBARS assays that show an increase of antioxidant capacity during maturation. The LODs ans LOQs obtained were also about 10 times lower than reported in literature. The carotenoid content was also evaluated by LL-USAE/UV-Vis in different tomatoes varieties. *Regional* variety present the high carotenoid level, followed by *campari* and *gordal*, and at last *grape*. This methodology was also applied to different processed food samples containing tomatoes derivatives. Highest carotenoids content were obtained in concentrated tomato foodstuffs.

Keywords: *Lycopersicon esculentum* L., lipophilic antioxidants, hydrophilic antioxidants, ripening; UHPLC; LL-USAE, method validation

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	α-TMT	tocopherol methyltransferases
	α-TTP	α-tocopherol transfer protein
	αTO$^{\bullet}$	α-tocopheroxyl radical
	αTOH	α-tocopherol
	λ	wavelength
	μ	linear velocity of the mobile phase
	ε	molar absorptivity
A	*A*	absorbance
	A	Eddy-diffusion coefficient
	AA	ascorbic acid
	AAO	abscisic aldehyde oxidase
	AAPH	azo-initiator 2,2'-Azobis(2-methylpropionamidine) dihydrochloride
	ABA	abscisic acid
	ABA2	alcohol dehydrogenase
	ACN	acetonitrile
	ADH	alcohol dehydrogenase
	AdKETO	zeaxanthin ketolase
	AKR	aldo/keto reductase
	AL	aldono-lactonase
	AOx	antioxidant
	AOx $^{\bullet}$	radical antioxidant
	APS	amino-propyl silane
	APX	ascorbate peroxidase
	AUC	area under the curve
	AVG	average
B	B	longitudinal diffusion coefficient
	BCC	β-carotene cleavage dioxygenase
	BEH	bridged ethylsiloxane-silica hybrid
C	*c*	speed of light
	c	concentration
	C	resistance to mass transfer coefficient
	C18	octadecyl
	^{3}CAR	carotenoid lowest excited triplet state
	CAT	catalase
	CEHC	carboxyethyl hydroxychromans
	CHYB	carotene β-hydroxylase
	CM	chorismate mutase
	CrtS	β-carotene ketolase
	CS	chorismate synthase
	CT	total cycle time

C	CtrISO	cynobacterial ζ-carotene desaturase, carotene isomerise
	CVDs	cardiovascular diseases
	CYP97A	β-ring carotene hydroxylase
	CYP97C	ε-ring carotene hydroxylase
D	DAHP	3-deoxy-D-arabino-heptulosonate 7-phosphate
	DAHPS	3-deoxy-D-arabinoheptulosonate-7-P synthase
	DHAA	dehydroascorbic acid
	DHAR	dehydroascorbic acid reductase
	DHQ	3-dehydroquinate
	DHQD	3-dehydroquinate dehydrogenase
	DHQS	3-dehydroquinate synthase
	DHQSDH	shikimate dehydrogenase/3-dehydroquinate dehydratase
	DHS	dehydroshikimate
	DKG	2,3-diketogulonic acid
	DLLME	dispersive liquid–liquid microextraction
	D_M	analyte diffusion coefficient
	DMAPP	dimethylallyl diphosphate
	DMPBQ	2,3-dimethyl-5-phytyl-1, 4-benzoquinone
	DNA	deoxyribonucleic acid
	d_p	particle size of the packing material
	DPPH	2,2-di(4-tertoctylphenyl)-1-picrylhydrazyl
E	E4P	erythrose 4-phosphate
	EDTA	ethylenediamine tetraacetic acid
	EPSP	5-enolpyruvylshikimate 3-phosphate
	EPSPS	5-enolpyruvylshikimate-3-P synthase
	ESI	electrospray Ionization
	EtOH	ethanol
F	F1 ou F2	flow rate
	FA	formic acid
	FAO	Food and Agriculture Organization of the United Nations
	FH	fluorescein
	FLR (FLD)	fluorescence detector
	FPP	farnesyl diphosphate
	FPS	farnesyl pyrophosphate synthase
G	GalDH	L-gulose dehydrogenase
	GalLDH	L-galactono-1,4-lactone dehydrogenase
	GDP	guanosine diphosphate
	GGP	GDP-L-galactose phosphorylase
	GGPP	geranylgeranyl diphosphate
	GGPS	geranylgeranyl diphosphate synthase
	GGuP	phosphodiesterase
	GK	D-glucurono kinase
	GLUT	glucose transporter
	GME	GDP-mannose-3',5'-epimerase
	GMP	GDP-mannose pyrophosphorylase

G	GPP	geranyl diphosphate
	GPP	L-galactose-1-phosphate phosphatase
	GPS	geranyl pyrophosphate synthase
	GPUT	D-glucuronate-1-phosphate uridylyltransferase
	GPX	glutathione transferases
	GR	D-glucuronate reductase
	GSH	selenium-dependent glutathione
	GuDH	L-gulono dehydrogenase
	GuLDH	L-gulono-1,4-lactone dehydrogenase
	GuPP	sugar phosphatase
H	H	hydrogen
	h	Planck constant
	H_2O	water
	H_2O_2	hydrogen peroxide
	HAT	transfer reactions model
	HDL	high-density lipoproteins
	HDVB	hydrophobic polystyrene-divinylbenzene copolymer
	HETP	height equivalent to a theoretical plate
	HGA	homogentisic acid
	HPLC	high performance liquid chromatography
	HPP	4-hydroxyphenylpyruvic acid
	HPPD	4-hydroxyphenylpyruvate dioxygenase
	HPT	homogentisate solanesyl transferase
	HSS	high strength silica
I	IMP	myo-inositol monophosphatase
	IPI	isopentenyl diphosphate isomerase
	IPP	isopentyl diphosphate
	IR	infrared
	IT-SPME	in-tube SPME
L	l	path length through the absorbing medium
	L	column length
	$L^{\bullet}$	lipid radical
	LL-USAE	liquid–liquid ultrasound assisted extraction
	L-AA	L-ascorbic acid
	LCYB	lycopene β-cyclase
	LCYE	lycopene ε-cyclase
	LDL	low-density lipoprotein
	LH	unsaturated fatty acids
	LLE	liquid-liquid extraction
	LLME	liquid–liquid microextraction
	LL-USAE	liquid–liquid ultrasound assisted extraction
	LOD	limit of detection
	$LOO^{\bullet}$	lipid peroxy radicals
	LOQ	limit of quantification
	LP	lipid peroxidation

L	LPL	lipoprotein lipase
M	MAV	mevalonate pathway
	MDA	malondialdehyde
	MDHA	ascorbyl radical monodehydroascorbate
	MDHAR	monodehydroascorbic acid reductase
	Me	matrix effect
	*ME	methylesterase
	MeOH	methanol
	MEP	methylerythritol phosphate pathway
	MEPS	microextraction by packed-sorbent
	METs	microextraction techniques
	M_{FS}	slope of fortified sample linear regression
	MIO	myo-inositol oxygenase
	MIPS	L-myo-inositol 1-phosphate synthase
	MPBQ	2-methyl-6-phytylplastoquinol
	MPBQ	2-methyl-6-phytylplastoquinol
	MPBQMT	dimethyl-phytylquinol methyl transferase
	mRNA	messenger ribonucleic acid
	M_{Sol}	slope of standards linear regression
	MT	thousand tonnes
	MWCNT	multi-walled carbon nanotube
N	n	frequency
	N_2O	nitrous oxide
	N_2O_3	dinitrogen trioxide
	NCDs	noncommunicable diseases
	NCED	9-cis-epoxycarotenoid dioxygenase
	NO	nitric oxide
	$^{\bullet}NO$	radical nitric oxide
	NO^+	nitronium
	$^{\bullet}NO_2$	radical nitrogen dioxide
	NO_2^-	ions nitrite
	N_{req}	required column efficiency
	NRP	non-radical products
	NSY	neoxanthin synthase
O	O_2	oxygen
	$^{\bullet}O_2^-$	superoxide
	$^{\bullet}OH$	hydroxyl radical
	$ONOO^-$	peroxynitrite
	ORAC	oxygen radical absorbance capacity
	ox-LDL	oxidised low-density lipoprotein
	OxS	oxidative stress
P	PAT	prephenate aminotransferase
	PDA	photodiode array
	PDS	phytoene desaturase
	PeP	phosphoenol pyruvate

P	PEP	polar enhanced polymer
	PGC	porous graphitic carbon
	PGI	phosphate isomerase
	PGM	phosphoglucomutase
	Phytyl-DP	phytyl diphosphate
	PK	phytol kinase
	PLE	pressurized liquid extraction
	PMI	phosphomannose isomerase
	PMM	phosphomannomutase
	PON-1	serum paraoxonase/arylesterase 1
	PPK	phytylphosphate kinase
	PSY	phytoene synthase
	PUFAs	polyunsaturated fatty acids
R	r1	radius for the larger internal diameter column
	r2	radius for the smaller internal diameter column
	RALDH	retinaldehyde dehydrogenase
	R-AX	retain anion exchange
	RCF	relative centrifugal force
	R-CX	retain cation exchange
	RDH	retinol dehydrogenase
	R	recovery percentage
	RF	radio-frequency
	RNS	reactive nitrogen species
	$ROO^{\bullet}$	peroxyl radicals
	ROS	reactive oxygen species
	RP	reversed phase
	RSD	relative standard deviation
S	S	concentration of target analytes in sample
	S3P	shikimate 3-phosphate
	SALLE	salting-out liquid–liquid extraction
	SAX	strong anion exchange
	SBSE	thestir-bar sorptive extraction
	SCX	strong cation exchange
	SD	standard deviation
	SD_{LC}	standard deviation of the lower concentration present
	SDME	single-drop microextraction
	SDVB	styrene-divinylbenzene
	S_F	concentration of target analytes in fortified sample
	SIL	silica
	SK	shikimate kinase
	SOD	superoxide dismutase
	SPE	solid–liquid extraction
	SPME	solid-phase microextraction
	Std	concentration of target analytes added to sample
	SVCT	vitamin C transporters

T	TAT	tyrosine aminotransferase
	TAP	total antioxidant potential
	TBA	thiobarbituric acid
	TBARS	thiobarbituric acid reactive species
	TC	tocopherol cyclise
	TCA	trichloroacetic acid
	TEAC	trolox equivalent antioxidant capacity
	TEP	1,1,3,3,-tetraethoxypropane
	TFME	thin film microextraction
	TMP	α-tocopherol-mediated peroxidation
	TMT	tocopherol methyltransferases
	TYRA	arogenate dehydrogenase
U	UDP	Uridine diphosphate
	UGDH	UDP-D-glucose dehydrogenase
	UGP	UDP-D-glucose pyrophosphorylase
	UHPLC	ultra-high performance liquid chromatography
	UPLC	ultra-high performance liquid chromatography from Waters
	US	ultrasound
	USAE	ultrasound assisted extraction
	UV	ultraviolet
	UV-Vis	ultraviolet-visible spectroscopy
V	VDE	violaxanthin de-epoxidase
	VLDL	very low-density lipoprotein
W	WHO	World Health Organization
Z	ZDS	ζ-carotene desaturase
	ZEP	zeaxanthin epoxidase

CHAPTER I

STATE OF THE ART

1.1. Introduction

Tomatoes originaly from the Andean region, arrived Europe around the 15th century. The native wild species were less attractive in size, shape and colour than the European cultivations selected over the times [1-3]. Nowadays, tomato is one of the most popular and extensively consumed vegetable crops worldwide, consumed either fresh or after being processed into various products [4, 5]. Tomato is composed by skin, pericarp, and locular contents with jelly-like parenchyma cells that surround the seeds. This fruit have a high water concentration, 5 to 10% of dry matter nearly half of reducing sugars and organic acids (mainly citric acid and malic acid) [6, 7].

According to Food and Agriculture Organization of the United Nations (FAO) data (FAOSTAT: www.faostat.org), from 2000 to 2010, Portugal produced a total of 12249.35 thousand tonnes (MT) of tomatoes, registering a gradual increase through the last decade (Figure 1.1). In 2010, with an area of 16000 Ha, Portugal was the 16th world tomato producer, with our neighbours, Spain, being the 8th, and the 1st place belonging to China. In 2012, tomato was the main Portuguese crop, with estimated values of ±408.5 million Euros, and production around 1392700 MT [7].

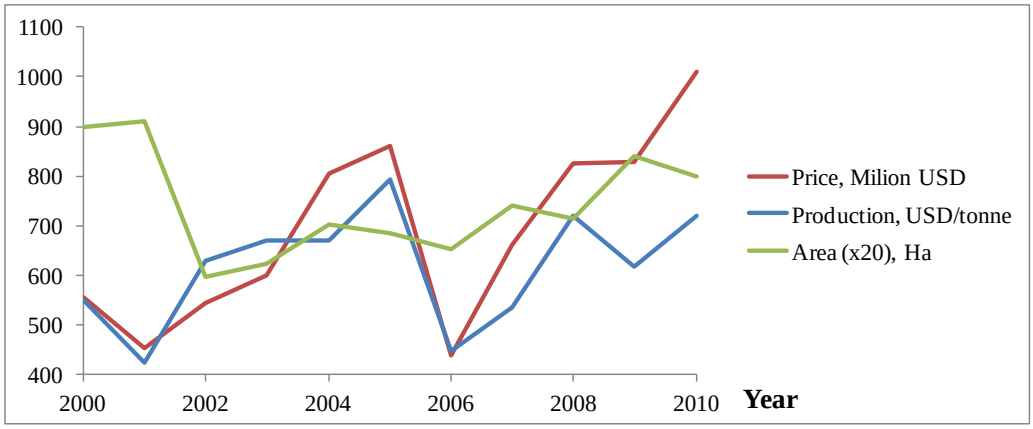


Figure 1.1. Tomato crop estimated from 2000 to 2010, for Portugal. Data from FAOSTAT.

The Mediterranean diet allows an increased intake of key nutrients, such as vitamins, minerals, antioxidant compounds and dietary fibre, with subsequent beneficial effects on health [8]. Its consumption is thought to reduce the risk of some cardiovascular diseases (CVDs) and certain types of cancer [4, 5, 7-10]. Tomato antioxidant content depends on the cultivar and also on agronomic and environmental conditions during cultivation. Antioxidants like provitamin A β-carotene, lycopene, vitamin C and vitamin E, will therefore be present in different levels, according to the specific abiotic conditions of each tomato [4, 5, 7-9].

CVDs are a group of disorders of the heart and blood vessels that includes atherosclerosis (coronary heart and cerebrovascular disease), peripheral arterial disease, rheumatic heart disease, congenital heart disease and deep vein thrombosis [11-13]. According to Chan (1998) [14] and Ross (1986) [15], atherosclerosis can be explained through the response-to-injury hypothesis as: "*a chronic inflammatory response to injury of the endothelium, which leads to an overproduction of free radicals, which promote oxidation of low-density lipoprotein (ox-LDL). ox-LDL induce endothelial expression and secretion of cytokines, prostacyclin and nitric oxide, growth factors and several cell surface adhesion molecules. In response to the growth factors and cytokines, smooth muscle cells proliferate in the intima, resulting in the narrowing of the lumen*" [14, 15].

According to World Health Organization (WHO) data (CVDs WHO Fact Sheet: http://www.who.int/cardiovascular_diseases/en/), CVDs are the major cause of death globally, being the first death cause of Noncommunicable Diseases (NCDs). In 2008, around 17.3 million people died from CVDs, representing 30% of all global deaths. As can be seen in Figure 1.2, 80 % of CVDs deaths take place in low- and middle-income countries, occuring almost equally in men and women (slightly higher in men).

Current theories on the pathogenesis of atherosclerosis suggest that when the balance between oxidants and antioxidants shifts in favour of the former, inflammation and oxidative stress (OxS) progressively damage arterial walls [11, 13]. Therefore dietary antioxidants are so relevant, although it is important to consider their sources in context of their availability. As defined by Devasagayam *et al.* (2004) [16], "*functional foods are those that provide more than simple nutrition, they supply additional physiological benefit to the consumer*". Tomato, a cheap and at hand source of antioxidants, can be seen as a functional food [7].

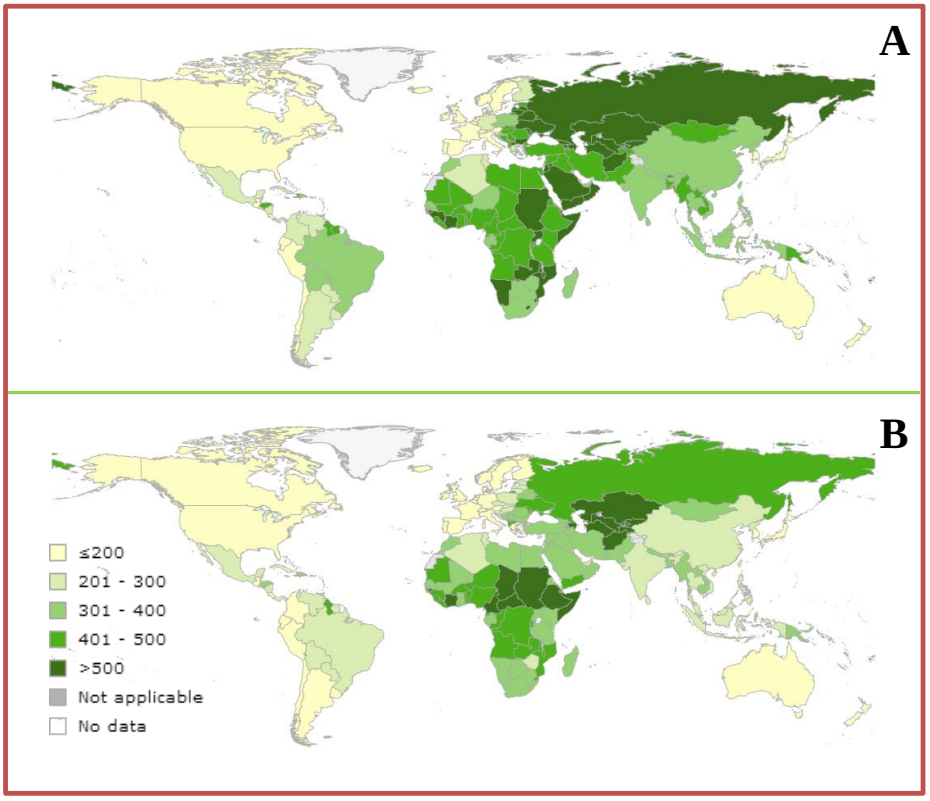


Figure 1.2. CVDs and diabetes, death rates per 100 000 population in 2008. **A** – Male; **B** - Female.[12]

1.1.1. Oxidative Stress (OxS)

According to Halliwell (2007) [17], free radical is defined as "*any specie capable of independent existence that contains one or more unpaired electrons*" [18-20]. In standard conditions O_2, *per se*, is stable and not harmful [21]. The activation of O_2 may occur by two different mechanisms, either by the reversal of the spin on one of the unpaired electrons, or by consecutive monovalent reduction (Figure 1.3), which can lead to formation of reactive oxygen species (ROS) [22].

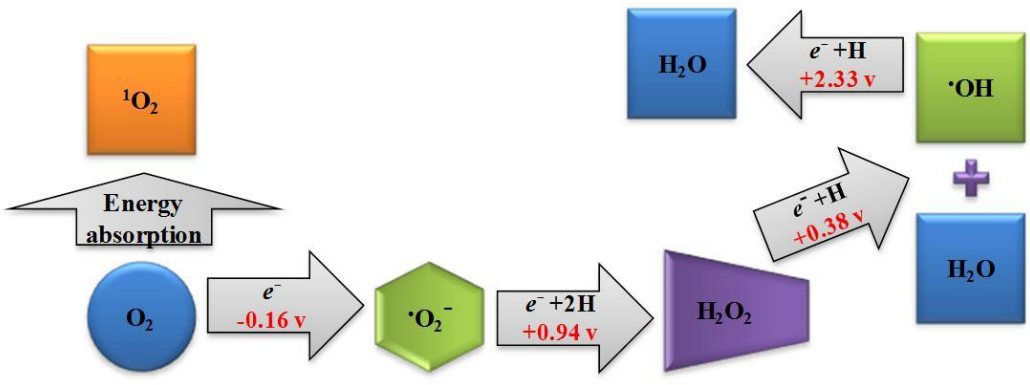


Figure 1.3. Reduction potentials for oxygen species, with formation of ROS. Adapted from Sharma (2012) [22] and Imlay (2003) [21].

Organic molecules with spin-paired electrons cannot transfer more than one electron at a time to O_2, and so O_2 cannot efficiently oxidise amino acids and nucleic acids [21, 23, 24]. To oxidise a non-radical atom or molecule, O_2 needs to react with a partner that provides a pair of electrons with parallel spins that fit into its free electron orbitals [22-24]. Due to spin restriction, molecular O_2 cannot accept four electrons at a time, which would produce H_2O, but instead undergoes the successive reductions described above and represented in Figure 1.3 [21-24]. Consecutive O_2 reductions leads to the formation of singlet oxygen, superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), which are all much stronger univalent oxidants than O_2 (Figure 1.3). Particularly, $^{\bullet}OH$ is the most reactive radical to a large amount of biomolecules [11, 21, 23]. In turn, although H_2O_2 is not a radical, its ability to generate $\cdot OH$ and its mobility makes it an important oxidant [24, 25]. In Table 1.1. are shown the most relevant chemical species of oxygen and classified according to their properties and reactivity.

Table 1.1. Classification of relevant chemical species of oxygen, according to their properties and reactivities. **Positive** and **negative** response. Adapted from Foyer (2009) **[26].**

Name	Symbol	Radical	Ion	ROS
Triplet oxygen	$^3O_2$			
Singlet oxygen	$^1O_2$			
Superoxide	$^{\bullet}O_2^-$			
Hydrogen peroxide	H_2O_2			
Hydroxyl radical	$^{\bullet}OH$			
Water	H_2O			
Hydroxide	OH^-			

In the biological systems a variety of radicals others than ROS can be generated. This is the case of the reactive nitrogen species (RNS) as nitric oxide ($\cdot NO$) and nitrogen dioxide ($\cdot NO_2$) radicals, peroxynitrite ($ONOO^-$) and its direct products (ONOOH and ROONO), dinitrogen trioxide (N_2O_3), nitrous oxide (N_2O), and the ions nitrite (NO_2^-) and nitronium (NO^+). NO is also an important specie, reacting with heme and heme-copper centres of a number of relevant biologic targets. Moroever, it has a favoured kinetic reaction with $\cdot O_2^-$ resulting in $ONOO^-$, which has distinct properties [11, 16, 22]. Most cells can produce $^{\bullet}O_2^-$, H_2O_2 and nitric oxide (NO) in a regulated manner to maintain homeostasis at the cellular level. It has been estimated that about 1% of the O_2 consumed by plants results in ROS, being their major sources the

mitochondrial respiration, followed by environmental stress stimulation (drought, salinity, chilling, metal toxicity, and UV-B radiation), and pathogens or drugs attacks [11, 16, 22, 25, 27].

Sies (1997) [28] define OxS as an "*imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage*", being this definition the most currently used [11, 16, 17]. In homeostasis, the generation of pro-oxidants in the form of ROS and RNS is under control through various levels of antioxidant defences [11, 16, 29]. This balance may be slightly tipped in favour of the oxidants so that there is continuous low-level oxidative damage in the human body – ageing [29, 30]. At high concentrations, ROS and RNS are extremely harmful to organisms, being able to inflict several forms of biological damage, including lipid peroxidation, protein oxidation, nucleic acids damage, enzyme inhibition, and apoptosis activation, that are a hallmark of several diseases, particularly cancer, CVDs and inflammatory and degenerative diseases [11, 22, 25]. Nevertheless, we must be aware that ROS and RNS are necessary for a healthy life. An example is the beneficial effect of a low to moderate concentration of ROS during exercise or the mechanisms of redox signalling [31-33]. Ultimately, the balance between ROS and RNS production, and antioxidant defences determines the degree of OxS (Figure 1.4) [11, 27].

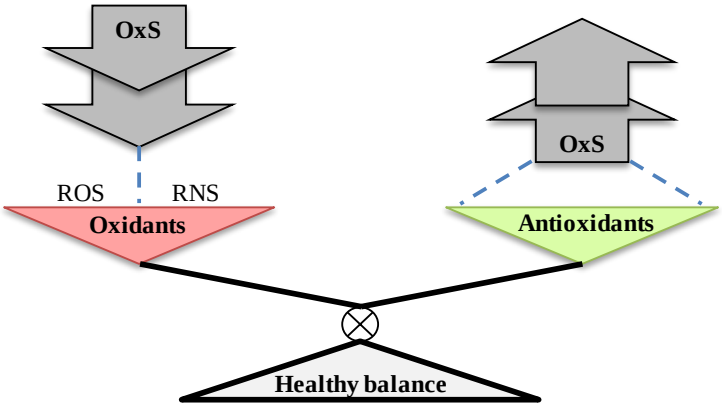


Figure 1.4. Oxidants and antioxidants balance/imbalance – OxS.

1.1.2. Lipid Peroxidation (LP)

OxS leads to cell damage, generating pro-oxidants in the form of ROS and RNS, shifting the balance significantly in favour of pro-oxidants (Figure 1.4) [22, 34].

The effect of ROS in the oxidative breakdown of polyunsaturated fatty acids (PUFAs) is well documented [24]. ROS and PUFAs can undergo to a highly damaging chain reaction, leading to both direct and indirect effects - LP [16, 24]. As reviewed by Niki *et al*. (2009) [35], LP was first studied in the food deterioration of in the 1930s. This deterioration is a consequence

of OxS, namely when membrane lipids, highly susceptible to free radical damage, react with ROS which in turn affects normal cellular functioning. LP also contributes to OxS through the production of lipid-derived radicals that can damage proteins and DNA [16, 19, 22]. According to Niki *et al*. (2009) [35], LP have three distinct mechanisms, (1) free radical-mediated oxidation, (2) free radical-independent non-enzymatic oxidation, and (3) enzymatic oxidation [35].

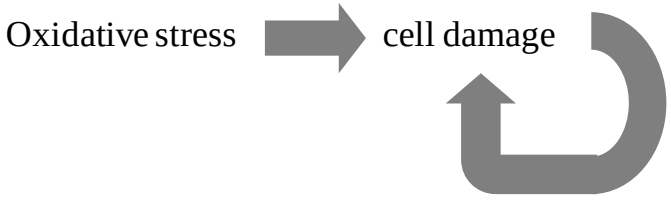


Figure 1.5. Illustration of relationship between oxidative stress and cell damage [19].

In the human body, lipid peroxides decomposition is not relevant due to the absence of transition metal ions [19]. Free transition metals like iron and copper are strong catalysts for oxidation reactions, in particular free iron. In biological forms like heme, haemoglobin, and myoglobin, iron appears to be safely sequestered [11, 24]. The iron transport protein transferrin as well as the storage protein ferritin may release iron upon low pH values or addition of reducing agents such as $^{\bullet}O_2^-$, from OxS. In excess $^{\bullet}O_2^-$ can provide enough iron for the initiation of LP [24]. Ferrous salts react with H_2O_2 to form $^{\bullet}OH$, through the Fenton reaction (Reaction I):

$$Fe(II) + H_2O_2 \rightarrow {}^{\bullet}OH + {}^{-}OH + Fe(III)\ [19] \qquad \text{(Reaction I)}$$

The hydrogen (H) atom can be considered as radical. Removing the hydrogen atom from a biological molecule leaves behind an unpaired electron on the atom from which the hydrogen was withdrawn, forming a radical. The greater the number of double bonds in a fatty acid side chain, the easier the removal of H, this make PUFAs particularly susceptible to LP, due to the presence of their methylene group [19, 36].

LP involves three different stages: (*i*) initiation, (*ii*) progression and (*iii*) termination. Initiation starts with the attack of unsaturated fatty acids (LH) by the $^{\bullet}OH$ radical, abstracting H from the LH, forming conjugated dienes, lipid radicals ($L^{\bullet}$) [19, 22, 37]:

Initiation:

$$^{\bullet}OH + LH \rightarrow H_2O + L^{\bullet} \qquad \text{(Reaction II)}$$

In the progression stage [11, 19, 37], the $L^{\bullet}$ formed is highly reactive, reacting with O_2 to form lipid peroxy radicals ($LOO^{\bullet}$). In turn, $LOO^{\bullet}$ can react with another LH forming a new $L^{\bullet}$, renewing the cycle, and propagating the chain reaction [22, 37]:

Propagation:

$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$ (Reaction III)

$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$ (Reaction IV)

Termination [11, 19, 37] occurs when the propagation is blocked, which can occurby two main mechanisms. One is the reaction between two radicals that leads to the formation of non-radical products (NRP) (Reaction V). The other mechanism is a reaction with an antioxidant (AOx), resulting in a more stable and less reactive radical ($AOx^{\bullet}$) (Reactions VI and VII) [11, 19, 37]:

Termination:

$LOO^{\bullet}/L^{\bullet} + LOO^{\bullet}/L^{\bullet} \rightarrow NRP$ (Reaction V)

$AntOH + LOO^{\bullet} \rightarrow LOOH + AOx^{\bullet}$ (Reaction VI)

$AntOH + L^{\bullet} \rightarrow LH + AOx^{\bullet}$ (Reaction VII)

As a result of LP, toxic by-products are formed, capable of cause effects in the area of generation or away from the original area (like second messengers) [16]. Recent studies [38, 39] have provided evidence that many LP products exert opposite effects depending on the conditions. At one hand, toxic effects for the cells, including atherogenic, apoptotic, and inflammatory effects have been reported [35]. LP contributes to the development of atherosclerosis, namely increasing oxidised low-density lipoprotein (oxLDL).

One the other hand, the metabolism of LP products, namely aldehydes, also constitutes a defence against the damage in the organism. Aldehydes can be removed by aldehyde reductase or glutathione transferases [24]. The cell damage due to ROS activity, that leads to LP, can be monitored through aldehydes levels, also giving an indication of OxS [11, 19, 22, 40]. An important degradation product obtained from cell membrane damage is malondialdehyde (MDA). This putative biomarker, can be determined by a colorimetric assay known as a thiobarbituric acid reactive species (TBARS) [11, 22, 24, 35].

1.1.3. Antioxidants

Protection from OxS leads to the development of a series of defence mechanisms: (i) preventative mechanisms, (ii) repair mechanisms, (iii) physical defences, and (iv) antioxidant defences [17, 35, 41]. Antioxidant defences, include multiple interactions of antioxidant compounds, antioxidant enzymes, damage-removal enzymes, and repair enzymes [17, 35].

Halliwell (2007) [17], defines an antioxidant as "*any substance that delays, prevents or removes oxidative damage to a target molecule*". It is known that antioxidants may improve health, through inhibition of oxidative damage, either directly, by scavenging or sequestering active species, or indirectly, by acting as a messenger. It is also suggested that the oxidition/reduction ratio of the different antioxidants can serve as a signal for scavenging mechanisms [16, 35, 42].

Antioxidants can act as a first line of defence, protecting against ROS formation. Examples include metal-ion-binding proteins or enzymes such as SOD, catalase (CAT) and selenium-dependent glutathione (GSH) peroxidase. Regarding to LP, a second line of defence occurs, resulting in the deactivation and, the chain-breaking of LP. Examples includes vitamin E (tocopherols) and vitamin C (L-ascorbic acid). A third line of defence that includes the metabolism of LP products, are for example aldehyde reductase, glutathione transferases (GPX), GSH peroxidases and cytochrome P-450 [11, 16, 24, 28].

Considering the nature of the antioxidants, they can be divided in non-enzymatic and enzymatic (Table 1.2). Enzymatic antioxidants include SOD, ascorbate peroxidase (APX), GPX, CAT and GSH peroxidase that act as the first line of defence, by chelating superoxide and various peroxides [11, 22, 23, 36, 41]. Non-enzymatic antioxidants include chain-breaking antioxidants as phenolics and tocopherol (primarily α-tocopherol), quenchers as carotenoids (primarily β-carotene and lycopene), ascorbate and flavonoids [11, 16, 22, 23, 28, 36]. Some antioxidants have more than one antioxidant function (scavenging or sequestering, acting as messenger, etc), as they interact with numerous cellular components and some can also act as enzyme cofactors [11, 22, 35, 36].

Table 1.2. Antioxidant defence in biological systems. Example of non-enzymatic and enzymatic antioxidants. Adapted from Sies (1994) [43].

Antioxidants	Remarks
Non-enzymatic	
α-Tocopherol	radical chain-breaking
β-Carotene	singlet oxygen quencher
Lycopene	singlet oxygen quencher
Ascorbate	diverse antioxidant functions
Bilirubin	plasma antioxidant
Flavonoids	plant antioxidant
Enzymatic	
Superoxide dismutases	CuZn enzyme, Mn enzyme, Fe enzyme
GSH peroxidades	enzyme family with peroxidase activity
Catalase	decomposition of hydrogen peroxide to water and oxygen
GSSG reductase	maintaining GSH levels

In biological systems we have to consider two environments, a lipophilic and a hydrophilic medium. Considering the biological activity, it is important to transfer oxidative damage from a lipophilic medium to a hydrophilic medium, maximising antioxidant activity, such as from the membrane to the cytosol or from lipoproteins to the aqueous phase of the plasma [11, 28]. From this point of view, antioxidants can be regarded as lipid-soluble (lipophilic) or water-soluble (hydrophilic). An antioxidant that combines both of these characteristics is the most efficient. α-Tocopherol is an example of an antioxidant that can interact with both a lipophilic and a hydrophilic medium. It can react with ROS, and is able to interact with water-soluble compounds like ascorbate, for its own regeneration [11, 28, 44]. α-Tocopherol tends to localise in membranes and lipoproteins, and is the major antioxidant in extracts prepared from LDL. Is probably the most efficient antioxidant in the lipid medium [11, 16, 28]. Other lipid-soluble antioxidants are carotenoids, which are quenchers in LP.

Biological systems contain considerably part of water, demonstrating the importance of water-soluble antioxidants. The concentration of these antioxidants in the extracellular space of the vascular wall may approximate the concentration in the lumen. Flavonoids and phenolics are examples of water-soluble antioxidants, but are poorly absorbed and extensively metabolised. One of the main water-soluble antioxidants, ascorbate, is derived from the diet and its concentration in interstitial fluid and lymph is similar to that in plasma (Table 1.3) [11].

Table 1.3. Non-enzymatic antioxidants in human plasma. Adapted from Sies and Stahl (1995) [45].

Antioxidant	Plasma contents
Water soluble	**µmol/L**
Ascorbate	30.00-150.00
Glutathione	1.00-2.00
Urate	160.00-450.00
Bilirubin	5.00-20.00
Lipid soluble	**µmol/L**
α-Tocopherol	15.00-40.00
δ-Tocopherol	3.00-5.00
α-Carotene	0.05-0.10
β-Carotene	0.30-0.60
Lycopene	0.50-1.00
Lutein	0.10-0.30
Zeaxanthin	0.10-0.20

Many studies, about diet-derived antioxidants, have attempted to establish a direct effect in health. Higher consumption of fruits and vegetables, sources of antioxidants, has been associated to a lower risk of CVD [11, 16, 26, 46] and cancer [16, 26, 36, 47]. Also, regular physical exercise has been found to increase the level of antioxidant defence and to decrease, at medium and long term, the rate of ROS production [16, 36, 46, 48].

Disturbance in the OxS balance, in any direction, causes health problems [16, 22, 26]. ROS are essential to all organisms, as is the case of animal cell apoptosis [26], and therefore, antioxidant over activity has a negative effect. Antioxidants also have the ability to act as pro-oxidants under certain conditions, depending on the biochemical context, for example α-tocopherol activity over LDL [11, 16, 26, 37]. These concerns lead to the necessity for identifications and quantifications of antioxidants present on functional foods. Foods from vegetable origin provide a complex mixture of natural substances with antioxidant capacity. Some of the most important naturally occurring plant substances include vitamins A, C and E, and also carotenoids and phenolic compounds [49]. Taking this into account, in the next section the lipophilic antioxidants, namely β-carotene, lycopene, α- and δ-tocopherol, and hydrophilic antioxidant ascorbic acid, will be reviewed.

1.2. LIPOPHILIC AND HYDROPHILIC ANTIOXIDANTS

1.2.1. Lipophilic Antioxidants

Carotenoids and tocopherols are the most important naturally occurring plant lipid-soluble antioxidants. In this section, two of the most important carotenoids, lycopene and β-carotene, will be addressed. After carotenoids, tocopherols, in particular α- and δ-tocopherol, will be addressed, since they are of the most relevant in biological systems [49, 50].

1.2.1.1. Carotenoids – Lycopene and β-Carotene

Carotenoids are natural pigments synthesised *de novo* by plants and some photosynthetic microorganisms. Chemically they are isoprenoids, eight isoprene (C_5H_8) units, resulting in a C_{40} polyene backbone, biosynthesised from two C_{20} geranylgeranyl diphosphate (GGPP) molecules. The characteristic feature of the polyene chain, consisting of 3 to 15 conjugated double bonds, is a conjugated system in which the π-electrons are delocalised along the entire polyene chain, conferring to carotenoids their unique molecular shape, chemical reactivity (e.g. making them susceptible to oxidative cleavage, and conferring them lipophilic proprieties), and light-absorbing properties (e.g. allowing them to absorb light with UV-vis absorption maxima between 400 and 570 nm) [51-57].

More than 600 carotenoids have been isolated from natural sources, but only about 40 (6 to 7%) are found in our daily foods [55]. Around 10 %, of the carotenoids can be metabolised to retinol and function as provitamin A, as they have a β-type non-substituted ring (β-ionone ring), along with one polyene chain (containing at least 11 carbon atoms). In mammals, carotenoids and vitamin A are important for vision, growth, cellular differentiation, morphogenesis, and several other cellular and physiologic functions. Some carotenoids have been identified in human plasma and tissues, including lycopene, β-carotene, α-carotene, lutein and β-cryptoxanthin, [10, 52, 54-56, 58, 59]. According to Gould (1992) [60], 21 different pigments carotenoid class have been identified and quantified in tomato fruits. Lycopene is the most dominant carotenoid found in tomatoes with lesser amounts of α-carotene, β-carotene, γ- carotene, ξ-carotene, phytoene, phytofluene, neurosporene, and lutein.

Structurally, carotenoids can be classified in two groups: (*i*) carotenes or carotenoids hydrocarbons, which are composed by only carbon and hydrogen (e.g., lycopene and β-carotene); and(*ii*) xanthophyls or oxygenated carotenoids, obtained by the addition of oxygen,

leading to the formation of epoxy, carbonyl, hydroxy, methoxy or carboxylic acid functional groups derivatives (e.g., violaxanthin – epoxy, canthaxanthin – oxo, zeaxanthin – hydroxy, spirilloxanthin – methoxy and torularhodin – carboxylic acid) [55, 56, 61, 62].

Carotenoids Biosynthesis

The biosynthetic pathway of the carotenoids in plants starts with a small C_5 (5 carbons) isoprenoid molecule, isopentyl diphosphate (IPP), derived from the methylerythritol phosphate (MEP) and mevalonate (MAV) pathways [63-65]. This small molecule may result in hundreds of other molecules, larger and more complex, with the biosynthesis pathway taking place within the plastids [65, 66].

The first biosynthetic phase is formed by the sequential and linear addition of three molecules of IPP to one molecule of dimethylallyl diphosphate (DMAPP), through enzymatic mediation. First addition results in geranyl diphosphate (GPP), which may lead to monoterpenes, while second addition generates farnesyl diphosphate (FPP), which may lead to sterols, sesquiterpenes or polyterpenes [63, 65, 67-69]. A third addition results in GGPP, which may lead to quinones, diterpenes, chlorophylls or tocopherols, and stop "growing" with phytoene (a C_{40}), which results in their condensation of two C20 GGPP molecules. Isomerisation and denaturation processes through enzymatic mediation, result in the formation of phytofluene, followed by ζ-carotene, neurosperene and ending with lycopene, an all C_{40} polyene backbone (Figure 1.6-A) [65-67, 69-72].

In a second phase, different paths result from lycopene that can lead to the formation of different molecular groups, such as provitamin A, retinol (vitamin A), xanthophylls or abscisic acid (ABA). The first transformation occurs through lycopene cyclases enzymes, lycopene β-cyclase (LCYB) and lycopene ε-cyclase (LCYE), resulting in γ-carotene and δ-carotene respectively [62, 65-67, 69-72]. The δ-carotene branch will result in α-carotene and then lutein, both stable and important biomolecules. In the other branch, γ-carotene, has a richer biosynthetic sequence, resulting in diverse biomolecules. γ-Carotene, through LCYE, can also be transformed into α-carotene, but its main product, β-carotene, is obtained through LCYB [65-67, 69, 70, 73].

β-Carotene may result in one or two molecules of retinol [53, 59, 67, 74-76], or can undergo another path, resulting in xanthophylls, like the ones found in the xanthophyll cycle, zeaxanthin, followed by antheraxanthin and violaxanthin [65-67, 71, 72, 77-79]. Neoxanthin result from violaxanthin, and can result in ABA through xanthoxin followed by abscisic aldehyde [71, 79, 80]. Another path from β-carotene results in the formation of important

antioxidant biomolecules, such as canthaxanthin, followed by astaxanthin and capsorubin [72, 81, 82]. This second phase can be seen in Figure 1.6-B.

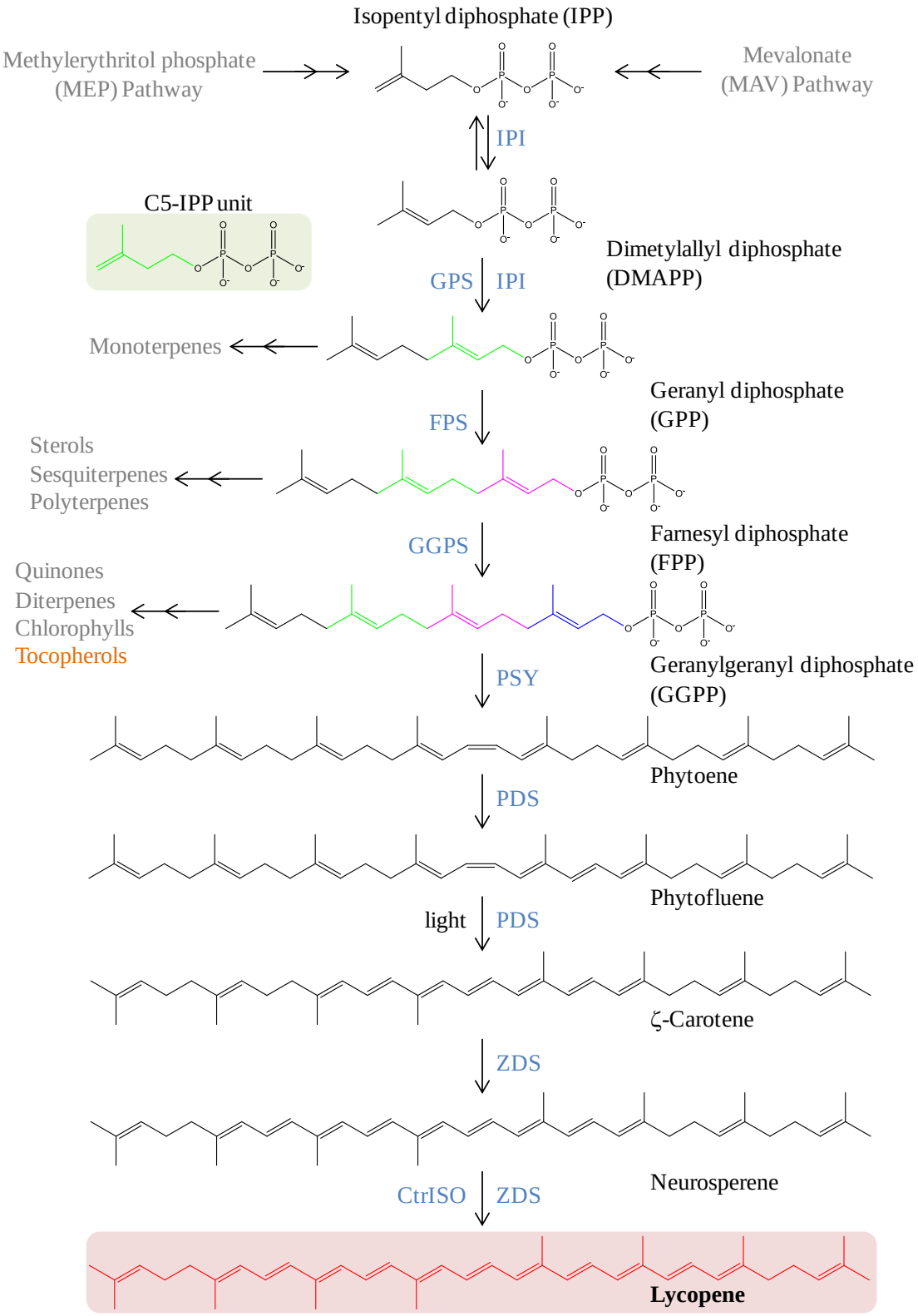


Figure 1.6-A. Biosynthetic sequence of the carotenoids in plants – *Lycopene biosynthesis* (*in blue*): IPI – isopentenyl diphosphate isomerase, GPS – geranyl pyrophosphate synthase, FPS – farnesyl pyrophosphate synthase, GGPS – geranylgeranyl diphosphate synthase, PSY – phytoene synthase, PDS – phytoene desaturase, ZDS – ζ-carotene desaturase, CtrISO – cynobacterial ζ-carotene desaturase, carotene isomerise [63-82].

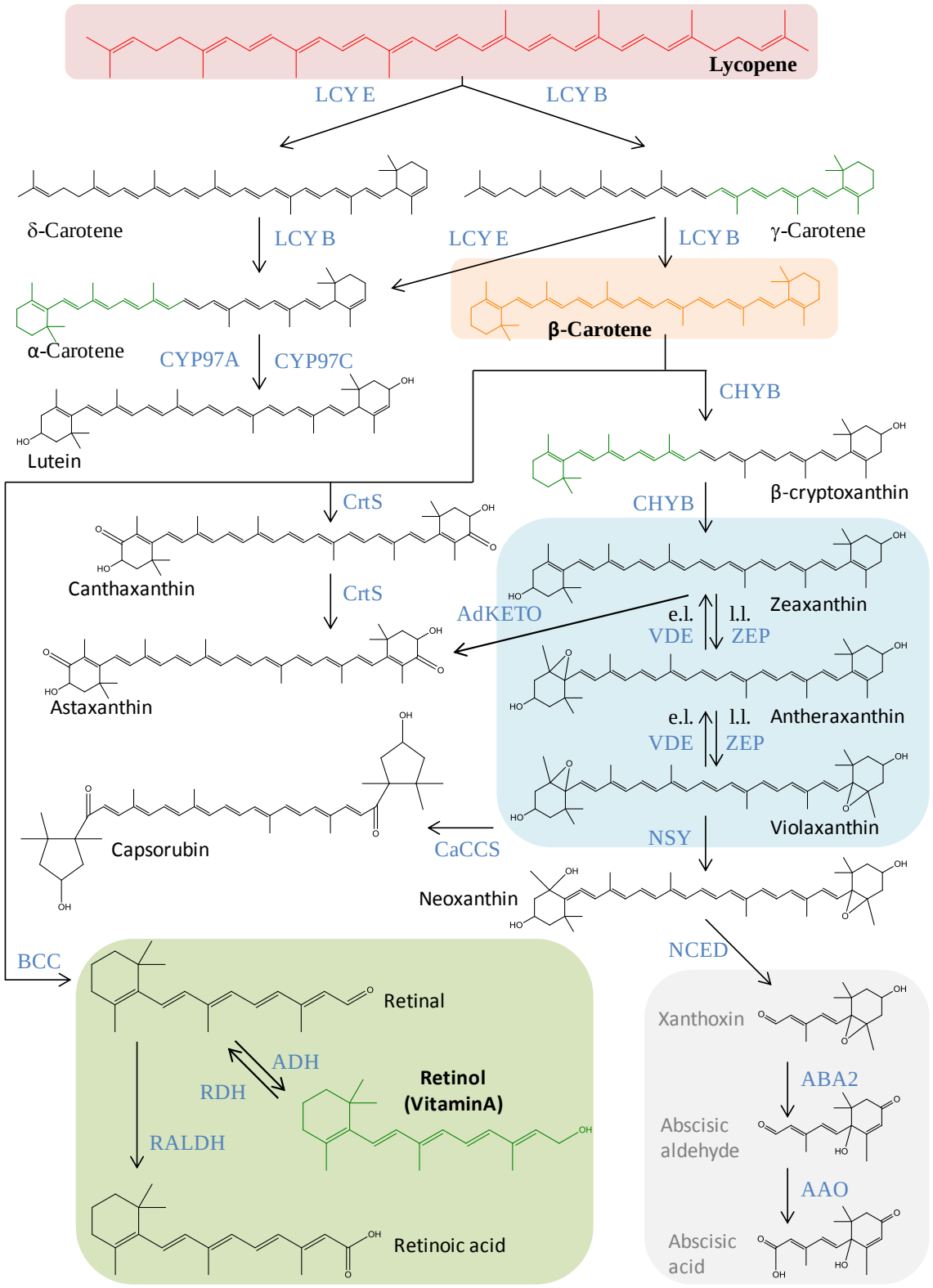


Figure 1.6-B. Biosynthetic sequence of the carotenoids in plants – *Lycopene derivatives* (*in blue*)**:** LCYE – lycopene ε-cyclase, LCY B – lycopene β-cyclase, CYP97 A – β-ring carotene hydroxylase, CYP97 C – ε-ring carotene hydroxylase, CHY B – carotene β-hydroxylase, VDE – violaxanthin de-epoxidase, ZEP – zeaxanthin epoxidase, AdKETO – zeaxanthin ketolase, NSY – neoxanthin synthase, NCED – 9-cis-epoxycarotenoid dioxygenase, ABA2 – alcohol dehydrogenase, AAO – abscisic aldehyde oxidase, CrtS – β-carotene ketolase, BCC – β-carotene cleavage dioxygenase, ADH – alcohol dehydrogenase, RDH – retinol dehydrogenase, RALDH – retinaldehyde dehydrogenase, e.l. – excess light, l.l. – low light [63-82].

Carotenoids Bioavailability

Humans cannot synthesise carotenoids *de novo*, and therefore depend on the diet for its uptake. Absorption and metabolism of carotenoids varies among animal species. In humans an appreciable amount of the carotenoids can be absorbed by the mucosal cells, and subsequently appear unchanged in the circulation and peripheral tissues or metabolised in the process of absorption (digestibility) [58, 59, 83].

According to Fernández-García *et al.* (2012) [59], bioavailability comprises accessibility for absorption, absorption, metabolism, transport and tissue distribution, and bioactivity. Carotenoids bioavailability schematisation is presented in Figure 1.7 [59].

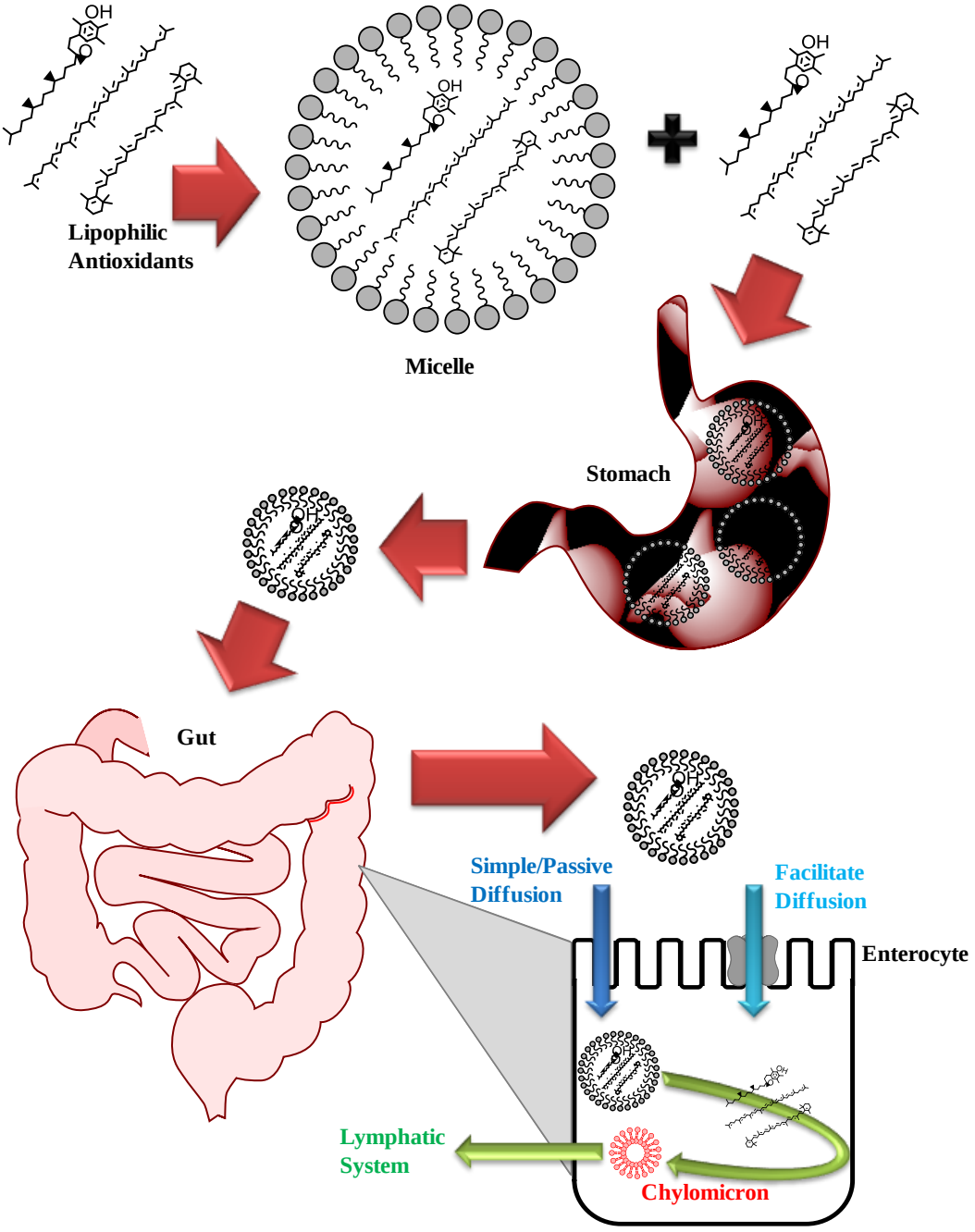


Figure 1.7. Scheme of digestion and absorption of lipophilic antioxidants. Adapted from Fernández-García *et al.* (2012) [59] and Story *et al.* (2010) [84].

Accessibility for the absorption of carotenoids includes modification over the food matrix, leading to absorption differentiation. This first step can involve a simple mastication or a more refined processing of the matrix. The aim is to facilitate biocompound release from the food matrix. This may occur in a chemical or a physical nature. It is well reported that the absorption of carotenoids is improved after processing, namely after maceration, and application of a thermal treatment in the presence of oil [59, 83, 85]. In the first case, carotenoids absorption is improved through the disruption of lycopene-protein complex and cell walls, and also through *trans* to *cis* isomerisation promotion (mainly for lycopene), improving the carotenoids release and solubilisation. Oil application also improves the carotenoids absorption, due to their fat-soluble character, and their contribution for micellisation [6, 59, 83-85].

The absorption of carotenoids occurs in the gut, where carotenoids (lipophilic compounds) undergo micellisation. In micelles carotenoids are stabilised by biliary salts. Additionally, micelles nature make them potentially accessible to the intestinal epithelium. Micellisation efficiency is highly influenced by the accessibility for absorption, where the initial presence of oil and the carotenoids release from the matrix is directly correlated. The oil uptake stimulates biliary secretions and pancreatic lipase levels, which in turn increases micellisation capacity. In addition, bioaccessibility appears to be increased by the consumption of long chain-triacylglycerides [59, 83, 84, 86].

The micelles approach the unstirred water layer of the apical side of the intestinal mucosal cells (enterocytes), and intestinal absorption of carotenoids occurs via passive diffusion or through facilitated transport. Carotenoids can be metabolised in the enterocytes. An example is provitamin A carotenoids that are partly converted to vitamin A (as retinyl esters), at it occurs with β-carotene, which is oxidatively cleaved by β-carotene cleavage dioxygenase (BCC's) [53, 58, 84]. In the enterocytes, both unmetabolised carotenoids and retinyl esters are incorporated into chylomicrons, being then transported across the basolateral membrane and secreted into lymph for followingdelivery to the blood [53, 58, 59, 84, 87].

In the blood, some chylomicrons degrade before reaching the liver due to the activity of lipoprotein lipase. Therefore part of the lipid content of these particles is released and absorbed into the endothelial tissue. Eventually chylomicrons are taken up by the liver where they are stored or re-excreted into circulation within very low-density lipoproteins (VLDLs). Once released into the circulatory system, they are transformed into low-density lipoproteins (LDLs) and finally into high-density lipoproteins (HDLs). The more non-polar carotenoids, such as β-carotene and lycopene, are predominantly found in LDL [58, 59, 87].

Carotenoids, being hydrophobic molecules, are expected to be restricted to hydrophobic areas in the cell, such as the inner core of membranes [52]. Adipose tissue and liver are the major

tissue storage for the carotenoids, although these compounds have also been identified in lungs, kidneys, cervix, prostate and most other tissues. In the tissues lycopene is metabolised and oxidised. Oxidation products (apocarotenoid) may be responsible for some of the biological activities attributed to lycopene. Several oxidised forms of lycopene and its polar metabolites have been isolated and identified [53]. Autoxidation of β-carotene leads to the formation of epoxides leading to the formation of ketones (β-apocarotenones) and aldehydes (β-apocarotenals). The apocarotenoids that have a central cleavage, result in 2 molecules of retinol [45, 53, 58, 87].

Lycopene

In ripe tomatoes lycopene is the most abundant carotenoid, accounting for more than 90 % of the natural pigments. Lycopene is present in higher content in the outer pericarp (skins contained about five times more than the whole tomato pulp), comprising approximately 80 to 90% of the lycopene level, about 3 to 5 mg of lycopene per 100 g of fresh weight, depending on climatic conditions and tomato variety [6].

The green colour of "immature green" tomatoes is a result of chlorophyll, the initial dominant pigment of tomato. When chlorophyll is reduced, the chloroplasts colour changes from green to white. Carotenoids in plants are synthesised *de novo* in nearly all types of plastids. During fruit development, the mRNA levels for the lycopene-producing enzymes PSY and PDS increase. During ripening, chlorophyll is degraded, and carotenoids take its place, converting lycopene into the β-carotene form, resulting in an orange tomato. Accumulation of lycopene begins at the 'breaker' stage of fruit ripening after the fruit has reached the 'mature green' stage. Lycopene β- and ε-cyclases decline and completely disappear, leading to the accumulation of lycopene during the ripening of tomatoes that are deposited in needlelike crystals into chloroplasts, ending β-carotene production. According to Ronen *et al.* (1999) [88], the total carotenoid concentration in tomatoes increases between 10- and 15-fold during fruit ripening. This change is due mainly to a 500-fold increase in the concentration of lycopene [6, 71, 86, 88].

In tomatoes and most biological systems, lycopene is present as all-trans lycopene, with a C_{40} carbon skeleton. This basic structure and carotenoid numbering scheme is illustrated the Figure 1.8 [52, 89]. This skeleton can be modified by cyclization at one or both ends of the molecule, and different geometric configurations are possible because of isomerism around C=C double bonds, (Figure A1) [52, 90, 91].

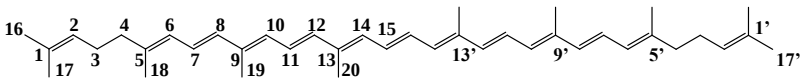


Figure 1.8. Chemical structure of lycopene.

An 11 conjugated double bonds linear system, is responsible for the unique characteristics of lycopene, as a consequence of $\pi \rightarrow \pi^*$ transitions, when one π electron of the polyene system is promoted to an unoccupied antibonding orbital (π^*). The energy between orbital's π/π^* is low, so the wavelength that results from the transition corresponds to light in the visible region, range of 400-500 nm, conferring the typical orange or red colour, as described in Table A1 [6, 52, 53, 84, 86, 91].

The polyene structure of lycopene, make them susceptible to attack by electrophilic species, and as a long hydrocarbon chain they are hydrophobic. Lycopene is also light and temperature sensitive. *Cis*- isomers are less stable than all-*trans* lycopene because they are more susceptible to oxygen-catalysed oxidation, acids, and some metallic ions such as Cu^{2+}, Fe^{3+}, and can undergo retro isomerism (heat favours the formation of *cis*-isomers) [6, 52, 84, 86, 87, 92, 93].

β-Carotene

β-Carotene is the second main carotenoid in tomato, accounting for 5 to 10% of total carotenoids in the ripe fruit. Its maximum levels occurs at the "breaker" stage (orange tomato) [88]. β-Carotene arises from lycopene through the action of LCY B (Figure 1.6-B) from carotenoids biosynthesis (Figures 1.6-A and 1.6-B), and can be metabolised to biologically important metabolites, namely retinol, and also xanthophylls (Figure 1.6-B) [6, 45].

β-Carotene has a C_{40} carbon skeleton, which differs from lycopene as it has a cyclization at both ends of the molecule (β,β-carotene). This basic structure and carotenoid numbering scheme is illustrated in Figure 1.9 [52, 89].

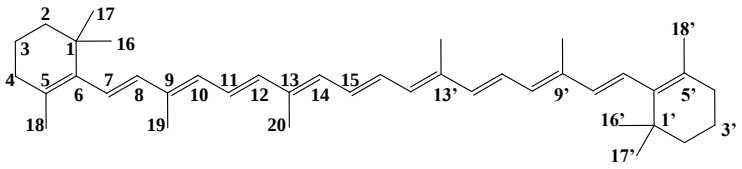


Figure 1.9. Chemical structure of β-carotene.

The β-ionone ring leads to a minor increase of energy between orbitals for $\pi \rightarrow \pi^*$ transitions, shifting the λ_{max} around 20 nm. The physical properties of lycopene and β-carotene are summarized in Appendix (Table A1). As in lycopene, the β-carotene structure make them

susceptible to attack by electrophilic species, not only in the linear chain (resulting in β-apocarotenones and β-apocarotenals), but also in the β-ionone ring (formation of epoxides). An important "attack", is the central cleavage resulting in apocarotenoid products, leading to the formation of 2 retinol molecules [45]. Antioxidant activity of β-carotene, namely singlet oxygen quenching, is approximately half of the lycopene, but the former is biologically relevant due to the ability to originate retinol molecules [6, 45].

Antioxidant Potential of Carotenoids

Carotenoids antioxidant characteristics confers them important biological function as efficient antioxidants, beinglycopene the most bioactive of all dietary carotenoids, despite the lack of a β-ionone ring. This confers to lycopene a high nutraceutical value. Mixtures of carotenoids, probably related to the specific positioning of different carotenoids in cell membranes, are more effective than the single compound. This synergistic effect is increased when lycopene or lutein are present. β-Carotene differs from lycopene by having two β-ionone rings, which leads to a slight decrease of antioxidant capacity, but on the other hand, confers the ability to generate two retinol molecules, with high biological importance [62, 86, 87, 94, 95].

When oxidised, the excited carotenoids have the ability to dissipate acquired energy through a series of rotational and vibrational interactions with the solvent, regenerating the original unexcited state. Paiva and Russell (1999) [62] state that the quenching activity of a carotenoid depends, mainly, on the number of conjugated double bonds of the molecule, and is influenced to a lesser extent by carotenoid end groups (cyclic or acyclic), or the nature of substituents in carotenoids containing cyclic end groups [62]. Carotenoids protect chlorophylls, proteins, lipids and DNA from non-radical ROS damage converting the resulting excess energy into heat via the carotenoid lowest excited triplet state (^{3}CAR) (Reaction VIII and IX) [52, 66, 96]:

$$CAR + SO \rightarrow {}^3CAR + O_2 \qquad \text{(Reaction VIII)}$$

$$^3CAR \rightarrow CAR + \text{heat} \qquad \text{(Reaction IX)}$$

The reactions of carotenoids with free radicals are much more complex than with non-radical ROS and depend mostly on the nature of the radical rather than the carotenoids. Three different reactions have been proposed between free radicals and carotenoids: electron transfer (Reaction X), adduct formation (Reaction XI), and hydrogen abstraction (Reaction XII) [52, 96].

Electron transfer: $CAR + R^{\bullet} \rightarrow CAR^{\bullet+} + R^{-}$ (Reaction X)

Adduct formation: $R^{\bullet} + CAR \rightarrow [CAR–R]^{\bullet}$ (Reaction XI)

Hydrogen abstraction: $R^{\bullet} + CAR \rightarrow CAR^{\bullet} + RH$ (Reaction XII)

The ability of carotenoids to act as antioxidants depends on several factors, such as carotenoid concentration, oxygen tension and the surrounding environment. The antioxidant mechanism involved still has not been elucidated but could involve the direct or indirect activation via oxidised products. Several oxidised forms of lycopene and polar metabolites have been isolated and identified, such as apo-6‘and apo-8‘-lycopenals in raw tomatoes, while 5,6-dihydroxy-5,6-dihydro-lycopene, and apo-6‘-, apo-8‘-, apo-10‘-, apo-12‘- and apo-14‘-lycopenals have been found in human plasma [76, 87, 94, 97]. It’s well known that the β-carotene central cleavage products retinol, and also excentric cleavage products, β-apocarotenals and β-apocarotenones, have been isolated and identified, although biological functions in mammals are unknown. Eroglu *et al.* (2012) [98] reported molecular modelling studies that confirmed that some β-apocarotenones can interact directly with the ligand binding site of retinoid receptors. After the detection of β-apo-13-carotenone present in human plasma, Eroglu suggested that “β-*apocarotenoids function as naturally occurring retinoid antagonists*” [98].

Recent studies indicate that carotenoids can also possess a pro-oxidative action depending on the factors involved. An example is the pro-oxidative activity of carotenoids when present at relatively high concentrations, as reported by Palozza *et al.* (2003) [99] for β-carotene, or Yeh and Hu (2000) [100] for lycopene and β-carotene, in cultured cells [87, 97].

Human Health and Carotenoids

Epidemiological studies, as well as laboratory experimentation, have shown that vegetables, fruits, tea and other foods with high antioxidant content, demonstrate a correlation with the lower prevalence of some pathologies. Geographically, the Mediterranean region, have a low incidence of chronic diseases like CVDs. Tomato, a component of the Mediterranean diet, is a fruit widely consumed either fresh or processed, making it an accessible source of nutraceuticals, as is the case with carotenoids [61, 101-109].

The bioactivity effects of tomato consumption are generally attributed to carotenoids, which are able to reduce the risk of certain types of cancer [57, 61, 87, 96, 101], erythema [5, 61, 96, 97], cataracts [5, 96], brain disorders [10, 109], and CVD [10, 96, 102, 105, 107-109]. Some of these pathologies arise from inflammatory processes caused by ROS and RNS, therefore OxS is observed to have an important role in their development and progression. Oxidation of cholesterol in arteries is discussed as one mechanism leading to CVD, like atherosclerosis (e.g.

LDL oxidation). Carotenoids, such as lycopene and β-carotene are highly reactive towards oxygen and free radicals, and this is the basis for carotenoids anti-oxidant activity in biological systems [10, 87, 96, 102, 105, 108].

Carotenoids may also exert their beneficial effects via other mechanisms, such as gap junction communication, cell growth regulation, modulating gene expression or immune response. Several studies [61, 102, 105] have reported a positive correlation between the intake of carotenoids and serum carotenoids levels. An inverse correlation has also been observed between plasma concentrations of cholesterol and triglyceride, prevention of platelet aggregation and thrombosis, increased levels of antioxidant enzymes (e.g. SOD), and decreased DNA damage [61, 102, 105, 107, 109].

Carotenoids can interact with polyphenols and vitamins E and C, the major antioxidative compounds of fruits and vegetables, as shown by reports of synergistic behaviours rising from its simultaneous presence in foods. Lycopene shows low protecting LDL activity when compared with its combination with other antioxidants, in particular vitamins C and E, proanthocyanins and selenium. In a mixture of dietary carotenoids and free radicals, it is the lycopene, as the most bioactive antioxidant, that is oxidised, in order to protect all the other carotenoids. An example of this synergistic behaviour is the lung cancer trial, where β-carotene revealed a negative effect rather than the expected protective benefit in a small subgroup in the study. Those affected were heavy smokers, and the reason is therefore appointed to a deficiency in vitamin C. Studies carried out at low carotenoids concentrations, in "normal" antioxidant conditions, tend to show protective effects, while higher concentrations tend to show the opposite effects, as a consequence of the ability of some carotenoids (e.g. β-carotene) to act as a pro-oxidant rather than antioxidant, at high concentrations and under high oxygen tension [61, 96, 101-103, 107, 110].

1.2.1.2. Vitamin E – α- and δ-Tocopherol

Structurally vitamin E consists of a 6-chromanol ring, similar to coumarins, but with the ketonic oxygen substituted by an alkyl C_{16} isoprenoid side chain [111-113]. This singular phenolic group is synthesised *de novo* by photosynthetic organisms, including higher plants, and is found in all green tissues but predominantly in seeds. Vitamin E was discovered in 1922 by Evans and Bishop [114], and can be classified in two groups: (*i*) tocopherols that comprehend four tocopherols: α-, β-, γ- and δ-tocopherol, differing by the number and position of alkyl groups; and (*ii*) and the corresponding tocotrienols: α-, β-, γ- and δ-tocotrienol, differing from tocopherols by having unsaturated side chains (Figure 1.10) [111, 112, 114-118].

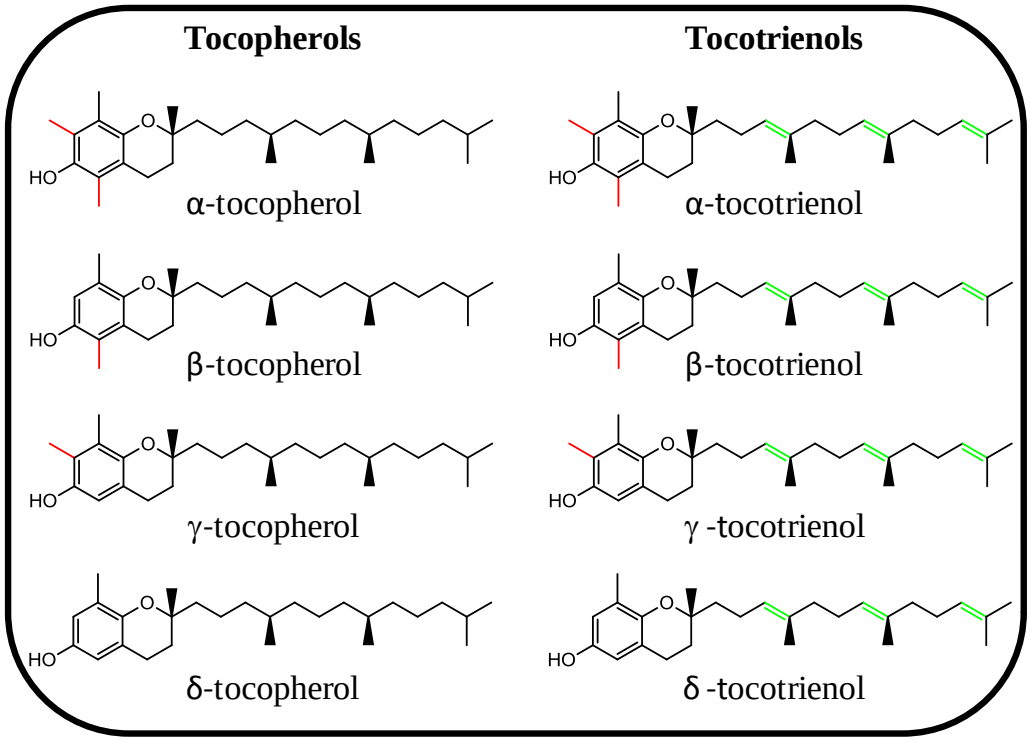


Figure 1.10. Naturally occurring isoforms of vitamin E. In **red**, differentiation between isoforms; in **green** differentiation between groups.

α-Tocopherol, due to its hydrophobic nature, is one of the most abundant lipid-soluble antioxidant along with γ-tocopherol, being involved in the protection of pigments, proteins, and polyunsaturated fatty acids against ROS, generated during photosynthesis. Together with carotenoid, they are stored in chloroplasts (especially α-tocopherol) [111, 112, 114, 116-118].

In the human diet, plant derived oils represent the major sources of vitamin E, containing the four tocopherols in different relative amounts. α-Tocopherol is, by far, the most bioactive isoform, and the most abundant in the human body. Concerning the antioxidant capacity of the different vitamin E isoforms, several studies have concluded that tocopherols and tocotrienols support a hydrogen-donating power in the order α > β > γ >δ. This was shown *in vivo*, although *in vitro* studies have also revealed that the antioxidant power was related to the conditions of the surrounding environment. According to Kamal-Eldin and Appelqvist (1996) [119], an example is the finding of a reversed order (δ>γ>β>α) antioxidant activity when tocopherols were placed in fats, oils, and lipoproteins solutions, and the same reversion can occur with a temperature change, from "physiological" 37º C to 100º C [37, 111, 114, 115, 119-121].

Tocopherols Biosynthesis

Tocopherols biosynthesis (Figure 1.11), results from the condensation of homogentisic acid (HGA), the final product of shikimate pathway, with phytyl diphosphate (Phytyl-DP) derived from chlorophyll *a* or GGPP (MEP and MAV pathway, respectively), resulting in 2-methyl-6-phytylplastoquinol (MPBQ). This process occurs mainly in chloroplasts [65, 116, 122-125].

The Phytyl-DP obtained from GGPP provides the long isoprenoid side chain of tocopherols. On the other hand, shikimate pathway begins with the condensation of phosphoenol pyruvate (PEP) and erythrose 4-phosphate (E4P) yielding 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP), and ends with the 4-hydroxyphenylpyruvic acid (HPP), catalyzed by 4-hydroxyphenylpyruvate dioxygenase (HPPD), producing HGA, the first step of the tocopherol-core pathway [126-128].

Structurally, MPBQ is the conjugation of a polar group (phenol) from HGA with an alkyl side chain from Phytyl-DP. MPBQ can be methylated to form 2,3-dimethyl-5-phytyl-1, 4-benzoquinone (DMPBQ). In turn, tocopherol cyclase (TC) converts MPBQ to δ-tocopherol and DMPBQ γ-tocopherol, which present a 6-chromanol ring and an alkyl C_{16} isoprenoid side chain. Finally, tocopherol methyltransferases (TMT) methylates the C5 of δ- and γ-tocopherol, originating β-tocopherol and α-tocopherol, respectively [65, 116, 122, 123].

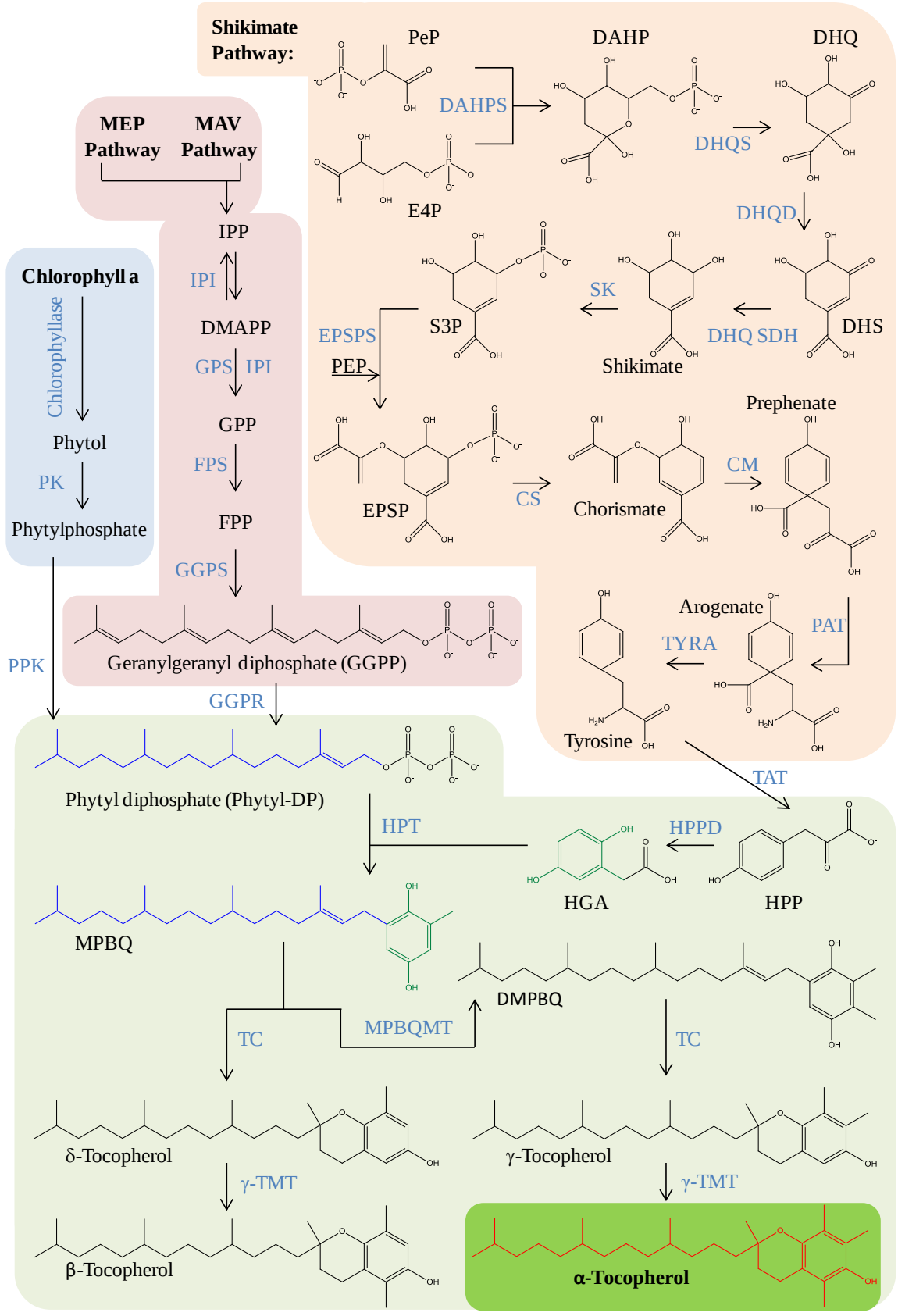


Figure 1.11. (continue next page)

Figure 1.11. Biosynthetic sequence of the tocopherols. ***Enzymes*** **(*in blue*):** PK – phytol kinase, PPK – phytylphosphate kinase, IPI – isopentenyl diphosphate isomerase, GPS – geranyl pyrophosphate synthase, FPS – farnesyl pyrophosphate synthase, GGPS – geranylgeranyl diphosphate synthase, DAHPS – 3-deoxy-D-arabinoheptulosonate-7-P synthase, DHQS – 3-dehydroquinate synthase, DHQD – 3-dehydroquinate dehydrogenase, DHQSDH – shikimate dehydrogenase/3-dehydroquinate dehydratase, SK – shikimate kinase, EPSPS – 5-enolpyruvylshikimate-3-P synthase, CS – chorismate synthase, CM – chorismate mutase, PAT – prephenate aminotransferase, TYRA – arogenate dehydrogenase, TAT – tyrosine aminotransferase, HPPD – 4-hydroxyphenylpyruvate dioxygenase, HPT – homogentisate solanesyl transferase, TC – tocopherol cyclase, γ-TMT – tocopherol methyltransferases, MPBQMT – Dimethyl-phytylquinol methyl transferase. ***Compounds*** **(*in black*):** IPP – isopentyl diphosphate, DMAPP – dimethylallyl diphosphate, GPP – geranyl diphosphate, FPP – farnesyl diphosphate, PeP – phosphoenol pyruvate, E4P – erythrose 4-phosphate, DAHP – 3-deoxy-D-arabino-heptulosonate 7-phosphate, DHQ – 3-dehydroquinate, DHS – dehydroshikimate, S3P – shikimate 3-phosphate, EPSP – 5-enolpyruvylshikimate 3-phosphate, HPP – 4-hydroxyphenylpyruvic acid, HGA – homogentisic acid, MPBQ – 2-methyl-6-phytylplastoquinol, DMPBQ – 2,3-dimethyl-5-phytyl-1, 4-benzoquinone [65, 116, 122, 125-128].

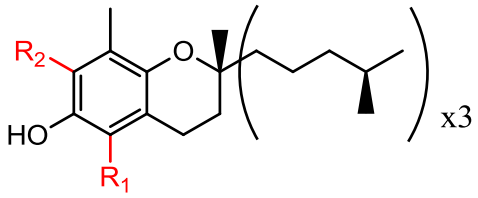
Tocopherols Bioavailability

Absorption and metabolism of tocopherols varies among animal species and humans. Bioactivity is also quite different from isoform to isoform, including among the same stereoisomers. An excellent example is the comparison among the American diet and European diet. Although the American diet is rich in γ-tocopherol, and the European diet rich in α-tocopherol, blood exams reveal an equal prevalence of the same isoform, α-tocopherol. In humans, severe vitamin E deficiency rarely occurs as a result of dietary deficiencies, it does however occur as a result of genetic abnormalities in the α-tocopherol transfer protein (α-TTP), and in extreme cases, could lead to neuromuscular abnormalities. In Europe, the recommended dietary intake of vitamin E is 12 mg/day, and in the USA 10 mg/day [110, 120, 129, 130].

The lipophilicity of vitamin E suggests an absorption and metabolism similar to carotenoids. The absorption efficiency will depends on food matrix, nature and the amount of macronutrients, activity of digestive enzymes and transport efficiency across the intestinal cell. Vitamin E supplements commonly use α-tocopheryl acetate instead of α-tocopherol, because the ester is much more air-stable than the phenol.However, this α-tocopheryl acetate is bioinactive, and must be hydrolyzed to α-tocopherol in the gut [110, 129, 130].

After ingestion, gastric enzymes as pepsin, will help to release some of the vitamin E from food matrices, incorporating them in dietary fats, if available, or mixing them with biliary and pancreatic secretions to form micelles, in the duodenum. In the gut, vitamin E is absorbed and secreted into chylomicrons particles that are transported to the liver via the lymphatic system. During circulation, lipids and vitamin E transference to peripheral tissues can occur by hydrolyzation mediated by endothelial bound lipoprotein lipase (LPL). Additionally, the excess

of chylomicrons leads to the conversion of cholesterol, along with vitamin E, to HDL. Up to this point, none of these steps exerts a preference for any particular isoform of vitamin E [110, 120, 129-133].



Tocochromanol type	R_1	R_2	α-TPP Binding	Vitamin E activity
α-tocopherol	CH_3	CH	100%	100%
β-tocopherol	CH_3	H	38%	25-50%
γ-tocopherol	H	CH_3	9%	8-19%
δ-tocopherol	H	H	15%	<3%

Figure 1.12. Tocochromanol structures and related activity. Adapted from DellaPenna and Pogson (2006) [65].

In the liver, chylomicron remnants are taken up by liver tissue, where α-TTP incorporates the isoforms of vitamin E into VLDL. α-TTP have higher affinity for tocopherols than for tocotrienols, and within tocopherols, present a higher affinity for α isoform (α > β > γ > δ). These affinities result in a differentiation of stereoisomer secretion onto VLDL, and consequently such preference is translated to the plasma and respective distribution to peripheral tissues. Only after passage through the liver α-tocopherol preferentially appear in the plasma. This makes α-TTP the major regulatory mechanism for controlling plasma α-tocopherol concentrations. VLDL is also catabolised by LPL, that carries vitamin E to LDL (major carrier of vitamin E to the peripheral tissues), and can be transferred again to HDL and tissues [110, 120, 129-133].

In humans, α-tocopherol range from 11 to 37 μM, whereas γ-tocopherol concentrations range within 2 and 5 μM, and tocotrienol levels are less than 1 mM. Even after the administration of supplemental vitamin E, these concentrations are limiting and cannot be raised more than 2- to 3-fold (±80 μM) [110, 120, 131]. α-Tocopherol increase is accompanied by a decrease in γ-tocopherol [130]. This α-tocopherol saturation is caused not only by α-TTP regulation, but also by a rapid replacement of circulating with newly absorbed α-tocopherol, resulting in a daily replacement of plasma α-tocopherol [110, 120, 130, 131].

In the liver, remaining vitamin E isoforms are actively metabolised through side-chain degradation to carboxyethyl hydroxychromans (CEHC), beginning with ω-hydroxylation, by

cytochromes P450 (CYP) followed by β-oxidation cycles, and eliminated via the bile or urinary excretion. α-Tocopherol is metabolised primarily to α-CEHC, whereas, γ- and δ-tocopherol are metabolised to γ-CEHC and δ-CEHC, respectively [110, 120, 131-133].

α-Tocopherol

α-Tocopherol is widely distributed in plant tissues, being more abundant in leaves than in fruits. A seed contains 10 to 20 times more vitamin E than plastids. The consistent presence of α-tocopherol in plastids, where its biosynthesis has it's highest expression, is due to the α-tocopherol contribution to membrane stability and it's ability to respond to a variety of abiotic stress, and protect the surrounding tissue, namely the photosynthetic system [9, 65, 116, 119, 124, 134-136].

Tomato is not a rich source of vitamin E, but is most bioactive isoform, α-tocopherol, has a significant presence, with the same level content of β-carotene (±0.1 to 1.8 mg / 100 g of fresh weight) [5, 9, 115]. The availability and occurrence of α-tocopherol in tomatoes is correlated with carotenoids. This occurs because part the biosynthesis, namely Phytyl-DP obtained from GGPP, is shared between both molecules. This correlation also occurs throughout fruit ripening, with a respective increase of α-tocopherol levels [9, 116, 124, 136, 137]. Regarding this, Chun *et al*. (2006) [115] reported that processed tomatoes have a higher α-tocopherol content than unprocessed tomatoes.

α-Tocopherol have an alkyl C16, isoprenoid side chain, with sequential three isoprene units attached, as can be seen in the Figure 1.13.

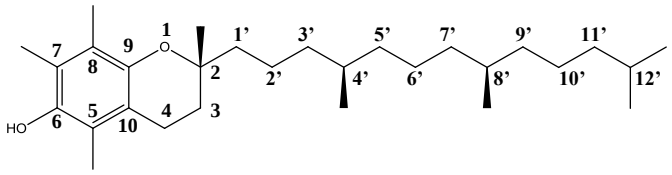


Figure 1.13. Chemical **s**tructure of α-tocopherol.

This saturated isoprenoid side chain differs from the unsaturated side chain of tocotrienols, being responsible for the the tocopherols lipophilicity. The other side of the α-tocopherol structure is a polar group, a 6-chromanol ring, similar to coumarins, consisting of a phenol attached to an oxygenated heterocyclic ring that is attached to the isoprenoid side chain. This phenol group is responsible for the differentiation between α-, β-, γ and δ isoforms (Figure 1.12), being the α- isoform trimethylated at the C5-, C7- and C8- on the chromanol ring. [89, 112, 133, 138]. The carbon of the oxygenated heterocyclic ring that binds the isoprenoid side

chain, C2, is a chiral centre. In tocopherol, the saturated side chains provide 2 extra chiral centres, at C4` and C8`, being RRR-α-tocopherol the most bioactive, and present at higher level due to α-TTP selectivity. C5 and C7 positions are associated with the enhancement of the antioxidant properties of tocopherols and the increases of solubility in lipid substrates. The dimethylation in C5 and C7 α-tocopherol, leads to a more potent hydrogen donor specie (Figure 1.12) [89, 111, 112, 133, 139]. Physical proprieties of α and δ-tocopherol can be seen in Table A2.

δ- Tocopherol

δ-Tocopherol is less abundant and distributed in the plants than α-tocopherol, and its higher concentration levels are found in seeds. In human plasma, δ-tocopherol is present at ±50 folds less than α-tocopherol, as a result of α-TTP selection in the liver, increasing δ-tocopherol metabolisation in the liver and subsequent excretion, although δ-tocopherol has a stronger dose-response [111, 136, 139, 140].

δ-Tocopherol monomethylated at C8, is the less substituted of the tocopherols. Although the physical-chemical differences with α-tocopherol are small, biologically they represent a big difference (Figure 1.12).

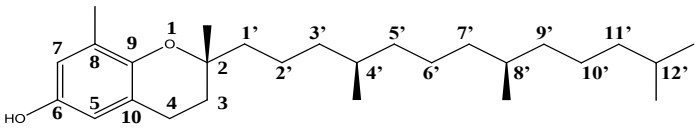


Figure 1.14. Chemical structure of δ-tocopherol.

δ-Tocopherol is the less bioactive (less than 3%) of tocopherols. This is mainly due to the not methylation of C5 and C7. In δ-tocopherol C5, the absence of a methyl group confers some stability to the surrounding medium influence (more stable to heat, alkali or acid) [111, 119, 136, 139].

Antioxidant Potential of Tocopherols

Tocopherols antioxidant capacity, namely *in vitro*, is well recognised. This behaviour is due totocopherols ability to donate their phenolic hydrogen, thus inactivating oxidative free-radicals, as the lipid free-radicals causing LP. The antioxidant strength of the different tocopherols isoforms *in vivo* occurs in the order α > β > γ > δ, but *in vitro*, this can change, depending on the chemical and physical parameters of the system. The donation of the phenolic

hydrogen to a lipid peroxy radical, results in the formation of a resonance stabilised tocopheroxyl radical, and indeed, the antioxidant capacity is linked to the 6-chromanol ring [119, 130, 133, 141].

This differences in the bioactivity between the tocopherols isoforms can be explained by the inductive effects related to the methylation at C5 and/or C7, and the stereoelectronic effects, from substituents *vs.* aromatic plane orientation (dihedral angle - θ). Structurally, α-tocopherol which has two methyl groups at C5 and C7, is expected to be a more potent hydrogen donor, than the other isoforms, that have only one or no methyl groups at C5 and/or C7, making δ-tocopherol the less potent hydrogen donor. The order (α > β = γ > δ) is in agreement with oxidation reduction potentials +0.273, +0.343, +0.348, and +0.405 volts, respectively, as reported by Kamal-Eldin and Appelqvist (1996) [119]. On the other hand, when they have not been methylated, C5 and C7 are electrophilic centres that can effectively trap ROS and RNS, as in the case of δ-tocopherol. For α-, β-, γ- and δ-tocopherols the θ at C2 is 21.0º, 21.4º, 19.8º and 20.8º respectively, as reported by Kamal-Eldin and Appelqvist (1996) [119]. Although there is an association between the reactivity and θ (the lower the angle, the higher the reactivity), it is noted that γ- and δ-tocopherols have slightly smaller θ values than α- and β-tocopherols. The reactivity of the tocopherols is highly influenced by their facility to donate their phenolic hydrogens. The presence of two methyl substituents at C5 and C7 in α-tocopherol, not only enhances its antioxidant activity, but also increases its lipophilic properties [119, 130, 133, 141].

The interest in α-tocopherol, lies in its antioxidant potential and its possible relationship with OxS, a major factor for atherosclerosis, CVDs, and cancer. *In vitro* and animal studies have shown that α-tocopherol has the ability to quench singlet oxygen and scavenge radicals, namely lipid peroxy radicals resulting from LP of PUFAs, at the termination phase of LP, as a chain-breaking antioxidant (Reactions XIII to XVI) [11, 110, 119, 134, 142]:

$$LOO^{\bullet} + \alpha\text{-TOH} \rightarrow \alpha\text{-TO}^{\bullet} + LOOH \quad \text{(Reaction XIII)}$$

$$L^{\bullet} + \alpha\text{-TOH} \rightarrow \alpha\text{-TO}^{\bullet} + LH \quad \text{(Reaction XIV)}$$

$$\alpha\text{-TO}^{\bullet} + \alpha\text{-TO}^{\bullet} \rightarrow \alpha\text{-TO-}\alpha\text{-TO} \quad \text{(Reaction XV)}$$

$$H^{+} + \text{ascorbate}^{-} + \alpha\text{-TO}^{\bullet} \rightarrow \alpha\text{-TOH} \quad \text{(Reaction XVI)}$$

In Reaction XIII and XIV, lipid peroxy radicals ($LOO^{\bullet}$ and $L^{\bullet}$) attack H from α-tocopherol (α-TOH), which results in a more stable radical, the α-tocopheroxyl radical ($\alpha\text{-TO}^{\bullet}$), and non-radical products (LOOH and LH) (Figure 1.15). $\alpha\text{-TO}^{\bullet}$ can be "neutralized" by reaction with other $\alpha\text{-TO}^{\bullet}$ and form dimmers (Reaction XV). α-Tocopherol can be restored by reduction

of the α-TO$^{\bullet}$ by redox-active reagents such as vitamin C (ascorbate) (Reaction XVI) [11, 119, 141, 142].

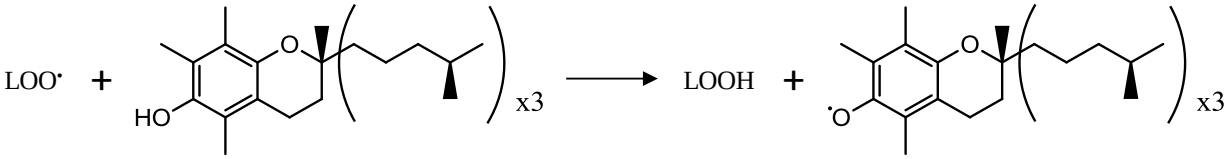


Figure 1.15. Representation of α-tocopherol reaction with lipid peroxy radicals, adapted from Schneider (2005) [141].

In atherosclerosis, α-tocopherol and its isomers are involved in the LDL oxidation. The LDL particle has a lipid core consisting of neutral lipids and a coat of polar lipids, and contains 6 to 12 molecules of α-tocopherol. A radical, attacking LDL can react with α-tocopherol, originating a α-tocopheroxyl radical that, can abduct hydrogen from lipid molecules and, trigger a new autoxidation chain reaction – α-tocopherol-mediated peroxidation (TMP) (Reaction XVII and XVIII) [11, 119, 141, 142].

$$\alpha\text{-TO}^{\bullet} + \text{LH} \rightarrow \alpha\text{-TOH} + \text{L}^{\bullet} \qquad \text{(Reaction XVII)}$$

$$\alpha\text{-TO}^{\bullet} + \text{LOOH} \rightarrow \alpha\text{-TOH} + \text{LOO}^{\bullet} \qquad \text{(Reaction XVIII)}$$

TMP do not occur under normal conditions, the α-tocopheroxyl radical would rather react with any other radical or a redox-active agent like ascorbate, before abstracting hydrogen from the lipids. A high concentration of oxidants or a low concentrations of antioxidants, leads to the consumption of α-tocopherol in LDL, and the consequent α-tocopheroxyl radical production and prevalence in LDL. In turn, this can lead to the occurrence of TMP. A high concentration of α-tocopherol leads to TMP due to a potential increase of α-tocopheroxyl radical production. As the α-tocopherol is less polar than the α-tocopheroxyl radical, this last can move within the particle where it can gain access to the core LH. Wade *et al.* (2012) [143], also suggest that α-tocopherol high concentration may lead to TMP on HDL, which is responsible for the removal of lipid peroxides from the oxidation sites, and PON-1 (responsible for lipid peroxides inactivation within HDL) inactivation due to reduction by α-tocopherol.[11, 118, 119, 141-143].

Tocopheroxyl radical can be reduced and restored to α-tocopherol. In addition, it can be oxidised to α-tocopheryl quinone which could be reduced to α-tocopherolhydroquinone (Figure 1.16) [65, 144].

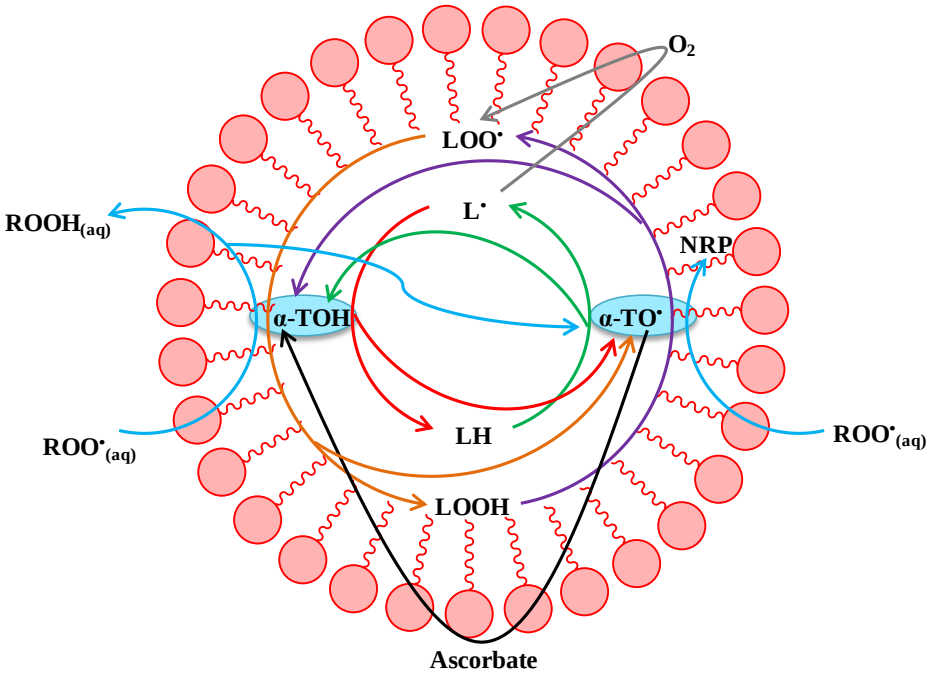


Figure 1.16. Aqueous peroxyl radical-mediated lipid peroxidation of LDL proceeds via TMP. Each colour represents 1 reaction. Adapted from Upston *et al.* (1999) [142].

Human Health and Tocopherols

Tocopherols can be seen from two perspectives, antioxidant and non antioxidant functions. As antioxidant, α-tocopherol stands out, not only for its antioxidant capacity, as well for its levels and presence in biological systems, making α-tocopherol the most bioactive of tocopherol isoforms. For the same reason, δ-tocopherol is the one that presents lower bioactivity [11, 141]. However, tocopherols bioactivity also involves other functions and inhibition of monocyte-endothelial cell adhesion, inhibition of platelet adhesion and aggregation, inhibition of cyclooxygenase-2 and 5-lipoxygenase (contribute to synthesis of inflammatory mediators such as prostaglandin E2 and leukotriene B4) and inhibition of SR-A and CD36 (inhibits the uptake of oxidised LDL into monocyte-derived macrophages) are other functions that have been reported for this compound [141]. Additionally, tocopherols can modulat of gene expression and cellular signalling [120, 129, 141, 145]. γ- and δ-tocopherol have been suggested to have stronger anti-inflammatory activities than α-tocopherol [132, 133], and have shown greater ability to reduce inflammation, cell proliferation, and tumour burden [132, 139].

The actions of vitamin E on cancer have been widely reported. High levels of α-tocopherol are associated with a decreased risk of developing prostate cancer, particularly among smokers [132, 146] while low levels are associated with hemorrhagic stroke mortality [132, 147].

1.2.2. Hydrophilic Antioxidants

Vitamin C and phenolic compounds are the most important naturally occurring plant water-soluble antioxidants. This section will addressed the vitamin C (mainly ascorbic acid), one of the most important vitamins for life [148, 149].

1.2.2.1. Vitamin C - L-Ascorbic acid (L-AA)

Surgeon James Lind found in the 1750's, a correlation between the scurvy disease and fresh citrus fruits. However, it was only in the 1920's that Albert Szent-Gyorgyi isolated a white crystalline compound that he named hexuronic acid, latter renamed ascorbic acid (AA) [150-152]. Hirst and Zilva found that AA oxidation resulted in a similarly active oxidation product, dehydroascorbic acid (DHAA) [152]. Later, Drummond proposed vitamin c for the anti-scurvy factor. Its structure was determined in 1932 by Haworth, and one year later Reichstein synthesised AA, initiating its industrial production [150-152].

AA has 3 main biological activities: (*i*) enzyme cofactor, (*ii*) radical scavenger, (*iii*) donor/acceptor in electron transport either at the plasma membrane or in the chloroplasts. ''Vitamin C'' is the generic descriptor for all compounds exhibiting qualitatively the biological activity of AA. L-ascorbic acid (L-AA) is the naturally occurring form of AA and has the most biological activity, followed by its oxidised product DHAA, by far more relevant than D-ascorbic acid as well as D- and L-isoascorbic acids. Indeed, L-AA / DHAA constitute a kind of redox pair, of major biological importance with a strong vitamin C activity. In fact, the activity of this pair reflects the Total Vitamin Ccontent [150, 151, 153-156].

L-AA, the main water-soluble non-enzymatic antioxidant within the body, is one of the most important vitamins for human nutrition, since it cannot be synthesised by humans. This is due to the lack of L-gulono-lactone oxidase, the last enzyme in the biosynthetic pathway of L-AA by animals. A daily intake of 10 to 15 mg/day of vitamin C (for an adult) is required, although a higher amount of 75 to 90 mg/day is recommended. Biosynthesised in all chlorophyll-containing plants and in the liver and kidney of most mammals, amphibians, reptiles, and most birds, the primary natural food sources of vitamin C are vegetables, fruits and animal organs. Although the nutritional composition of a fruit at harvest can vary widely depending on cultivar, maturity, climate, soil type, and fertility, the concentration of L-AA in tomato ranges between 10 to 25 mg / 100 g of fresh weight [5, 150, 151, 155-158].

L-AA has a five-member lactone ring configuration, with an enediol group, double-bond between C2 and C3, with an adjacent carbonyl group. The delocalisation of the π-electrons over

the C2-C3 stabilises the molecule and causes the hydrogen of the C3 hydroxyl to become highly acidic. In fact, the pKa_1 value is 4.18 and corresponds to oxidation at C3, in aqueous solution results in L-ascorbate. The pKa_2 value is 11.6, with the dissociation of the second hydroxyl at C2, resulting in DHAA. Physiological pH level, with the exception of gastric acid or lysosomes, is "between" pKa_1 and pKa_2, meaning that in the human body L-AA is generally present as L-ascorbate. This makes L-ascorbate an excellent reducing agent [37, 150, 151].

L-AA structure (Figure 1.17), shows two chiral centres at C4 and C5. D-isoascorbic acid is the stereoisomer of L-AA, differing at C5. C4 is responsible for the differentiation between L-AA and D-ascorbic acid. L-AA is differentiated from L-isoascorbic acid by C4 and C5.

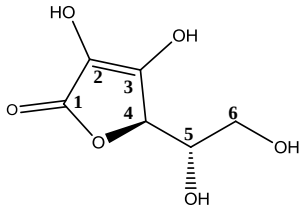


Figure 1.17. Chemical structure of L-ascorbic acid.

The bioactivity of stereoisomers of L-AA is negligible, representing a vitamin C activity lower than 5% [150, 151, 159]. L-AA physical proprieties are summarised in Table A3.

L-AA Biosynthesis

In animals, L-AA biosynthesis occurs mostly in the liver and the kidneys. These are both the glycogen-storing organs, whose breakdown results in D-glucose, the starting point of biosynthesis. L-AA was first reported in rats, synthesised *de novo* in the hexuronic acid pathway, with the occurrence of a configuration inversion. D-glucose-6-P can be metabolised to GDP-mannose. In animals GDP-mannose transforms in L-gulose, and this one results in L-gulonic acid. In plants GDP-mannose is metabolised to L-galactose, which can be transformed in L-galactonic acid. GDP-mannose and its epimerization product, GDP-L-galactose, can be metabolised to Me-D-galacturonate, and finally to L-galactonic acid. D-glucose-6-P can also engage two other routes, one for animals obtaining D-glucose1-P which is transformed to L-gulonic acid. The other route is through L-*myo*-inositol-1-P and *myo*-inositol, results in D-glucuronic acid. In plants these products are metabolised to Me-D-galacturonate in Golgi apparatus, and in animals results in L-gulonic. L-gulonic acid and L-galactonic acid are transformed in the lactones L-gulono-1,4-lactone and L- galatono -1,4-lactone, respectively, the

two immediate precursors of L-AA. All these processes are mediated by enzymes, as can be seen in Figure 1.18 [150, 160-164].

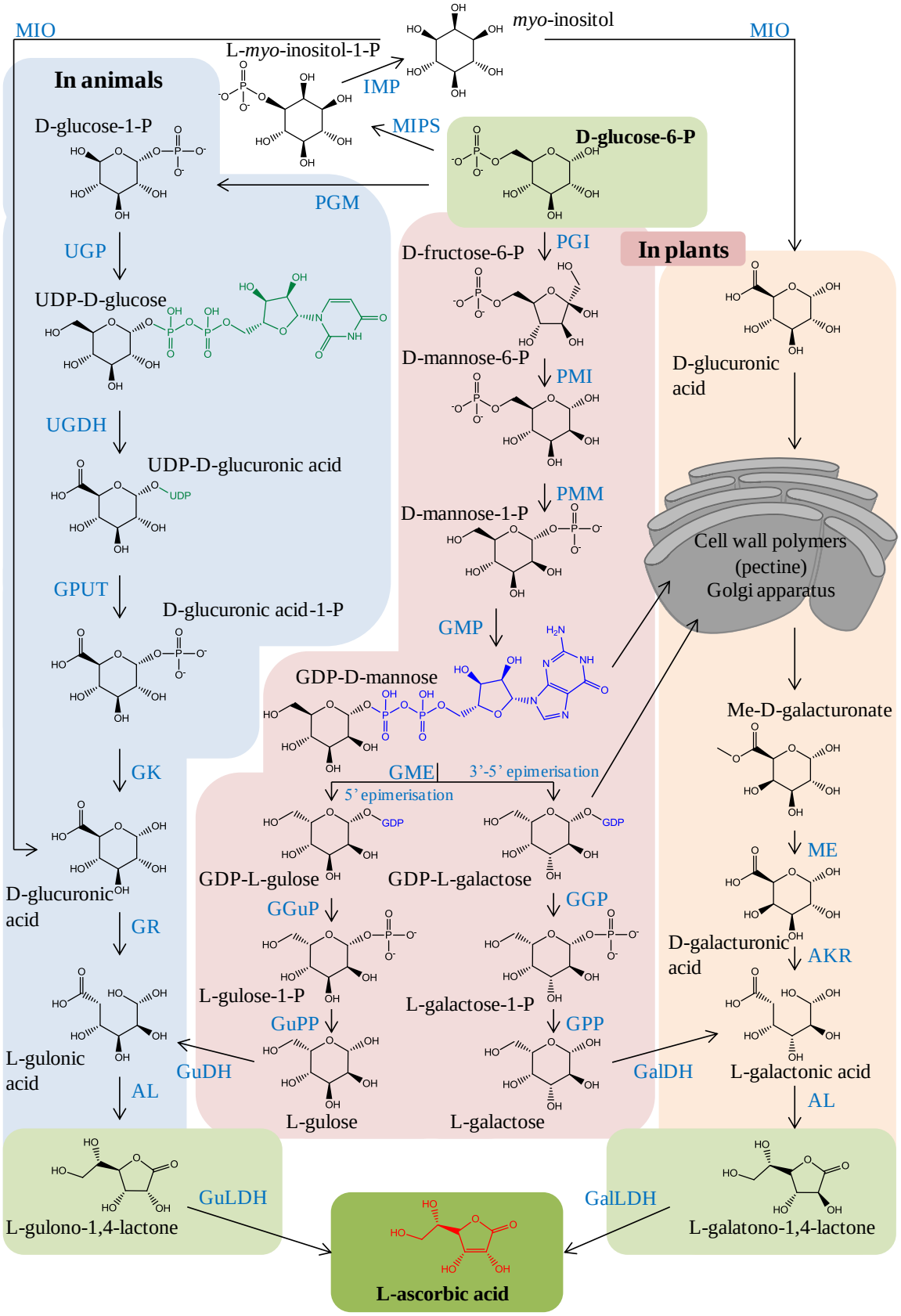


Figure 1.18. (continue next page)

Figure 1.18. Biosynthetic sequence of the L-Ascorbic Acid. ***In Animals* (*in left-centre*):** PGM – phosphoglucomutase, UGP – UDP-D-glucose pyrophosphorylase, UGDH – UDP-D-glucose dehydrogenase, GPUT – D-glucuronate-1-phosphate uridylyltransferase, GK – D-glucurono kinase, GR – D-glucuronate reductase, AL – aldono-lactonase, GuLDH – L-gulono-1,4-lactone dehydrogenase, GGuP – phosphodiesterase, GuPP – sugar phosphatase, GuDH – L-gulono dehydrogenase; ***In Plants* (*in right-centre*):** *ME – methylesterase, AKR – aldo/keto reductase, GalLDH – L-galactono-1,4-lactone dehydrogenase, GGP – GDP-L-galactose phosphorylase, GPP – L-galactose-1-phosphate phosphatase, GalDH – L-gulose dehydrogenase; ***Common to plants and animals* (*in centre*):** MIPS – L-*myo*-inositol 1-phosphate synthase, IMP – *myo*-inositol monophosphatase, MIO – *myo*-inositol oxygenase, PGI – phosphate isomerase, PMI – phosphomannose isomerase, PMM – phosphomannomutase, GMP – GDP-mannose pyrophosphorylase , GME – GDP-mannose-3',5'-epimerase **[160-166]**.

L-AA Bioavailability

Ingestion of fresh fruits and vegetables with a minimum processing optimises vitamin C intake. The food composition is also relevant. An example is that more L-AA may be absorbed when it is taken with food, which leads to a longer gastrointestinal transit time [150, 157, 159].

Vitamin C is absorbed through the lumen of the intestine and renal tubules by enterocytes and renal epithelial cells, respectively, and enters tissue cells by active transport (sodium-dependent vitamin C transporters - SVCT), and by a passive absorptive pathway, through sugar carriers belonging to the glucose transporter (GLUT) family (Figure 1.19). After absorption, plasma L-AA levels increase within 15 to 20 min of ingestion, most of which are absorbed into peripheral tissues. Almost all tissues, with the exception of erythrocytes, absorb and accumulate vitamin C, as the tissue concentration is much higher than the plasma concentration. Intracellular incorporation of vitamin C is also carried out by SVCT 1 and 2. DHAA is reported to be removed rapidly from plasma by erythrocytes, through SCVT and GLUT, and immediately reduced to L-AA. "Ascorbate recycling" consists in this vitamin C transformation and transport, a process of critical importance for L-AA bioactivity, that removes potentially harmful DHAA and maintains a reservoir of L-AA. Excess of L-AA is excreted through urine 3 to 6 h after intake. The main metabolites are the L-AA oxidised form DHAA, which can in turn be oxidised to 2,3-diketogulonic acid (DKG), and in minor amounts to oxalic acid [150, 159].

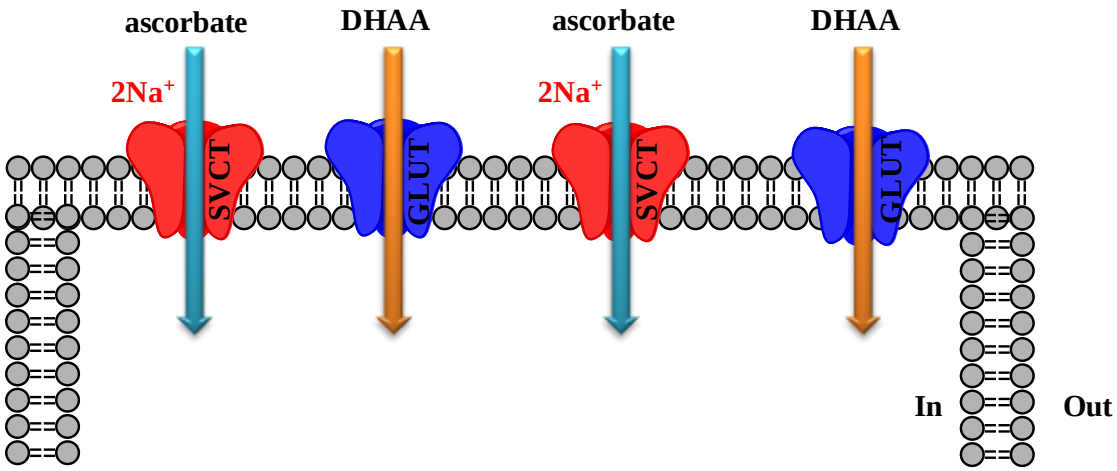


Figure 1.19. Cell transporters for uptake of ascorbate and DHAA. Adapted from Fenoll *et al.* (2011) [159].

Antioxidant Potential of L-AA

Vitamin C, can be considered an efficient antioxidant, in particular, an efficient free radical scavenger. At physiological pH, L-AA is largely ionized to ascorbate. From a biological point of view, the main importance of ascorbate is the ability to terminate radical chain reactions by disproportionation of ascorbate to the non-toxic, non-radical products DAHH and DKG, reducing the population of potentially damaging ROS (Figure 1.20).

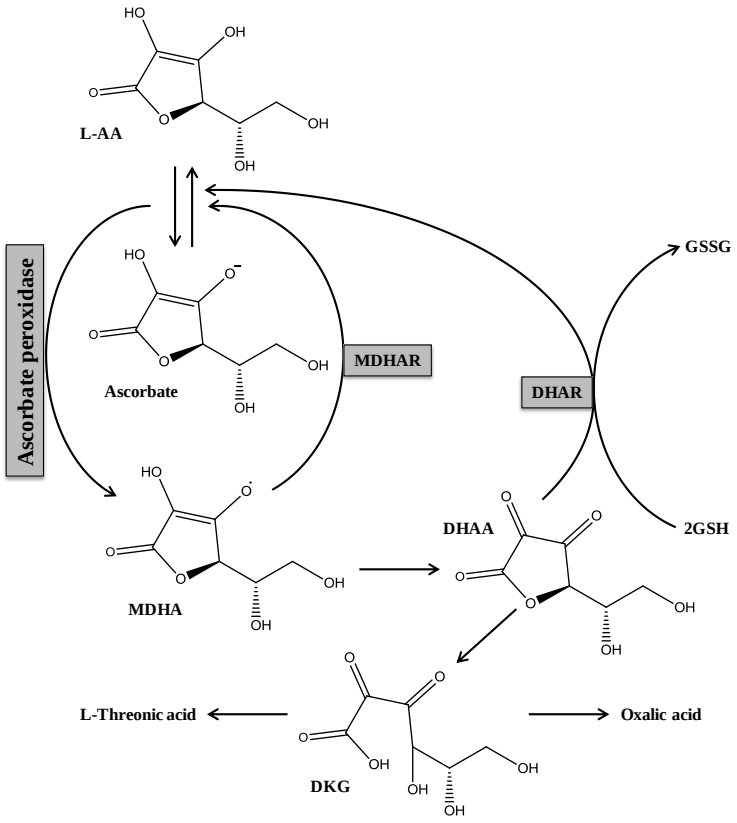


Figure 1.20. L-AA/ascorbate recycling and oxidations products. Adapted from Davey *et al.* (2000) and Majumder and Biswas (2006) [150, 162].

These oxidations are greatly favoured, in addition to ROS, by the presence of oxygen and metal ions. One electron donated by ascorbate results in the ascorbyl radical monodehydroascorbate (MDHA), which can regenerate into ascorbate or can be further oxidised to DHAA. DHAA can also regenerate into ascorbate or oxidised to DKG, which has no biological activity. Finally, DKG breaks to oxalic and L-threonic acid [148, 150, 153, 154, 159].

L-AA/ascorbate can react with $^{\bullet}O_2^-$, but that reaction results in H_2O_2, an active compound in OxS and LP. L-AA/ascorbate can also react with H_2O_2, and $^{\bullet}OH$, eliminating these two ROS, as shown in reactions XIX to XXI [150, 151, 159]:

$$^{\bullet}O_2^- + 2H^+ + \text{ascorbate} \rightarrow 2\ H_2O_2 + \text{DHAA} \quad \text{(Reaction XIX)}$$

$$H_2O_2 + 2\text{ascorbate} \rightarrow 2H_2O + 2\text{MDHA} \quad \text{(Reaction XX)}$$

$$^{\bullet}OH + + \text{ascorbate} \rightarrow H_2O + \text{MDHA} \quad \text{(Reaction XXI)}$$

Ascorbate, donate electrons to a wide range of substrates. One of the most important features is its involvement in the regeneration of the lipophilic antioxidants, mainly α-tocopherol (Reaction XXII), but also carotenoids [150, 159]:

$$\alpha\text{-TO}^{\bullet} + \text{ascorbate} \rightarrow \alpha\text{-TOH} + \text{MDHA} \quad \text{(Reaction XXII)}$$

The recycling of L-AA/ascorbate is important due to the consumption through reactions of vitamin C, or a relative, easy and quick (3 to 6 h) depuration. In order to maintain a stable and available level of vitamin C in the body, this recycling is fundamental. Oxidation products MDHA and DHAA can be recycled again to L-AA/ascorbate, with the aid of monodehydroascorbic acid reductase (MDHAR) and dehydroascorbic acid reductase (DHAR), respectively, but also MDHA can react with itself, as shown in Figure 1.20 and Reactions XXIII to XXV [150, 159, 165]:

$$2\ \text{MDHA} \rightarrow \text{ascorbate} + \text{DHAA} \quad \text{(Reaction XXIII)}$$

$$\text{MDHA} + \text{NADPH} \rightarrow \text{ascorbate} + \text{NADP} \quad \text{(Reaction XXIV)}$$

$$2\text{GSH} + \text{DHAA} \rightarrow \text{GSSG} + \text{ascorbate} \quad \text{(Reaction XXV)}$$

At physiological pH, DHAA is unstable and if not recycled decomposes to DKG, which breaks to oxalic and L-threonic acid [154, 159].

Under some conditions, especially in the presence of transition metal ions, ascorbate has a pro-oxidant effect. An example is the reaction of ascorbate with Fe(III), that results in MDHA,

but also Fe(II). This Fe(II) may react with H_2O_2: the Fenton reaction (Reaction I) [150, 151, 153, 159]:

$$Fe(III) + ascorbate \rightarrow Fe(II) + MDHA \quad \text{(Reaction XXVI)}$$

$$Fe(II) + H_2O_2 \rightarrow {}^{\bullet}OH + OH + Fe(III) \quad \text{(Reaction XXVII)}$$

Human Health and L-AA

In 1970, Linus Pauling was the first to suggest the crucial importance of vitamin C in the maintenance of a healthy immune system [150, 159].

A considerable number of metabolic reactions require vitamin C as a cofactor. Vitamin C its involved in the synthesis of epinephrine from tyrosine, the synthesis of norepinephrine, the conversion of tyrosine to the neurotransmitter dopamine and further hydroxylation to adrenaline and noradrenaline. Vitamin C also serves as an electron donor for enzymes, including those necessary for hydroxylation of proline and lysine in collagen during its synthesis [11, 151, 156, 159].

It is well reported that Vitamin C has a protective role against some types of cancer, diabetes and ageing. The antioxidant capacity of L-AA and the involvement in the production of the powerful vasodilator nitric oxide, along with the ability to improve arterial function, suggests that L-AA has a protective effect against atherosclerosis. It has been shown that long term deficiency appear tofavour an increase risk for CVDs, being also noted an inverse relationship between plasma levels of vitamin C and ischemic heart disease [11, 150, 159].

1.3. Aims

The aim of this study, is to develop extraction methods and consequent analysis by LL-USAE/UHPLC-PDA and LL-USAE/UHPLC-FLR in order to determine the lipophilic (lycopene, β-carotene, α-tocopherol and δ-tocopherol) and hydrophilic (L-AA) antioxidants in ripe tomato, from *gordal* variety. Through LL-USAE/UV-Vis, were determinated the total carotenoids content in different tomatoes varieties (*gordal, regional, campari* and *grape*), in different processed food samples containing tomatoes derivatives (ketchup, tomato paste and concentrated tomato paste) and in four ripening stages (“immature green”, “full growth”, “breaker” and “ripe”) of tomato from *gordal* variety.

In order to evaluate total antioxidant potential (TAP) in four ripening stages ("immature green", "full growth", "breaker" and "ripe") of tomato from *gordal* variety, ORAC and TBARS assays were developed and applied.

All the procedures were optimized, with LL-USAE/UV-Vis and LL-USAE/UHPLC-PDA/FLR methodologies submitted to validation. The work design can be divided in three steps, as can be seen in Figure 1.21.

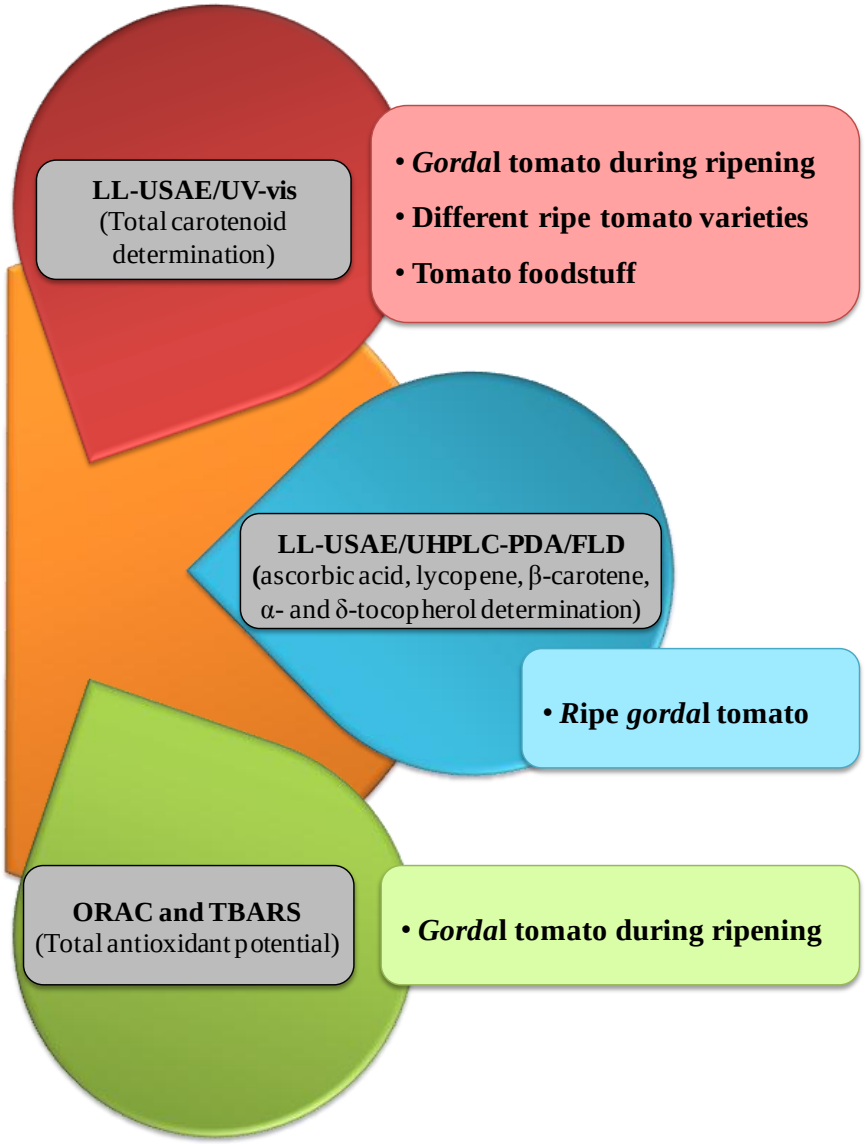


Figure 1.21. Design of the work aims.

CHAPTER II

ANALYTICAL METHODOLOGIES

2.1. Antioxidant Tests

Antioxidant capacity, gives us indications about the biological potential of target substances or matrices. A wide range of assays can be used for the assessment of antioxidant capacity. The evaluation of TAP using one chemical reaction seems to be rather unrealistic, and so, an evaluation of the antioxidant activity by a number of different methods is recommended, rather than depending on the results of a single method [9, 167].

Antioxidants tests can be classified according to: (i) the nature of the reducing agent (using organic radical producers or metal ions for oxidation), (ii) the type of transfer involved in the reaction (atom or electron transfer), (iii) the principle of the method (spectrometry, electrochemical techniques or chromatography), and (iv) the type of measurement, direct or indirect [9, 167, 168].

In order to evaluate the TAP, Rice-Evans and co-workers developed the Trolox equivalent antioxidant capacity (TEAC) assay, Sanchez-Moreno suggested the 2,2-di(4-tertoctylphenyl)-1-picrylhydrazyl (DPPH) assay, Cutler and Cao developed the oxygen radical absorbance capacity (ORAC) assay, Yagi and co-workers modified TBA test to thiobarbituric acid reactive substances (TBARS) assay, and many other assays are used, differing from each other [167-170].

In order to check the TAP of *Solanum lycopersicum* L. from gordal variety during ripening, the ORAC and TBARS assays were used.

2.1.1. ORAC

ORAC can be classified as a hydrogen atoms transfer reactions model (HAT). These reactions are very fast and generally occur through peroxyl radicals ($ROO^{\bullet}$), like the carbon-centered radicals, generated by the azo-initiator 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) that reacts with oxygen to produce $ROO^{\bullet}$. To monitor the reaction, a florescent probe, like fluorescein (FH), is used. $ROO^{\bullet}$ can react either with fluorescein (Reaction XXVIII) or directly with the antioxidant(s) analysed (Reaction XXXI). From the reaction of $ROO^{\bullet}$ with fluorescein, non-fluorescent products, like fluoresceinyl radical, are produced (Reactions XXVIII and XXIX). This radical can also react with antioxidants and regenerate florescent compounds (Reaction XXX). With time, the antioxidant is consumed, leading to a rapid oxidation of fluorescein. All these reactions occur at a physiological pH and temperature.

At the same time, fluorescence is measured in the maximum possible number of cycles for about one hour [167, 171, 172].

$$ROO^{\bullet} + FH \rightarrow ROOH + F^{\bullet} \quad \text{(Reaction XXVIII)}$$

$$F^{\bullet} + ROO^{\bullet} \rightarrow ROOF \quad \text{(Reaction XXIX)}$$

$$F^{\bullet} + AH \rightarrow FH + A^{\bullet} \quad \text{(Reaction XXX)}$$

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet} \quad \text{(Reaction XXXI)}$$

$$A^{\bullet} + ROO^{\bullet} \rightarrow ROOA \quad \text{(Reaction XXXII)}$$

AAPH give a steady flux of peroxyl radicals, and fluorescein, the florescent probe, is gradually degraded. The monitoring of this decay can be achieved making use of the area under the curve (AUC), as follows:

$$AUC = (0.5 + f1/f0 + f2/f0 + f3/f0 + ... + fi/f0) \times CT \quad \text{(Equation 1)}$$

Where f0 is the initial fluorescence reading, f1, f2, f3 and fi are the fluorescence readings at the cycles 1, 2, 3 and i; CT is the total cycle time in minutes. A factor of 0.5 is added to normalize AUC. Equation 1 is used for samples and for trolox (is used as standard), therefore, ORAC values are expressed as micromoles of Trolox equivalent per gram of sample (μmol TE/g) [167, 171, 173, 174]. Li *et al.* (2013) [106] reported ORAC values for different tomatoes ranging between 10.47 and 13.76 μmol TE/g.

2.1.2. TBARS

The peroxidation of polyunsaturated fatty acids (PUFAs), is accompanied by the formation of conjugated diene structures, MDA being one of the main products, and for that reason, it is chosen as a biomarker for OxS induced LP [19, 175-177]. Due to its simplicity and cheapness, a thiobarbituric acid (TBA) test is a popular assay. It is based on the particular reaction of TBA with conjugated diene species. MDA is the main reactive specie, but is not the only one, as so, it is more correct to designate this test focused on LP, as thiobarbituric acid reactive substances – TBARS [19, 177, 178].

The reaction of MDA with TBA, at high temperatures (95 to 100º C) and low pH, results in a pink chromogen, MDA-TBA_2 adduct (Reaction XXXIII), with an absorbance at 532 nm and fluorescence at 553 nm. Trichloroacetic acid (TCA) can be added to maximise MDA recovery.

In TBARS temperature and pH conditions, 1,1,3,3,-tetraethoxypropane (TEP) is hydrolysed, releasing MDA, therefore, TEP solution is used as a standard MDA, and consequently, results are frequently expressed as μmol MDA equivalents. [19, 177, 179, 180].

$$MDA + 2TBA \rightarrow MDA\text{-}TBA_2 \qquad \text{(Reaction XXXIII)}$$

Reactivity is dependent on temperature, pH, concentration of TBA and time. The maximum colour intensity is obtained with 60 min reaction at 95-100º C. Chain-breaking antioxidants also lower TBA reactivity [19, 177].

2.2. Extraction Techniques for Carotenoids, Tocopherols and L-AA

For qualitative and quantitative studies of bioactive compounds from various plant parts such as leaves, stems, flower and fruits, a proper extraction method is crucial. Conventional or classical sample extraction procedures, are mostly based on liquid-liquid (LLE) or solid–phase extractions (SPE). Non-conventional methods, due to decreased use of synthetic and organic chemicals, and reduced operational time, are more environmentally friendly (green chemistry). Also, non-conventional methods tend to have a better yield and quality of extraction, and are able to be miniaturised [181-183].

Recently several extraction techniques emerged as environmentally friendly. In order to determinate lipophilic and hydrophilic antioxidants in different tomato varieties and tomato foodstuffs, two extraction techniques were used: microextraction by packed-sorbent (MEPS), and liquid–liquid ultrasound assisted extraction (LL-USAE).

2.2.1 MEPS

SPE current needs for miniaturisation had led to new microextraction techniques (METs), such as fibre solid-phase microextraction (SPME), stir-bar sorptive extraction (SBSE) and thin film microextraction (TFME). All of these are characterised by diffusion of the analytes mediated by stirring. With a diffusion of the analytes mediated by flow-through, we have the in-tip SPME, in-tube SPME (IT-SPME), and in-needle SPME (e.g. MEPS) [183-185].

MEPS, first introduced by Abdel-Rehim [186], can be considered a miniaturised SPE, namely because both are based on the same sorbent chemistry, but scaling down the reagent and

sample volumes. In MPES, 1 to 4 mg of sorbent (stationary phase) is tightly packed inside a cylinder (BIN), which is crossed by a syringe (able to process sample volumes from 10 µL up to 1000 µL). The samples loaded to the sorbent are retained according to their chemical properties, particularly their polarity. Elution of the analytes is due to results from a higher affinity with the solvent used, than to the sorbent. As we can see, extraction depends on the affinity between sorbent, analyte and solvents. Figure 2.1 shows MEPS experimental process, up to six sequential steps are characterised: (*i*) sorbent conditioning, (*ii*) equilibration, (*iii*) sample loading, (*iv*) washing, (*v*) elution, (*vi*) sorbent regeneration. MEPS sorbent, depending on the complexity of the matrix being processed, can be used up to about 100th times [183, 187, 188].

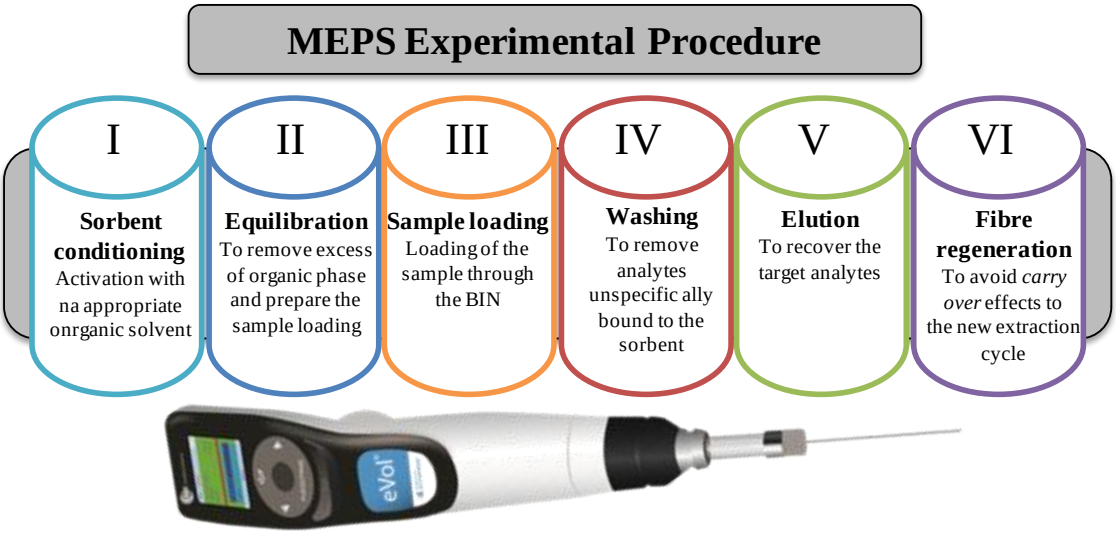


Figure 2.1. Steps of MEPS experimental procedure, and electronic pipette eVol® MEPS. Adapted from Pereira *et al.* (2014) [183].

In MEPS the sorbent chosen must have enough affinity towards the target analyte, but not too much, to avoid permanent retention of the analyte when the sample passes through the solid support. For a more appropriate extraction, there is a wide range of MEPS sorbent materials, with different chemistries, such as C2 – ethyl; C8 – octyl; C18 – octadecyl; HDVB – hydrophobic polystyrene-divinylbenzene copolymer; PEP – polar enhanced polymer; PGC – porous graphitic carbon; SAX – strong anion exchange; SCX – strong cation exchange; SDVB – styrene-divinylbenzene; SIL – silica; R-AX – (retain) anion exchange; R-CX – (retain) cation exchange; APS – amino-propyl silane; C8/SCX – a C8 combined with SCX. Therefore, MEPS range of applications is very broad, covering the extraction of hydrophobic analytes from aqueous matrices (reversed phase extraction), polar analytes from non-polar organic solvents (normal phase extraction), and charged analytes from aqueous or non-polar organic samples (mixed mode and ion exchange extraction) [183, 187, 188].

In the beginning, MEPS analysis was applied in blood, plasma and urine samples. Currently, applications have a broader range, like other biological matrices, such as exhaled breath, but also vegetal products, water, among others [183, 188]. Adam *et al.* (2012) [189] reported L-AA determination in beverages, through a MEPS silica sorbent, using methanol as elution solvent. Shen *et al.* (2009) [190], reported high retentions of β-carotene with the C18 and C30 sorbents using isopropanol/ethyl acetate/water (1:1:1, v/v) as a solvent. Sagratini *et al* (2012) [190], reported the extraction of α-tocopherol and β-carotene from olives through SPE silica cartridge and elution with n-hexane:ethyl acetate mixture (9:1).

Another development of MEPS was on operation mode, ranging from a simple manual Hamilton syringe to the semiautomatic format using the electronic pipette eVol® MEPS or the online versions. The automatisation allows a slightly accurate control of the flow during the whole methodology, minimises user intervention, increases the analytical performance and introduces new automation and high-throughput possibilities to the methodology [183, 188].

2.2.2. LL-USAE

During the search for non-conventional methods, newly developed sample preparation techniques emerged, namely liquid–liquid microextraction (LLME), pressurized liquid extraction (PLE), salting-out liquid–liquid extraction (SALLE), single-drop microextraction (SDME), dispersive liquid–liquid microextraction (DLLME), and liquid–liquid ultrasound assisted extraction (LL-USAE) [185, 191].

Ultrasound (US), a pressure wave with high frequency beyond human hearing, above 20 kHz, passes through a medium by creating compression and expansion. It and can be used to aid in emulsification, homogenisation, freezing, crystallisation, filtration, drying and extraction. When ultrasound is used for extraction purposes, the extraction is denominated ultrasound assisted extraction (USAE). USAE has been used in analysing contaminants in soil, animal tissues, and food packaging materials, demonstrating that it could increase the extraction yield of targeted compounds [181, 192]. The basis of USAE is the ultrasonic cavitation, which means production, growth and collapse of bubbles. Cavitation results from sound waves, mechanical vibrations, which in the case of ultrasounds, the high frequency generates and collapses bubbles of high pressure and temperature, heating and a cooling rate above 10^{10} K/s. This occurs in the presence of liquid, in a liquid-liquid or in a liquid-solid phase. This mechanical effect facilitates the destruction of material surface, namely matrix or disruption of the cells walls, which

enhances solvent penetration into the matrix, and the release of disruption products to the solvents [181, 182, 192-194].

LLE, the most commonly used procedure for separation of a chemical species, is based in a two-phase system where the analyte and interferences are distributed between the two immiscible liquid phases in contact with each other. USAE combined with LLE results in LL-USAE, were the extraction of the analytes from matrix is enhanced by ultrasounds. Other advantages of USAE combination, is the possible use of less and not such "aggressive" solvents for the extraction [185, 192, 195].

The increased stability provided by USAE towards bioactive compounds has lead to a systematic use of USAE, namely associated with LLE [182, 191]. Azmir *et al.* (2013) [181], reported the widespread use of USAE to a wide range of bioactive compounds with different solvents, while Eh *et al.* (2012) [193], and Xu *et al.* (2013) [196], reported lycopene extraction with USAE, claiming a low degradation and isomerisation, and a high yield of lycopene extracted. Xu *et al.* (2013) [196], states that extraction at 30 ºC for 30 min gave the best results. Prado *et al.* (2014) [182] and Shen *et al.* (2014) [197] also reported different extractions assays for carotenoids involving USAE. USAE was also used by Sun *et al.* (2010) [198] to extract β-carotene, reporting a higher stability than classical methods. Le *et al.* (2012) [199] and Bae *et al.* (2013) [200] reported the extraction of vitamin C through USAE, with higher levels of vitamin C, and also less degradation of the same.

2.3. Analytical Techniques for Identification and Quantification of Carotenoids, Tocopherols and L-AA

Analytical chemistry is applied throughout industry, medicine, and academy, being qualitative, quantitative, or both simultaneously. It is applied in many research areas, such as biochemistry, biology, geology, physics, and the other sciences. Analytes can be measurement on mass or volume. Gravimetric, volumetric, electroanalytic, spectroscopic methods and chromatography are the main and basic methods, existing also other miscellaneous methods [201].

In spectrochemical methods, light and other forms of electromagnetic radiation are widely used for measurements. Its basic core, is measuring the amount of radiation produced or absorbed by target molecular or atomic species, and according to the region of the electromagnetic spectrum used or produced in the measurement, we can classify the method as γ-

ray, X-ray, ultraviolet (UV), visible (Vis), infrared (IR), microwave, and radio-frequency (RF) [201].

Electromagnetic radiation, a form of energy that is transmitted, can be described as a wave with properties of wavelength, frequency, velocity and amplitude. It also has propriety particles, and so, photons or quanta can be seen as discrete packets of energy or particles of electromagnetic radiation, that can be quantified by Equation 2 [201]:

$$E = h\nu = hc/\lambda \qquad \text{(Equation 2)}$$

Where E is the energy, *h* is the Planck constant, and *c* is the speed of light (in vacuum), revealing that the energy and momentum of a photon depend only on its frequency (ν) or its wavelength (λ) [201].

The occurrence of electronic transition between different energy levels of chemical species, caused by radiation (photons), is one of the most interesting and useful interactions in spectroscopy. The effect of radiation on matter, in this case, leading to electronic transition, depends of the nature of the matter (our target molecular or atomic species) and the radiation (as seen in Equation 2, characterised by the wavelength). Wavelength range define spectrum regions, such as the UV region (occurs at 180-380 nm), and the Vis region (occurs at 380-780 nm). In spectroscopy, these two regions can be analyzed together UV-Vis (180-780 nm). The information about the analyte is observed by measuring the electromagnetic radiation emitted as it returns to the ground state or by measuring the amount of electromagnetic radiation absorbed as a result of excitation [201].

Beer-Lambert law (Equation 3), relates the effect of radiation transmitted to a solution and the absorption by the analytes present. Absorption capacity characterise the analyte, whose response is proportional to its concentration [201].

$$A = \varepsilon lc \qquad \text{(Equation 3)}$$

Where *A* is the absorbance, c is the concentration of the absorbing species (M), l is the path length through the absorbing medium (cm), and ε is the molar absorptivity, whose units are the inverse of c and l, (M^{-1} cm^{-1}) [201]. Sequential measurements of absorbance *versus* wavelength will results in an absorption spectrum. If the electronic transitions occurs with radiation in the range of 180-780 nm, with atoms or molecules being excited, and after returns to the ground state, we will have an UV-Vis spectrum, and if the excited species still have an excess energy

after relaxing to the ground state, giving up their excess energy as photons the result be will result in fluorescence or phosphorescence.

2.3.1. UV-Vis

The electronic transitions are specific to molecular interactions, therefore, the energy necessary for its occurrence is characteristic of the analyte. The basic components of analytical instruments for absorption, as well as for emission and fluorescence spectroscopy, are remarkably similar in function and in general performance requirements regardless of whether the instruments are designed for UV, visible or IR radiation. Analysing the UV-Vis spectrum of the sample, we can obtain information about its structure and concentration [201].

2.3.2. UHPLC-PDA and UHPLC-FLR

In recent years, the subject of green chemistry has gained increasing attention, not only because of environmental grounds, but mostly for economic reasons. The demands of high sample output in a short time frame have given rise to high efficiency and fast liquid chromatography. At present, most analytical methods recommended by pharmacopoeias are based on chromatographic techniques, of which high performance liquid chromatography (HPLC) is the most common. The first commercially available ultra-high performance liquid chromatography (UHPLC) system was introduced by Waters in 2004 (UPLC®) (Figure 2.2). In the recent years the use of UHPLC for analysing pharmaceuticals and biopharmaceuticals has increased significantly, and also UHPLC analyses were applied to many other different analytes. The UHPLC implementation occurred first in industry, with the transferrable ability of the works in the stated HPLC to UHPLC, due to the sharing the same chromatographic conditions, as in the example Equation 4, for "flux transfer", where F_2 and r_2 are the flow rate and the radius for the smaller internal diameter column, and F_1 and r_1 are the flow rate and the radius for the larger internal diameter column [185, 202-204]:

$$F_2 = F_1 (r_2/r_1)^2 \qquad \text{(Equation 4)}$$

The easiest way to reduce the solvent consumption is to reduce the run time, and a fast and efficient separation can easily be achieved by using a short column packed with smaller particles, (Equation 5). The column length, L, required for a given separation is proportional to the particle size, where N_{req} is the required column efficiency for a given separation and d_p is the particle size of the packing material [185, 202, 203]:

$$L = 2N_{req} \times d_p \quad \text{(Equation 5)}$$

This reduction of particle size, at sub-2 µm, allows an increase the flow rate (reduce the run time), but this leads to an increase in backpressure. High flow rate also results in frictional heating between the mobile phase and the stationary phase. In order to minimise these setbacks, the internal diameter of the column can be reduced, and also, new ultra-high pressure resistant systems are necessary in order to profit fully from the advantages of the use of sub-2 µm particles. Nováková and Vlcková (2009) [185] reported that a 1.7 µm particle packed column will generate 27 times higher pressure than a 5 µm particle packed column [185, 202, 203].

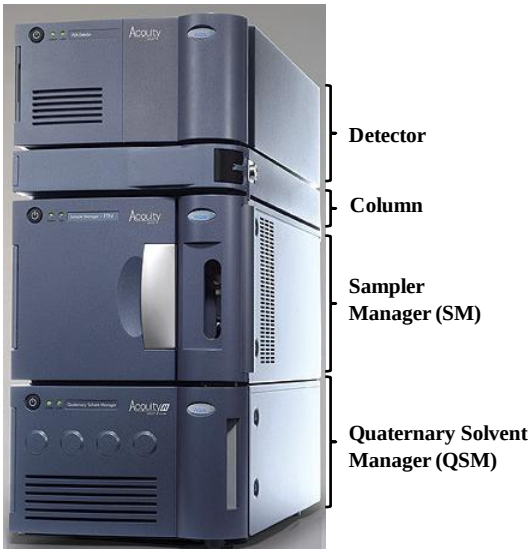


Figure 2.2. ACQUITY UPLC H-Class System, from Waters.

Two other advantages emerged from these new parameters, the first being smaller mobile phase volumes and faster mobile phase flows, low injection volumes are now necessary, typically 0.5 to 2.0 µL [203, 204]. The second advantage is presented by the analysis of the van Deemter equation (Equation 6) for the relationship between the height equivalent to a theoretical plate (HETP) and linear velocity (µ) [185, 203, 204]:

$$H = Ad_p + (BD_M/\mu) + (Cd^2{}_p\mu/D_M) \quad \text{(Equation 6)}$$

HETP is denoted as H where A represents Eddy-diffusion coefficient, d_p the particle size of the stationary phase, B the longitudinal diffusion coefficient, µ the linear velocity of the mobile phase, D_M the analyte diffusion coefficient and C the resistance to mass transfer coefficient. From this relationship between HETP and linear velocity we see that for $d_p < 2$ µm, the use of

high linear velocities does not influence the values of HETP. So, short columns packed with sub-2 µm particles reduce the time of analysis without affecting efficiency or resolution, and simultaneously require less mobile phase and sample injection volumes.

Columns packed with sub-2 µm particles in UHPLC have also emerged in a powerful approach particularly because of the higher resolution. Reversed phase (RP) columns are the most currently used, among them, Acquitty High Strength Silica (HSS) T3 and the Acquitty C18 with a bridged ethylsiloxane-silica hybrid (BEH) adsorbent are the most widely used (Figure 2.3). Rivera *et al.* (2011) [56], reports, that UHPLC is a promising tool for carotenoid analysis. So far, HSS C18 and T3 and BEH C18 stationary phases have been successfully used to separate several carotenoids [56]. Chebrolu *et al.* (2012) [205], also reported the determination of β-carotene, lycopene and vitamin C from grape fruits. Chauveau-Duriot *et al.* (2010) [206], reported the determination of tocopherols and β-carotene from plasma and milk. Therefore, HPLC methods are considered the best choice for the determination of lipid-soluble vitamins, and are adapted to UHPLC [56, 185, 202-204].

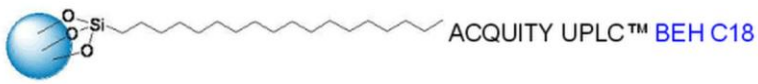


Figure 2.3. Representation of adsorbent in Acquitty BEH C18.

The analysis in UHPLC requires high sensitivity detectors. UHPLC coupled with electrospray ionisation (ESI) tandem mass spectrometry (MSMS) seems to be a method of choice in bio-analytical applications, but due to is cost most commercial UHPLC instruments are equipped with a photo diode array (PDA), a modified UV detector, but a fluorescence detector (FLR or FLD) is also accessible (Figures A2 and A3) [185, 203].

CHAPTER III

EXPERIMENTAL

3.1. Material and Reagents

The materials and equipments used are listed in Table 3.1, and the reagents in Table 3.2.

Table 3.1. Equipments and materials used in this work (ordered by supplier).

Equipment	Supplier
Ultrapure water purification system	Millipore Corporation (USA)
Milli-Q® Direct 8 (18 MW cm, 23º C)	
Polytetrafluoroethylene (PTFE) membrane filters – 0.22 µm	
Semi-automatic syringe for MEPS	SGE Analytical Science (Australia)
eVol® XR Electronic Syringe	
Sorbent and needle support for MEPS (BIN)	
C2, C8, C18, M1, SIL	
Sorbent and needle support for MEPS (BIN)	Thermo Scientific (Australia)
C18, PGC, R-AX, R-CX, PEP	
Microcentrifuge	
Espresso Personal microcentrifuge	
UHPLC chromatographic system	Waters (USA)
ACQUITY UPLC H-Class System, with Quaternary Solvent Management	
Analytical column for UHPLC	
ACQUITY UPLC® BEH C18 (15 mm × 2.1mm, 1.7 µm particle size)	
UHPLC detectors	
ACQUITY UPLC® Photodiode Array (PDA) Detector	
ACQUITY UPLC® Fluorescence (FLR) Detector	
Single channel adjustable volume micropipettors	Orange Scientific (Belgium)
TIPOR-V^{+} (10, 200 and 1000 µL)	
pH meter	Metrohm (Switzerland)
691 pH Meter	
Analytical balance	OHAUS Corporation (Switzerland)
OHAUS Pioneer precision balance PA114	
Fluorescence reader	Perkin Elmer (Belgium)
VICTOR^{3} Multilabel Plate Counter 1420	
Spectrophotometer UV-Vis	
LAMBDA 25	
Eppendorfs (1.5 mL)	VWR International (Portugal)
Vials (1.5 mL)	
Hellma® absorption cuvettes, ultra Micro	Labodidactica, Lda (Portugal)
Suprasil® quartz, limit 200-2.500 nm spectral range, pathlength 10 mm, chamber volume 160 µL	
Ultrasonic Cleaner	Branson company (USA)
B2510E-DTH, 100W, 40KHz	
Block heating system	Frilabo (Portugal)
Grant QBD1	

Table 3.2. Reagents, solvents and salts used in this work (ordered by supplier).

Reagents	Remarks	Supplier
Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)	98%	Fluka
Fluorescein	-	JMGS
n-Hexane	AR, 95%	Lab-Scan
Acetonitrile (ACN)	HPLC grade, 99.99%	Fisher Scientific
Methanol (MeOH)	HPLC grade, 99.99%	
Potassium chloride (KCl)	PA-ACS, 99.5-100.5%	Riedel.de Haen
Hydrochloric acid (HCl)	37%	
PSA/C18/MgSO4 (25mg/25mg/150mg)	DisQuE	Waters
PSA	DisQuE	
EDTA (Ethylenediaminetetraacetic acid)	98-101%	Merck
Glacial acetic acid	PA, 99.8%	
Sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$)	99-102%	
Formic acid (FA)	PA-ACS, 98%	Panreac Química
L-Ascorbic acid	99%	
meta-Phosphoric acid (MPA)	33.5-36.5%	
Sodium chloride (NaCl)	PA-ACS, 99.5%	
Sodium phosphate dibasic dehydrate ($Na_2HPO_4.2H_2O$)	PA, 99%	
Sodium hydroxide (NaOH)	PA-ACS, 98%	
Ethanol (EtOH)	absolute PA, 99.5%	
TBA (2-Thiobarbituric acid)	98%	Sigma-Aldrich
TCA (Trichloroacetic acid)	99-100.5%	
TEP (1,1,3,3-Tetraethoxypropane)	96%	
Lycopene	from tomato, 90%	
β-Carotene	HPLC, 95%	
(±)-α-Tocopherol	HPLC, 96%	
(+)-δ-Tocopherol	90%	
Carbon nanotube, multi-walled	95%	
Graphene oxide	2 mg/mL in H_2O	
AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride)	granular, 97%	

3.2. Solutions

Stock solutions of lycopene, β-carotene, α- and δ-tocopherol standards were prepared with ethanol (EtOH) to 12.43 µg/mL for lycopene, and 1000 µg/mL for remaining standards. L-AA stock solution was prepared according to Spínola *et al.* (2012) [207], in 3% MPA–8% acetic acid–1 mM EDTA solution to 200 µg/mL.

For ORAC assays, a solution of fresh PBS was prepared mixing, 8 g of NaCl, 0.20 g of KCl, 1.432 g of $Na_2HPO_4.2H_2O$ and 0.272 g of $NaH_2PO_4.H_2O$ with 800 mL of ultrapure water. The pH was adjusted to 7.4.

3.3. Sampling and Sample Pre-treatment

Madeira island belongs to the Portuguese Archipelago of Madeira, located on the Atlantic Ocean between 32º38'N and 16º55'W, 660 km west off coast of North Africa. It has a characteristic subtropical weather, with an annual medium temperature between 20º and 25º C, and average 500 mm precipitation. The tomato plantation under study (3450m^2 exploration area) is located at 32º40'N, 16º55'W and 280 m altitude, in the south coast of Madeira, in Funchal, as shown in Figure 3.1.

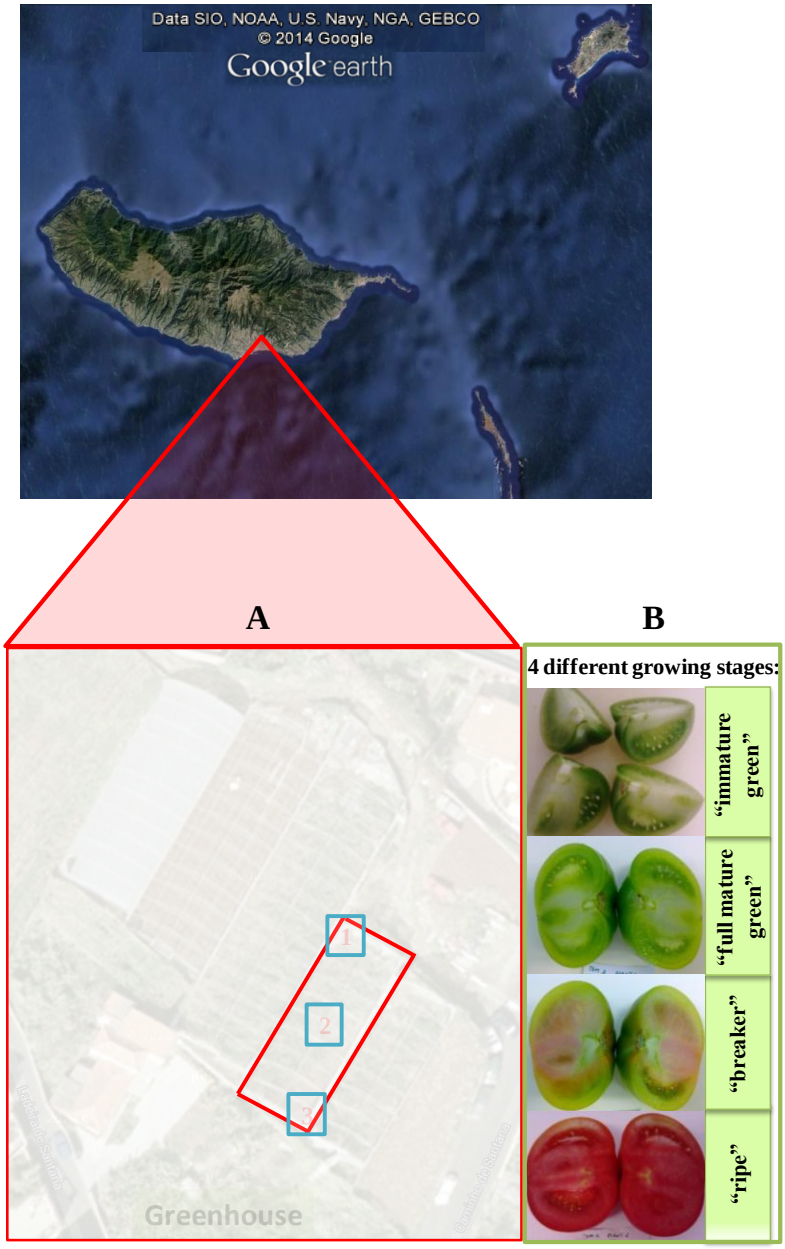


Figure 3.1. Tomato sampling: (A) Location of tomato plantation under study (greenhouse), with identification of the 3 sampling zones ("1", "2" and "3"); (B) Exemplification of the 4 different ripening stages of tomato analyzed, "immature green", "full mature green", "breaker" and "ripe".

About 500 g of tomato from *gordal* variety at different ripening stages, "immature green", "full mature green", "breaker" and "ripe" were collected in the greenhouse (Figure 3.1). About 100 g of ripe tomato from each *regional*, *campari* and *grape* varieties were collected in local market. The samples were mashed, aliquoted and immediately stored at -80º C. The aliquots were used once (single use), to prevent sample degradation.

Different processed food samples containing tomatoes derivatives were analysed, four ketchup samples (Continente, Heinz, Perdiz and Pingo Doce), two tomato paste (Guloso and Compal) and three concentrated tomato samples (Guloso from can, Guloso from tube and Compal from tube). All samples were aliquoted and immediately stored -80º C.

3.4. Evaluation of TAP of Tomatoes from *Gordal* Variety through ORAC and TBARS Assays

For evaluation of antioxidant potential, ORAC and TBARS assays were used. Due to the sensitive nature of target antioxidants (light and O_2 sensitive), the use of aliquots occur just once with the samples being handled in the absence of light and remaining at room temperature the shortest time.

3.4.1. ORAC Assay

ORAC assays was adapted from Bernaert *et al.* (2012) [173]. ORAC design is represented in Figure 3.2: in a Greiner 96 well Cellstar plate, black with flat bottom, each microplate was properly marked from T1 to T4 and added 25 µL of the trolox solution (standards linear regression). In the wells marked from A1 to A4, it was added 25 µL of sample diluted 1000 times (filtrated 0.20 µm). For control (wells marked as CTRL), 25 µL of 10 mM phosphate buffer at pH 7.40 was used. For blank (wells marked as PBS), 200 µL of 10.00 mM phosphate buffer at pH was pipetted. With exception of blank wells, 150 µL of fluorescein solution (10.00 nM) was added to all wells. After 30 minutes of incubation at 37º C, 25 µL AAPH (153.00 mM) was added to all wells, with exception of blank. Exterior wells and spaces between wells were filled with water, functioning as a thermal reservoir.

The values of fluorescence (λ_{Exc}. 485 nm, λ_{Em}. 520 nm) were subsequently determined every 90 s, for about one hour through Victor3 Multilabel Plate Counter 1420 fluorescence reader.

	1	2	3	4	5	6	7	8	9	10	11	12
A	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O
B	H_2O	T1	T1	T1	T1	T1	T2	T2	T2	T2	T2	H_2O
C	H_2O	T3	T3	T3	T3	T3	T4	T4	T4	T4	T4	H_2O
D	H_2O	T5	T5	T5	T5	T5	T6	T6	T6	T6	T6	H_2O
E	H_2O	PBS	PBS	PBS	PBS	PBS	CTRL	CTRL	CTRL	CTRL	CTRL	H_2O
F	H_2O	A1	A1	A1	A1	A1	A2	A2	A2	A2	A2	H_2O
G	H_2O	A3	A3	A3	A3	A3	A4	A4	A4	A4	A4	H_2O
H	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O

Figure 3.2. ORAC design for a 96 well plate: **T1**, **T2**, **T3**, **T4**, **T5** and **T6** represents 25 µL of Trolox in different concentrations; **PBS** represents 200 µL of phosphate buffer (blank); **CTRL** is the control with 25 µL of PBS solution; **A1**, **A2**, **A3** and **A4** represents 25 µL of sample. With exception of PBS (blank), to all the other wells is added 150 µL of fluorescein solution, and after incubation, 25 µL of AAPH.

The data matrix was used to determinate the average (AVG), standard deviation (SD) and relative standard deviation (RSD). Values with RSD < 20% were used in order to obtain AUC. A standards linear regression was obtained from the AUC results, and applied to samples, with results expressed as µmol TE/g sample, TE as trolox equivalents [173].

3.4.2. TBARS Assay

TBARS assays were performed according to Senphan and Benjakul (2012) [208]. A 100 mL stock solution of 3.75×10^{-1}% TBA (w/v), 15% TCA (w/v) in 0.25 M HCl was prepared (TBA/TCA/HCl solution). 1.20 g of sample was mixed with 3 ml of a TBA/TCA/HCl solution. To prepared six different TEP concentrations of 1.0, 5.0, 7.5, 10.0, 15.0 and 20.0 nmol/mL necessary for standards linear regression, it was added to 3 mL of TBA/TCA/HCl solution the corresponding TEP (1 µmol/mL) volumes of 1.50, 7.50, 11.25, 15.00, 22.50 and 30.00 µL. For the blank assays it was used 3 mL of TBA/TCA/HCl solution. Each solutions prepared were divided into 3 eppendorfs of 1.5 mL (n=3) and heated at 95º C, for 10 min, to develop a pink colour. After cooling to room temperature, it was centrifuged with a relative centrifugal force (RCF) of 3300×g, for 20 min. The absorbance of the supernatant was measured at λ = 534 nm through UV-Vis (Figure 3.3). TBARS was calculated and expressed as nmol MDA/g sample, being MDA malondialdehyde [208].

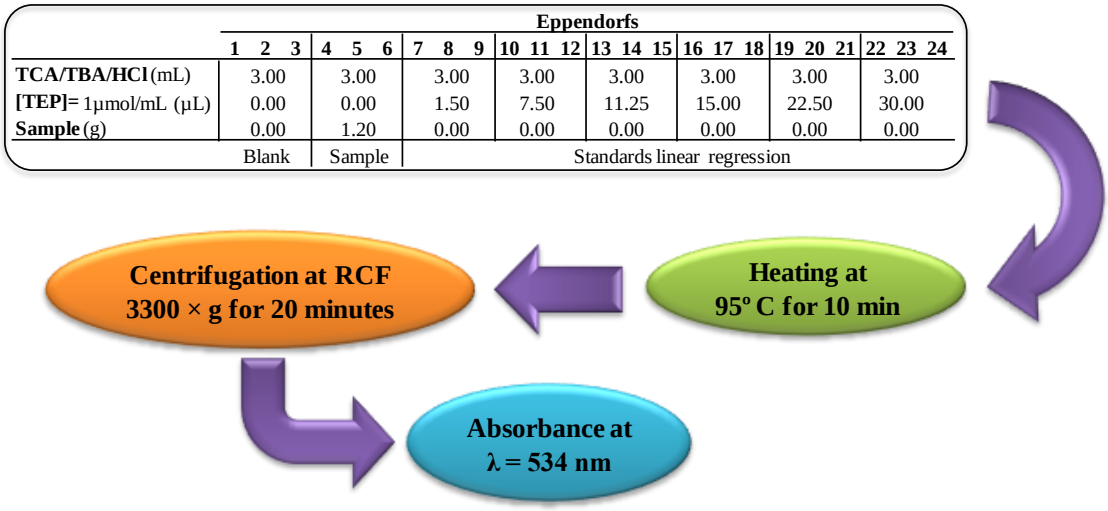


Figure 3.3. TBARS procedure.

3.5. Extraction Procedures and Analysis of Lipophilic and Hydrophilic Antioxidants

The aim of this work was the determination of lipophilic (β-carotene, lycopene, α- and β-tocopherol) and hydrophilic (L-AA) antioxidants in tomato varieties and tomato foodstuff. For that, it were used two different extraction procedures (MEPS and LL-USAE), and the target antioxidants were analysed through UV-Vis, UHPLC-PDA and UHPLC-FLR.Several experimental procedures, namely the extraction conditions, were assayed to obtain an optimized methodology for the target antioxidants.

3.5.1. Optimization of Extraction Procedures

eVol® MEPS

For the extraction of lipophilic and hydrophilic antioxidants MEPS was selected as extraction technique, because it allows the extraction and separation of lipophilic and hydrophilic analytes. For MEPS procedure, different experimental parameters were optimized: (*i*) sorbent (stationary phase) used (n=10), (*ii*) sorbent conditioning and sorbent regeneration reagent mix (n=3), (*iii*) volume and number of repetitions for sample loading (n=3), and (*iv*) volume and number of repetitions for elution (n=3). MEPS optimization was performed with C18 sorbent fibre from SGE, sorbent conditioning and fibre regeneration was carried out with an acetonitrile (ACN) - methanol (MeOH) solution 4:1 (v/v) followed by formic acid (FA) 0.10% (H_2O/0.1%

FA) for equilibration. The sorbent was loaded with 5×50 µL of sample, followed by washing (in the case, the first extraction – hydrophilic analytes) with 2×100 µL H_2O/0.1% FA. Elution of lipophilic analytes was performed with 2×100 µL of hexane. MEPS optimization was developed as shown in Figure 3.4.

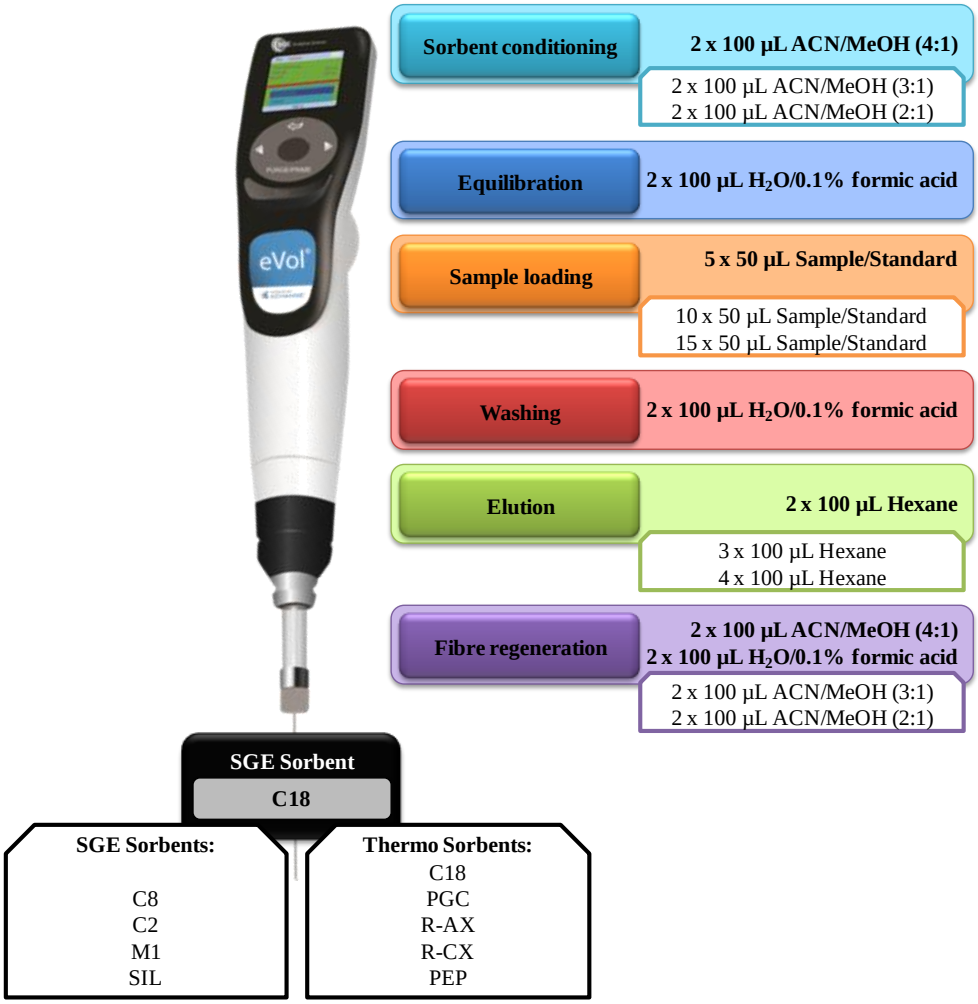


Figure 3.4. eVol® MEPS extraction and optimization procedure.

LL-USAE

The last step of MEPS optimization reveals a setback that led to substitution of MEPS by LL-USAE. In order to maximize the extraction yield, ultra sound has joined into procedure, along with one or two clean ups. Briefly, 0.50 g of sample was diluted (1:10) with 5 mL of ACN/MeOH (4:1, v/v), submitted to sonification for 30 minutes (temperature maintained between 25 and 30º C). After that, 1 mL of supernatant was collected to 3 eppendorfs (*n*=3) and mixed with 20.00 mg of PSA/C18/$MgSO_4$ (1:1:6; w/w), then submitted to centrifugation (RCF of 3300×g) for 15 minutes. The supernatant was collected and submitted to a final filtration (0.20

µm) before analysis. Matrix for carotenoids analysis was obtained by adding a second cleanup with multi-walled carbon nanotube (MWCNT) (10 mg), immediately before the PSA/C18/$MgSO_4$ cleanup. Several parameters were tested and optimized: (*i*) extraction solvent (MeOH, EtOH, ACN, ACN/MeOH 4:1, MeOH/EtOH 4:1), (*ii*) time of sonification (0, 15, 30 and 45 minutes), and (*iii*) cleanup salts (PSA, graphene oxide, MWCNT and PSA/C18/$MgSO_4$). Extraction optimization parameters can be seen in Figure 3.5.

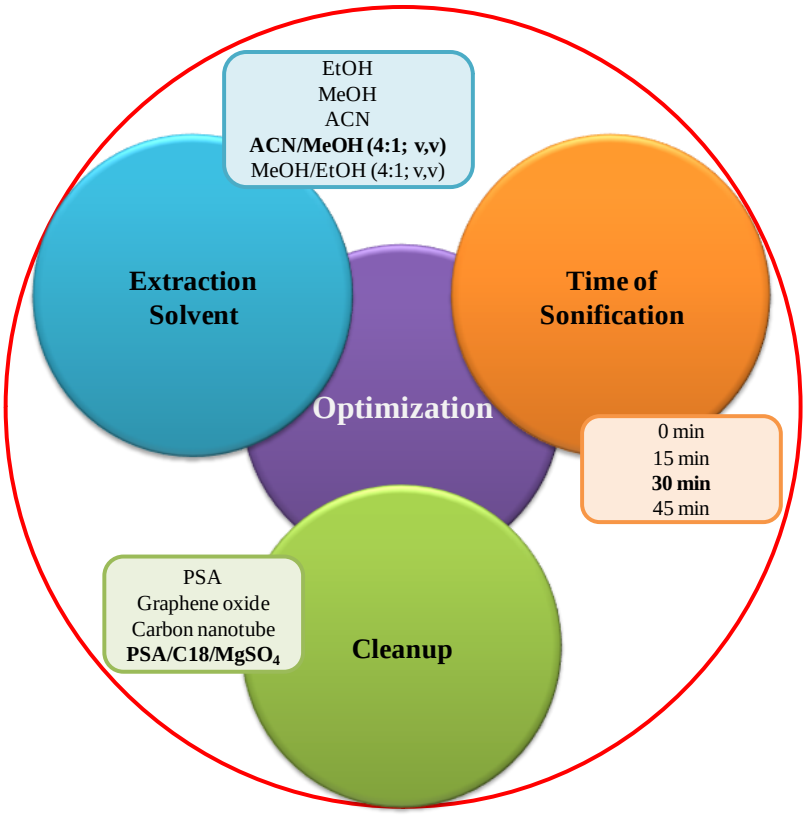


Figure 3.5. LL-USAE optimization.

3.5.2. Optimization of Analytical Methods

UV-Vis

Determination of carotenoids through UV-Vis analysis is well established in literature. In this work, a method based on the mean absorption coefficients and mean absorption wavelength - Method of Mean, as presented by Biehler *et al.* (2010) [51] was used. Since 90% of the carotenoids in the diet and human body are represented by β-carotene, α-carotene, lycopene, lutein, and cryptoxanthin (in the case of tomato, mainly β-carotene and lycopene), they present molar mass, molar absorption coefficient and absorption wavelength with similar values, so, average values for determination of total carotenoids can be applied [51].

Molar absorptivity (ε) for lycopene and β-carotene has to be determinated, and from the average, estimate ε for total carotenoids. ε was obtained from application of Equation 3 to standards linear regression. UV-Vis optimization was performed using lycopene and β-carotene.

UHPLC-PDA and UHPLC-FLR

UHPLC-PDA and UHPLC-FLR optimization was performed after a deep literature research about this subject. Several parameters were tested and optimized: (*i*) mobile phase (ACN, MeOH and H_2O/0.1% FA), (*ii*) flow rate, and (*iii*) run time. All runs were performed at 30º C using an ACQUITY UPLC® BEH C18 Column, with PDA® wavelength set at 450 nm for carotenoids and at 245 nm for L-AA. The FLR® was set at λ_{Exc} 296 nm and λ_{Em} 330 nm, for tocopherols [9, 56, 61, 89, 103, 206, 207, 209-213]. UHPLC-PDA and UHPLC-FLR optimization was performed using standards of lycopene, β-carotene, α- and δ-tocopherol, and L-AA.

3.6. Validation of LL-USAE/UV-Vis Method for Total Carotenoids Determination

After optimization, the validation of the analytical method LL-USAE/UV-Vis, with the best conditions obtained, was assessed through the evaluation of: (*i*) selectivity, (*ii*) linearity, (*iii*) sensibility by limits of detection (LOD) and limits of quantification (LOQ), (*iv*) intra- and inter-day precision, (*v*) accuracy and (*vi*) matrix effect, (Figure 3.6) [201, 207].

The selectivity of the method was assessed by the absence of interfering peaks in UV-Vis spectrum at the wavelength established for total carotenoids analysis, at 450 nm. [201, 207].

Linearity was evaluated by external standard addition method, through analytes standards linear regression (n=3), using 8 different concentrations, applying the least-squares method, obtaining the respective correlation coefficient (r^2) [201, 207].

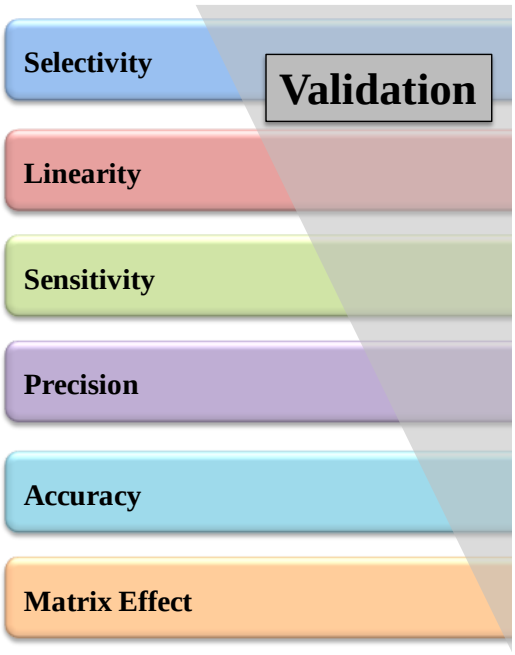


Figure 3.6. Parameters considered on the validation of analytical methods.

Sensitivity of the method is assessed through determination of LOD and LOQ, obtained from the linear regression [201, 214]. LOD is the smallest concentration that can be reported as detected with a certain level of confidence, this level of confidence is related to the confidence factor, in the case, "3", that factor, corresponds to a 98.3% confidence level. LOD was determinated by the Equation 7:

$$LOD = 3 \times SD_{LC} \qquad \text{(Equation 7)}$$

were SD_{LC} is the standard deviation of the lower concentration present in the standards linear regression [201, 214]. LOQ is the concentration at which quantitative results can be reported with a high degree of confidence. The only difference to LOD is the confidence factor, "10", this increase from 3 to 10 aims to prevent a random fluctuation of the blank reading, leading to a false negative, and ensure that the result is a "real" one [201, 214]. LOQ was determinate by the Equation 8:

$$LOQ = 10 \times SD_{LC} \qquad \text{(Equation 8)}$$

These parameters were calculated for each analyte from the standard solutions used to obtain the corresponding calibration curves, using the LL-USAE/UV-Vis developed method.

Precision is a function of concentration and it was calculated by dividing the standard deviation (SD) by the means of concentration to obtain the coefficient of variation, which when

expressed on a percentage basis gives the RSD. For method precision assessment, 3 concentrations (Table A4), one at low level (LL), other at medium level (ML) and the last, at high level (HL), were evaluated four times (*n*=4). Four trials were executed in the same day, resulting in intra-day precision and this give the repeatability, other four trials were executed in not consecutive days, resulting in inter-day precision, that give us the reproducibility. These parameters were calculated for lycopene and β-carotene standards, and applied the method of mean, as so, values correspond to total carotenoids, not individual carotenoids.

Accuracy was evaluated through recovery study and expressed as recovery percentage (R, %). Three different standard concentrations LL, ML and HL were evaluated (*n*=3) in standard solution and fortified sample, it is also used a sample evaluation. Recovery percentage is given by Equation 9 [201, 207, 215]:

$$R(\%) = (S_F - S) / Std \qquad \text{(Equation 9)}$$

were R(%) is the recovery percentage, S_F is the concentration of target analytes in fortified sample, S is the concentration of target analytes in sample and Std is the concentration of target analytes added to sample. In this particular case, S was a tomato matrix without carotenoids (removed by MWCNT, see Figure 5.11), and so carotenoids concentration was zero, consequently, equation can be simplified:

$$R(\%) = S_F / Std \qquad \text{(Equation 10)}$$

Matrix effect (ME, %), with a tolerance range between 80 and 120%, is the effect on an analytical method caused by all other components of the sample. Matrix effect percentage was determinate through the Equation 11 [201, 207, 215]:

$$ME(\%) = m_{Sol}/m_{FS} \qquad \text{(Equation 11)}$$

Were ME is the matrix effect, m_{Sol} is the slope of standards linear regression, and m_{FS} is the slope of fortified sample linear regression.

Application of LL-USAE/UV-Vis

After validation, the LL-USAE/UV-Vis method was applied for determination of total carotenoid content in different tomatoes varieties (*gordal, regional, campari* and *grape),* different processed food samples containing tomato derivatives (ketchup, tomato paste and

tomato concentrate) and in four ripening stages ("immature green", "full growth", "breaker" and "ripe") of tomato from *gordal* variety.

In the evaluation of the four ripening stages of *gordal* tomato, it was applied the second derivative to the UV-Vis data obtained in each assay, enabling observation of small yet significant variations in absorbance between 446 and 452 nm.

3.7. Validation of LL-USAE/UHPLC-PDA and LL-USAE/UHPLC-FLR Methods for Lipophilic and Hydrophilic Antioxidants

With the best conditions optimized for LL-USAE and UHPLC-PDA/FLR methods, LL-USAE/UHPLC-PDA was validated, for β-carotene, lycopene determination in ripe tomato from *gordal* variety. The LL-USAE/UHPLC-PDA method validation was not completed for the L-AA due to equipment breakdown. Also for ripe tomato from *gordal* variety, LL-USAE/UHPLC-FLR method was submitted to validation, for α- and δ-tocopherol determination. The selectivity of the method was assessed by the absence of interfering peaks in UV-Vis spectrum at the wavelength established for β-carotene, lycopene analysis, at λ = 450 nm, and λ = 245 nm for L-AA analysis, whereas for tocopherols, it was assessed by the absence of interfering peaks in fluorescence spectra with λ_{Exc} = 296 nm and λ_{Em} = 330 nm [201, 207].

Linearity was evaluated by external standard addition method, through analytes standards linear regression (n=3), using 6 different concentrations (Table A5), applying the least-squares method, obtaining the respective correlation coefficient (r^2) [201, 207].

Sensitivity of the method is assessed through determination of LOD (Equation 7) and LOQ (Equation 8), obtained from the linear regression [201, 214]. These parameters were calculated for each analyte from the standard solutions used to obtain the corresponding calibration curves, using the LL-USAE/UHPLC-PDA and LL-USAE/UHPLC-FLR developed methods.

For method precision assessment (as RSD), 3 concentrations (from Table A5), one at low level (LL), other at medium level (ML) and the last, at high level (HL), were evaluated four times (n=4). Four trials were executed in the same day, resulting in intra-day precision and this give the repeatability, other four trials were executed in not consecutive days, resulting in inter-day precision, that give us the reproducibility.

Accuracy was evaluated through recovery study (Equation 9 for tocopherols, and Equation 10 for carotenoids), with evaluation (n=3) in S_F and Std of three different standard concentrations levels corresponding to the LL, ML and HL. Matrix effect was obtained through Equation 11.

Application of LL-USAE/UHPLC-PDA and LL-USAE/UHPLC-FLR

LL-USAE/UHPLC-PDA was applied for determination of β-carotene, lycopene and L-AA in ripe tomato from *gordal* variety. LL-USAE/UHPLC-FLR was also applied for determination of α- and β-tocopherol determination in ripe tomato from *gordal* variety.

CHAPTER IV

RESULTS AND DISCUSSION

4.1. Evaluation of TAP of Tomatoes from *Gordal* Variety through ORAC and TBARS Assays

To evaluate the TAP, ORAC procedure was applied to tomato samples at four ripening stages: (*i*) immature green, (*ii*) full mature green, (*iii*) breaker, and (*iv*) ripe. ORAC values organized by maturation stages are summarized in Figure 4.1, where can be see the distribution of the results.

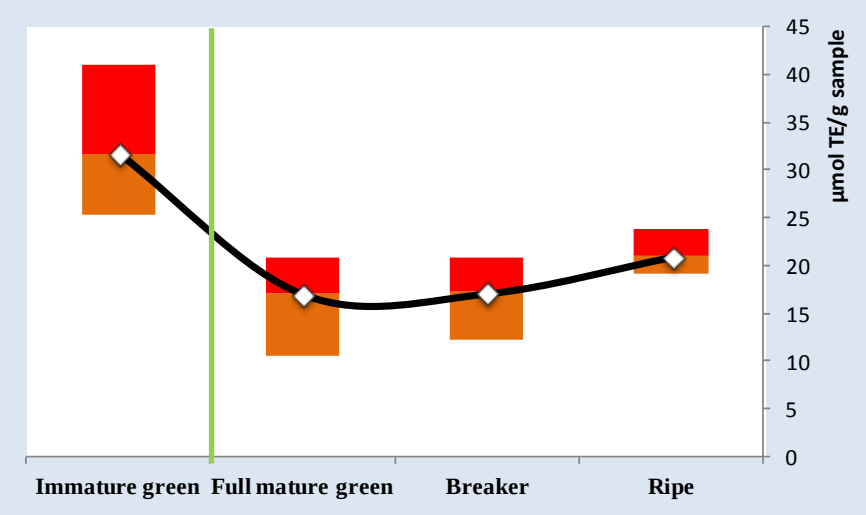


Figure 4.1. ORAC values (µmol TE/g sample) for different ripening stages. Vertical green line indicates the end of tomato growth.

The amplitude of the ORAC values obtained decreases during ripening (Figures 4.1 to 4.3), this was expected, as metabolic activity decrease along the ripening and metabolic processes tend to stabilize. The high values of ORAC for "immature green" stage, can possibly be explained by the low fruit size and low water content, resulting in higher concentrations of constituents. The last three stages of tomato have similar sizes and water content, and so, ORAC assays reveal a slight increase of TAP during ripening, following the increase of carotenoid content, L-AA and tocopherols [216, 217]. After full growth, ORAC values range from 17.17 µmol TE/g tomato at full mature green, to 21.06 µmol TE/g at ripe stage. These values are about twice those reported in literature [106].

TBARS assay was also applied to tomato samples at different ripening stages. The results are summarized in Figure 4.2.

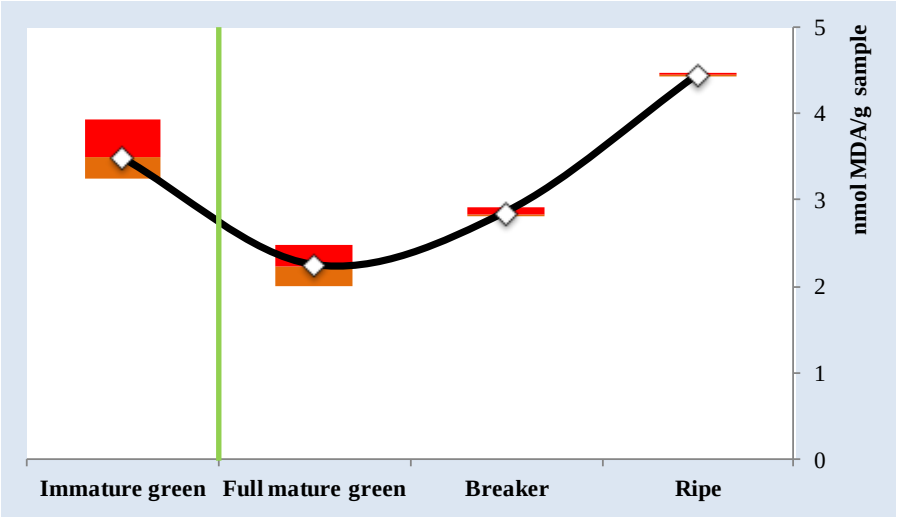


Figure 4.2. TBARS values (nmol MDA/g sample) for different ripening stages. Vertical green line indicates the end of tomato growth.

For the last three stages TBARS indicates an expected increase of LP due to alterations in physical properties of the lipid matrix in membranes and changes in the activity of membrane-bound enzymes, leading to accumulation of peroxidised lipids in membranes that occur during maturation and ripening of tomato fruit [218, 219].

The results of ORAC and TBARS showed a similar behaviour (Figure 4.3). Considering only the last three maturation stages, an increase of TBARS values corresponds to an increase of ORAC values, in other words, even with the increase of LP, leading to a reduction of theavailable antioxidants, there is an increase in antioxidant capacity, caused probably due to the increase of relevant antioxidants, namely carotenoids such as lycopene (Figure 4.16).

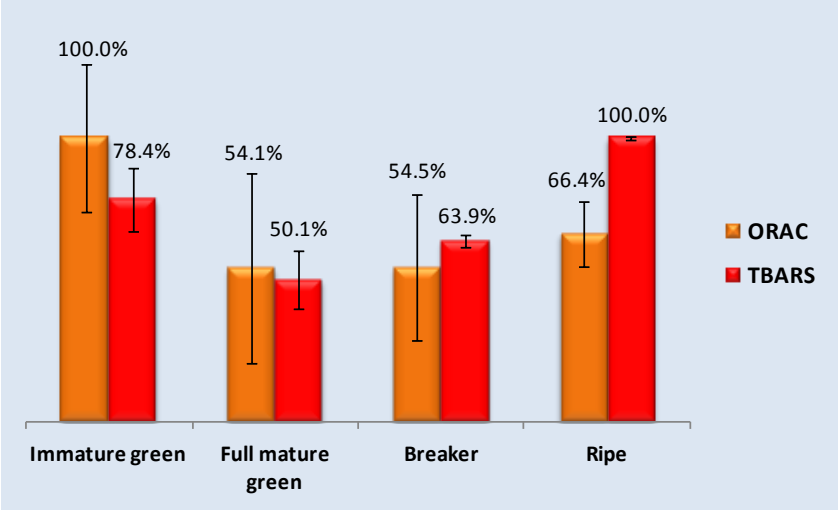


Figure 4.3. TBARS and ORAC relative values at different ripening stages.

4.2. Extraction Procedures and Analysis of Lipophilic and Hydrophilic Antioxidants

4.2.1. Optimization of Extraction Procedures

eVol® MEPS

The initial conditions used in MEPS were C18 sorbent fibre, sorbent conditioning and fibre regeneration with 2×100 µL of ACN/MeOH (4:1; v/v), equilibration with 2×100 µL of H_2O/0.1% FA. Sample loading was 5×50 µL, followed by washing with 2×100 µL H_2O/0.1% FA, and finally, elution was performed with 2×100 µL of hexane.

eVol® MEPS optimization was assessed analyzing the results obtained for each parameter assayed (Figure 4.4), through respective elution in step *v* (elution) of MEPS experimental procedure (Figure 2.1). Different combinations of ACN/MeOH (v/v) were used for sorbent conditioning and fibre regeneration optimization (step *i*), in combinations of 4:1, 3:1 and 2:1. Combination of 2:1 ACN/MeOH (v/v) give the best results elution results (Figure 4.4-A). The number of repetitions in sample loading (step *iii*) was optimised: 2, 5 and 10 repetitions, with 5 and 10×100 µL of sample loading presenting similar results, and so, 5 times was chosen to minimize extraction steps and sample and solvents usage (Figure 4.4-B). From elution optimization (step *v*), 2 and 3 repetition of hexane were tested, with 2×100 µL of hexane having the best result for elution (Figure 4.4-C).

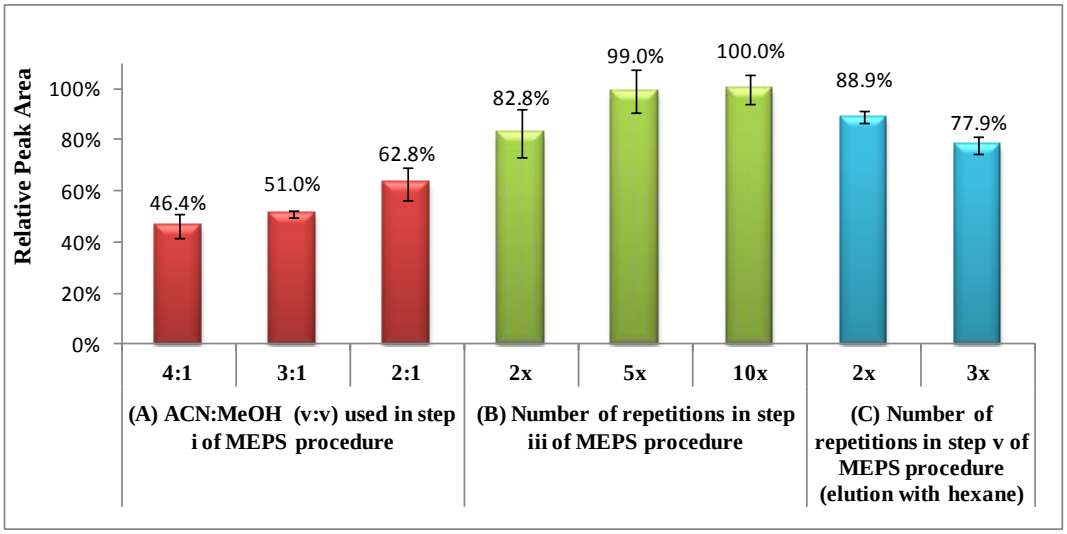


Figure 4.4. Elutions results as relative peak area from MEPS parameters optimized: (A) Sorbent conditioning and Fibre regeneration (step *i*) with different ACN and MeOH solutions (ACN:MeOH; v/v); (B) *n* repetitions of sample loading (step *iii*) (n×100 µL); and (C) Elution (step *v*) with *n* repetition of hexane (*n*×100 µL).

The last optimized parameter was the sorbent. This optimization was performed with the best conditions obtained for different parameters. Ten sorbents commercially available were tested, 5 from SGE (C2, C8, C18, M1, SIL) and 5 from Thermo Fisher Scientific (C18, PGC, R-AX, R-CX, PEP).

Carotenoids are verysensitive and reactive (being the most difficult antioxidant to extract from the five evaluated in this work), and so evaluation was carry out through UV-Vis analysis for carotenoids content. From SGE, C18 and SIL retrieved the best results (Figure 4.5). Regarding the Thermo Fisher Scientific fibres, the best results were obtained using R-AX, R-CX and PEP (Figure 4.5). The results showed a higher precision with C18 sorbent, either from SGE or Thermo (Figure A4.)

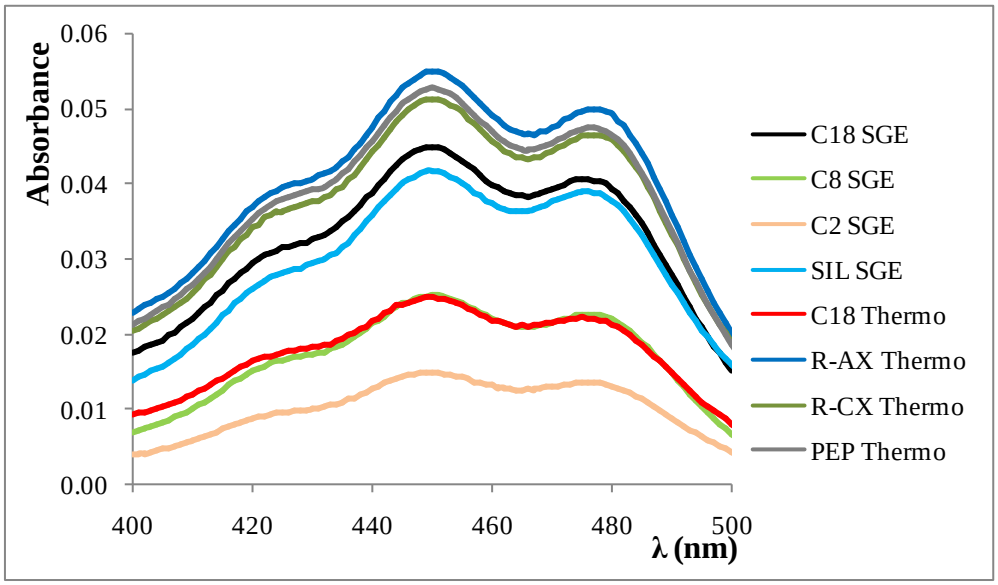


Figure 4.5. Representative UV-Vis spectrum for carotenoids obtained by MEPS extraction with different sorbents.

Using ultra-micro cuvettes of 160 µL, ideal for the low elution volumes obtained in MEPS, it was possible to analyze the extracts prior to drying in N_2 stream. Comparing the results for carotenoids (λ = 450 nm) prior to hexane drying (through UV-Vis) and after hexane drying and resuspension in ACN:MeOH (4:1; v/v) (through UHPLC-PDA/FLR), results showed that there was no linearity in the method, indicated that losses in hexane drying were not uniform (Figure 4.6).

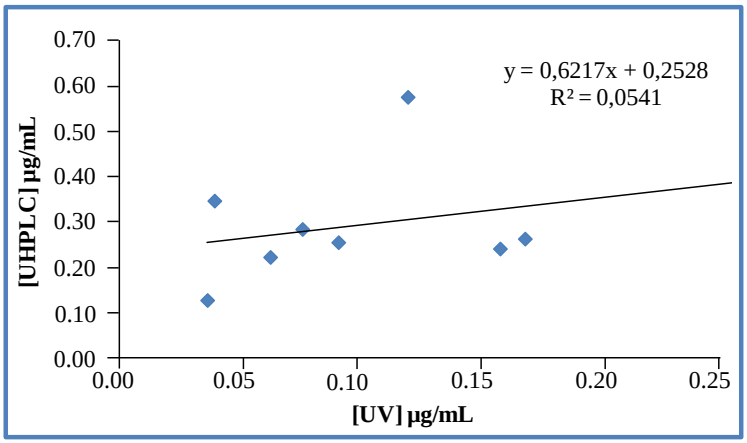


Figure 4.6. Evaluation of carotenoids content in the same MEPS extraction, prior to hexane drying (through UV-Vis; λ = 450 nm), and after hexane drying (through UHPLC; λ = 450 nm).

A sample was processed by MEPS and injected in UHPLC-PDA/FLR and a diluted sample (same dilution as the MEPS sample injected) was injected directly in UHPLC-PDA/FLR. The resulting chromatograms are shown in Figure 4.7. By comparing the two extracts, we can see that MEPS also reveals a very low recovery for carotenoids and tocopherols, and therefore, MEPS extraction procedure was discarded.

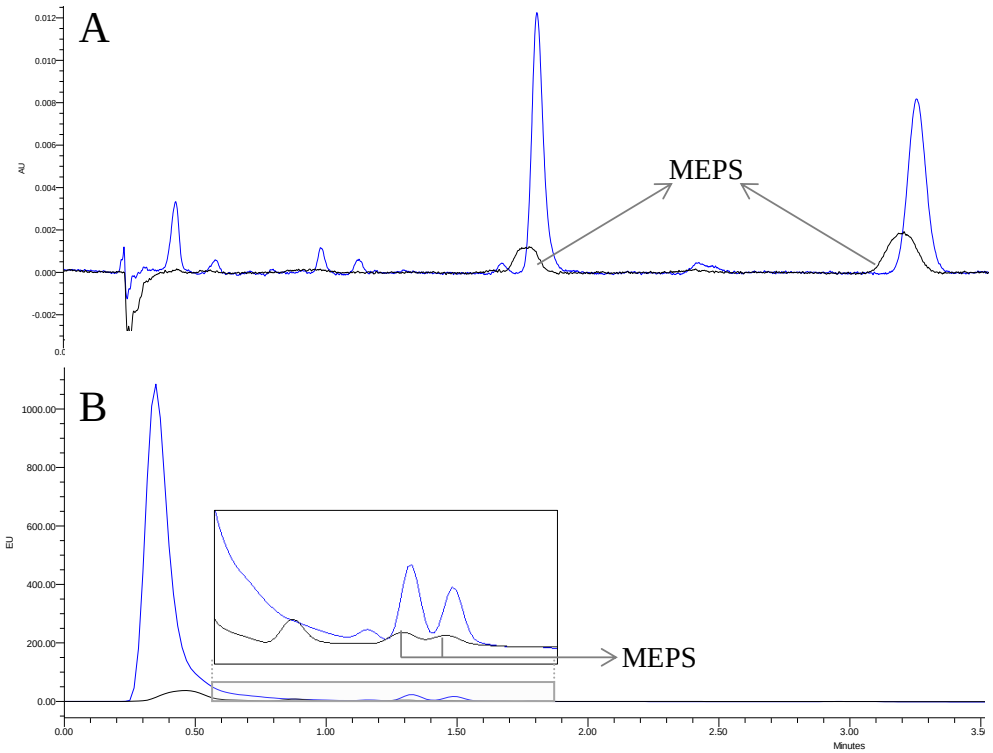


Figure 4.7. Comparison between tomato MEPS extract and direct injection. (**A)** Cromatogramas obtained with PDA detector at λ = 450 nm (carotenoids); (**B)** Cromatogramas obtained with FLR detector at λ_{Exc}296 nm and λ_{Em} of 330 nm (tocopherols).

LL-USAE

In order to select the best extraction solvent, several organic solvents (ACN, MeOH, EtOH, MeOH:EtOH, ACN:MeOH) were tested and compared (Figure 4.8). As shown, ACN/MeOH (4:1; v/v) gives the best results.

In addition to contribute to increase the extraction efficiency, ACN:MeOH also promotes protein precipitation [220].

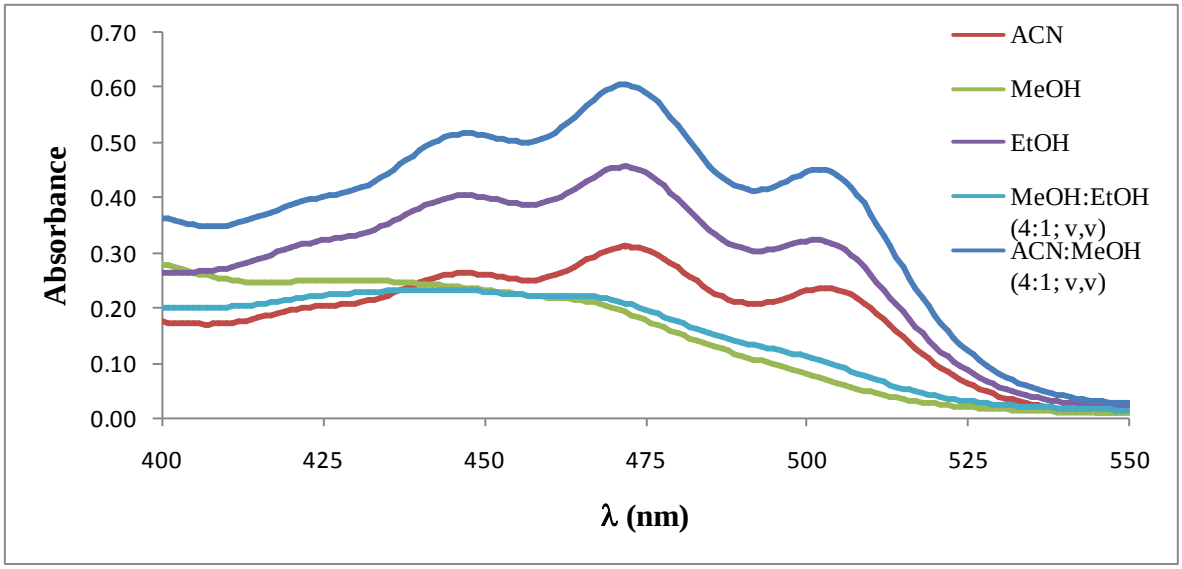


Figure 4.8. Representative spectra obtained with LL-USAE by UV-Vis. Comparison of different extraction solvents used.

Optimization of sonification time was evaluated through UHPLC-PDA and UHPLC-FLR. Sonification for 30 min present the best results (Figure 4.9), in agreement with that reported by Xu *et al.* (2013) [196]. Water bath in sonification was maintained between 25 and 30º C, wich were the best temperatures for carotenoids extraction using sonification, as reported by Xu *et al.* (2013) [196] and Sun *et al.* (2010) [198].

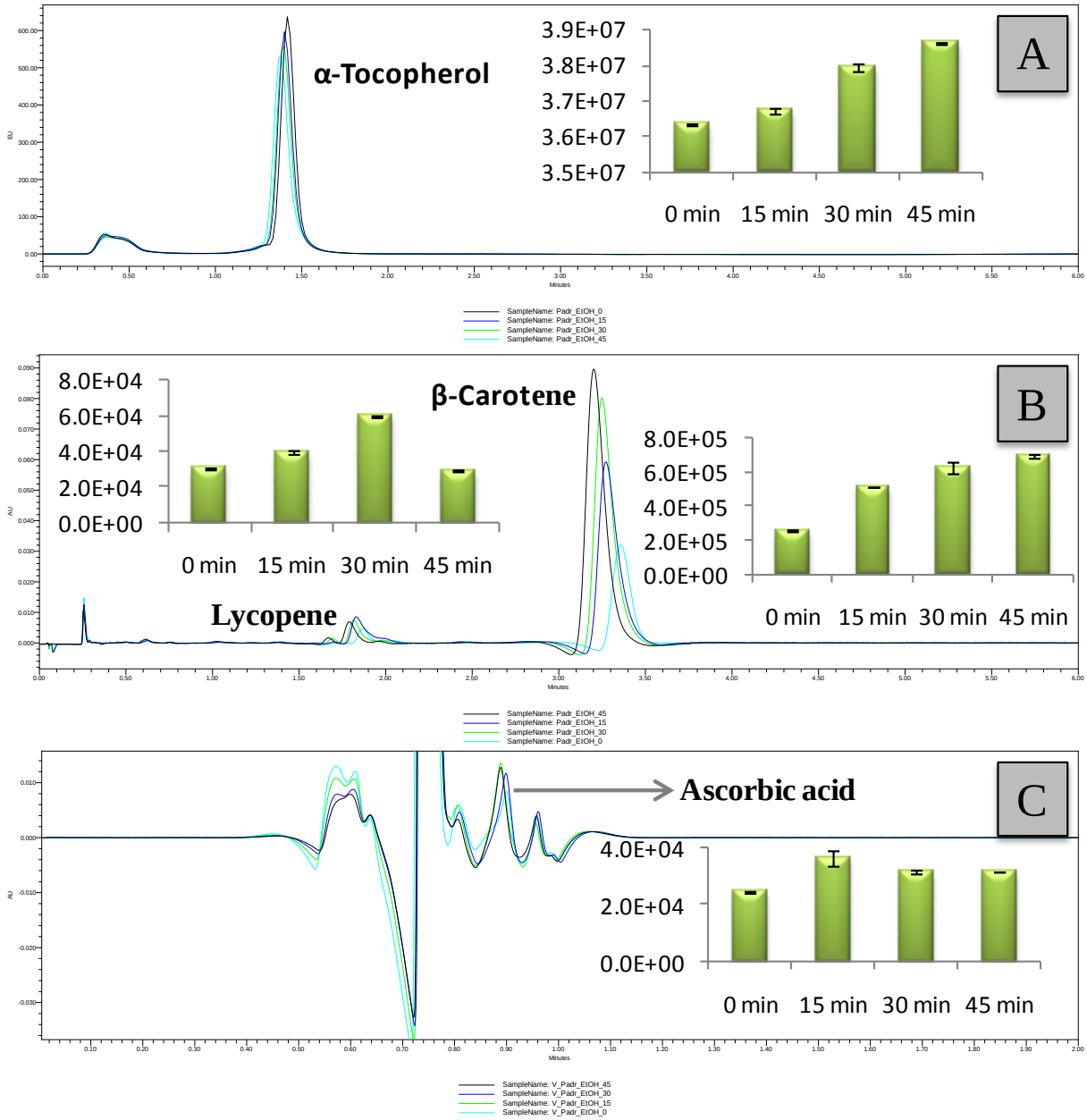


Figure 4.9. Effects of US extraction time on LL-USAE efficiency. UHPLC-FLR analysis: (**A)** α-tocopherol solution; UHPLC-PDA analysis: (**B)** carotenoids solution; (**C)** L-AA solution.

Evaluation of the efficiency of the use of cleanup salts and MWCNTs in LL-USAE was realized through UHPLC-PDA and UHPLC-FLR. The results are summarized in Figure 4.10. PSA, graphene oxide, MWCNT and PSA/C18/$MgSO_4$ were tested and compared with direct sample injection. MWCNT reveal high affinity to carotenoids, retaining them indefinitely. PSA and graphene oxide present similar results, while the best results, the closest to the sample direct injection, were obtained using PSA/C18/$MgSO_4$.

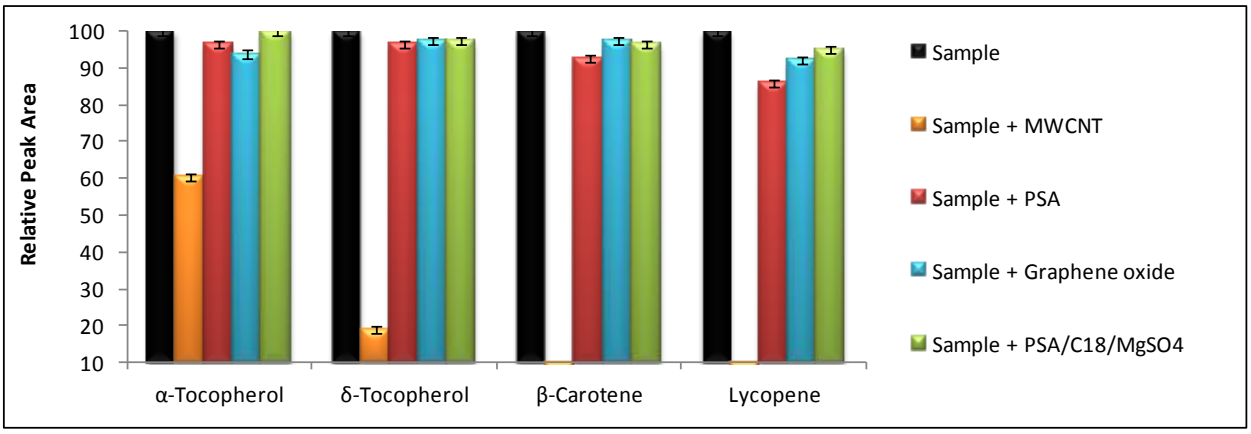


Figure 4.10. Influence of salts and MWCNTs on clean-up procedure.

The best optimization conditions for LL-USAE are summarized in Figure 4.11.

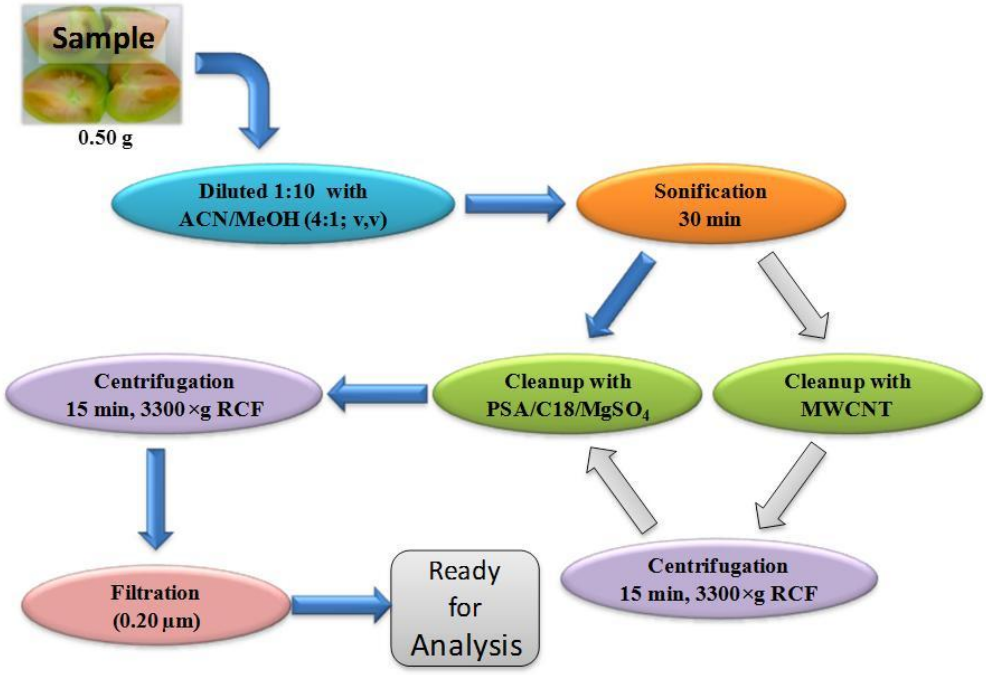


Figure 4.11. Extraction design for LL-USAE.

4.2.2 Optimization of Analytical Methods

Optimization of UV-Vis analysis procedure for total carotenoids and optimization of UHPLC-PDA and UHPLC-FLR analysis procedure for lipophilic and hydrophilic antioxidants was accomplished.

UV-Vis

For determination of total carotenoids through UV-Vis analysis, the method of means was used. For that, molar absorptivity (ε) for lycopene ($\varepsilon_{lycopene}$) and β-carotene ($\varepsilon_{\beta\text{-carotene}}$) was determinate from application of Equation 3 (ε = A/lc) to different solutions. The linear regressions were built with the obtained ε for each concentration. $\varepsilon_{lycopene}$ and $\varepsilon_{\beta\text{-carotene}}$ were obtained using the linear regression for each carotenoid, and ε for total carotenoids ($\varepsilon_{total\ carotenoids}$) was estimated from the average results:

ε Linear regression for lycopene: y = 145248.83 + 0.80; r^2 = 0.981

$\varepsilon_{(lycopene)}$ = 145249.80 M^{-1} cm^{-1}

ε Linear regression for β-carotene: y = 133338.23 + 0.84; r^2 = 0.997

$\varepsilon_{(\beta\text{-carotene})}$ = 133339.08 M^{-1} cm^{-1}

$\varepsilon_{(total\ carotenoids)}$ = 139294.44 M^{-1} cm^{-1}

The validation of this method in tomato using only lycopene and β-carotene for carotenpods quantification, is reinforced by the UHPLC-PDA analysis at λ = 450 nm (Figure 4.9), that shows two dominant peaks in the chromatogram of tomato sample, corresponding to the carotenoids lycopene and β-carotene.

UHPLC-PDA and UHPLC-FLR

Previously to the UHPLC-PDA/FLR optimization, a dee literature research allowed to obtain starting procedures close to the final method design [9, 56, 61, 89, 103, 206, 207, 209-213]. It is reported for L-AA, the use of water as the main solvent for mobile phase, with flow rates around 0.250 mL/min, and using C18 UHPLC columns (best results for BEH C18 column). The UHPLC-PDA system at λ = 245 nm is the most commonly used for L-AA detection. For carotenoids a wide range of solvents are used as mobile phase, but two of them persists in most of the methods reported in literature: ACN and MeOH, with a proportions of 3 to 4 volumes of ACN to 1 of MeOH. Also C18 UHLPC columns are commonly used, with flow rate around 0.500 mL/min. UHPLC-PDA was also used for carotenoids detection at λ = 450 nm. For tocopherols the FLR is more sensitive, with λ_{Exc} around 296 nm and λ_{Em} around 330 nm. The mobile phase solvents and flow rates reported in the literature for tocopherols were very similar to the conditions described for carotenoids.

The first assays were carry out in order to obtain the simultaneous separations of lipophilic and hydrophilic antioxidants in a single run, using ACQUITY UPLC® BEH C18 Column at 30º C, UHPLC-PDA at λ = 450 nm for carotenoids and λ = 245 nm for L-AA, and

FLR with λ_{Exc} = 296 nm and λ_{Em} = 330 nm, for tocopherols. Flow rate was set at 0.500 mL/min, using H_2O/0.1% FA for 2 min, followed by a mobile phase gradient as described in Figure 4.12.

This gradient was created in order to guarantee a smooth transition from lipophilic to hydrophilic conditions. L-AA peak was eluted too close of solvent front. In addition some carryover was observed. This drawback was overcome by decreasing the flow rate to 0.200 mL/min for hydrophilic conditions, and increase the delay time for 1 min. The results obtained for target antioxidants using the conditions optimized (Figure 4.12) were interesting.

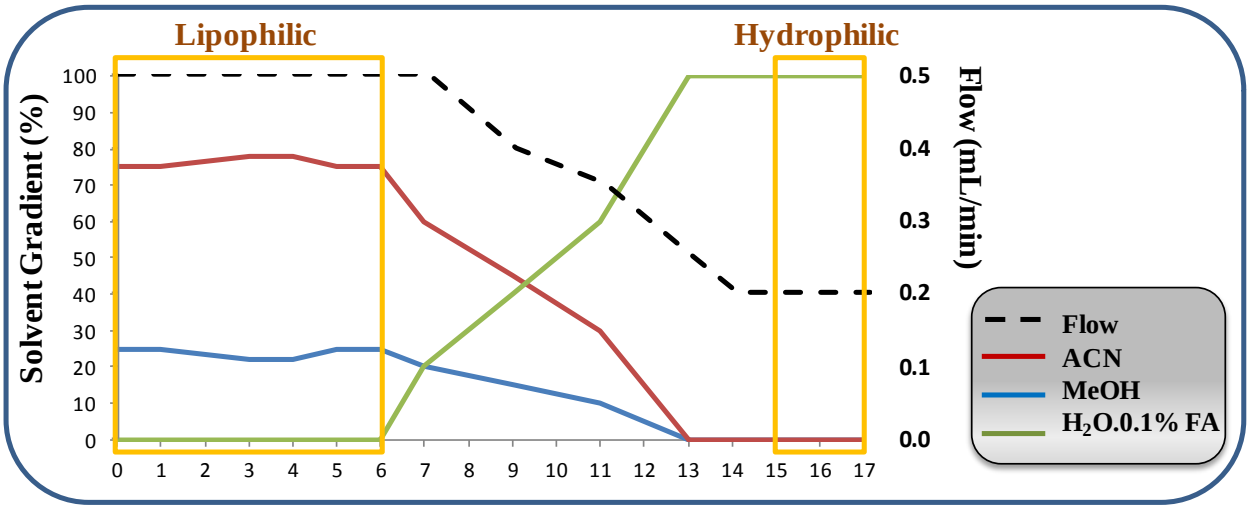


Figure 4.12. Gradient conditions and flow rate variation during the UHPLC chromatographic run.

Keeping a high flow rate for hydrophobic antioxidants (0.500 mL/min) allows a short run time, which is very important due to instability of most of the antioxidants at room temperature.

FLR revealed a high sensitivity and selectivity, giving the nature of fluorescence phenomena. PDA allowed the confirmation of peak identification due to the possibility of using the UV-Vis spectra for carotenoids and L-AA (Figure 4.13).

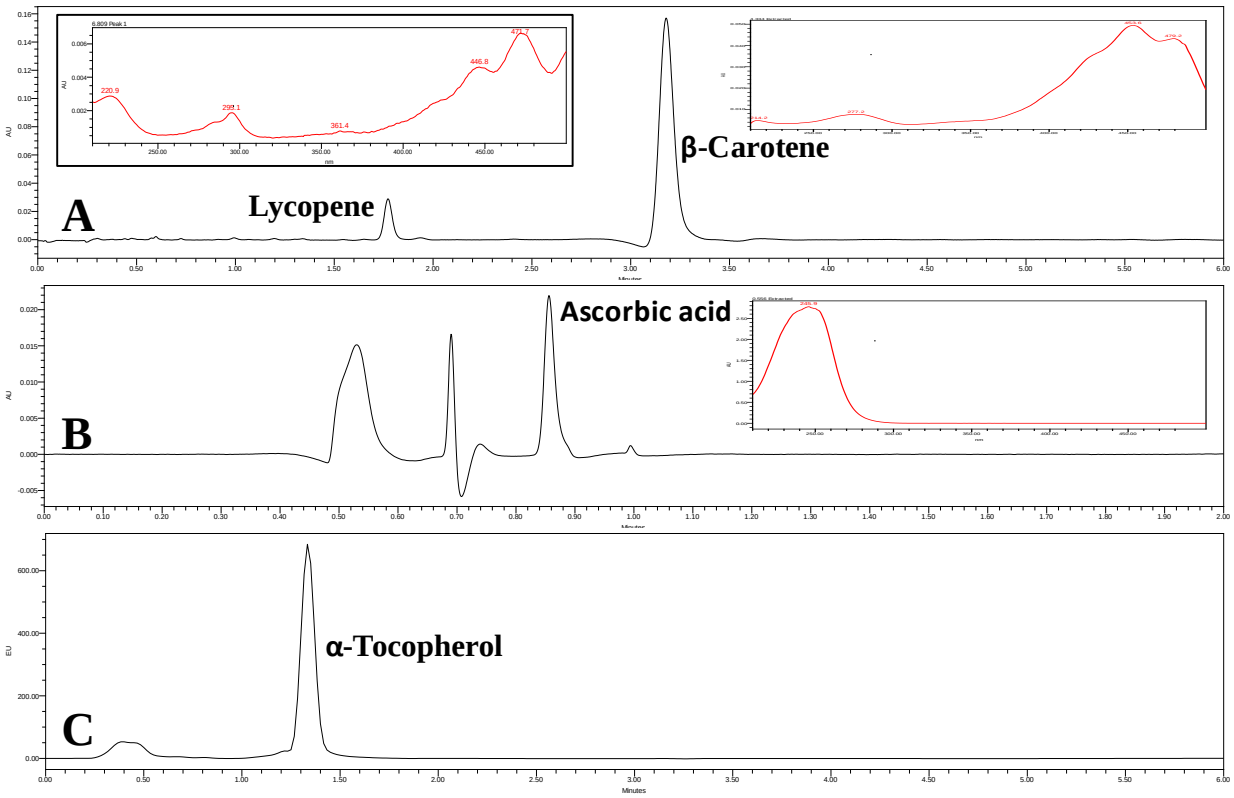


Figure 4.13. Representative chromatograms obtained with: (**A)** UHPLC-PDA at λ = 450 nm for carotenoids (lycopene and β-carotene standards), with corresponding UV-Vis spectrum; (**B)** UHPLC-PDA at λ = 245 nm for L-AA standard, with corresponding UV-Vis spectrum; (**C)** UHPLC-FLR with λ_{Exc} = 296 nm and λ_{Em} = 330 nm for α-tocopherol standard.

After the analytical and extraction techniques optimization, the validation of the methods LL-USAE/UV-Vis, LL-USAE/UHPLC-PDA and LL-USAE/UHPLC-FLR was performed. Following this, the methods were applied todifferent tomato varieties and tomato foodstuff samples.

4.3. Validation and Application of LL-USAE/UV-Vis Method for Total Carotenoids Determination

With the optimized conditions, LL-USAE/UV-Vis was submitted to validation and then it was applied to the determination of total carotenoid content in different tomatoes varieties (*gordal*, *regional*, *campari* and *grape*), in four tomato ripening stages (“immature green”, “full growth”, “breaker” and “ripe”) and different processed foods containing tomatoes derivatives (ketchup, tomato paste and tomato concentrate).

4.3.1. Validation of the LL-USAE/UV-Vis Analytical Method

Validation of the LL-USAE/UV-Vis analytical methodology was performed in terms of selectivity, linearity, sensibility by limits of detection (LOD) and limits of quantification (LOQ), intra- and inter-day precision, accuracy and matrix effect.

Selectivity

LL-USAE/UV-Vis, aimed the determination of total carotenoids. In carotenoids, the energy between orbital's π/π^* is low, so the wavelength resulting from the transition correspond to light in the visible region, up from of 400-500 nm. In vegetable samples, the dominant substances with this wavelength range, are carotenoids and chlorophyll. As chlorophyll (a and b) presents two major absorbance peaks between 650 and 660 nm, and between 400 and 440 nm, the presence of chlorophyll will not influence the results if we choose 450 nm for carotenoids quantification [51, 221]. In addition, UHPLC-PDA chromatograms of ripe *gordal* tomato obtained at 450 nm shows only two dominant peaks, corresponding to lycopene and β-carotene.

Linearity

Linearity was evaluated by external standard addition method, through analytes standards linear regression (n=3), in 5 different concentrations for lycopene and β-carotene, respectively, as shown in Table A4. The least-squares method, enable to obtain the correlation coefficient, LOD and LOQ. Taken advantage of the high affinity of carotenoids to MWCNTs and attending to carotenoids sensitivity to temperature and light, sample was submitted to cleanup with MWVNT, resulting in a matrix without carotenoids. Standard addition method was carried out in this matrix and not in fresh sample. For each standard, a calibration curve was obtained, as can be seen in Figure 4.14.

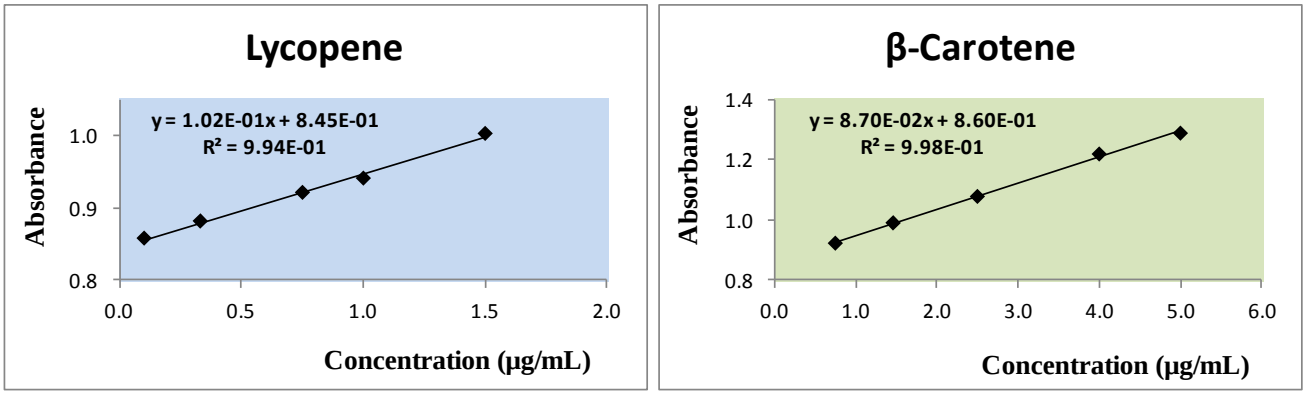


Figure 4.14. Calibration plots for lycopene and β-carotene by LL-USAE/UV-Vis.

From calibration curves, linearity was estimated as the correlation coefficient, and LOD and LOQ were determinated. The results are summarized in Table 4.1. All standards present high linearity with the correlation coefficient above 0.993. Concentrations in tomato samples [5, 115], but also in human serum studies [110, 206, 222, 223] reported in literature, are about 10 times higher than the LOD and LOQ obtained in LL-USAE/UV-Vis, meaning that LL-USAE/UV-Vis have the advantage to quantify carotenoids content 10 times lower than the levels usually found in biological samples.

Table 4.1. Validation parameters of LL-USAE/UV-Vis for β-carotene and lycopene determination. LDR – limit range; r^2 – correlation coefficient; LOD – limits of detection; LOQ – limits of quantification.

	Linearity		**Sensibility**				**Precision (%)**			**Matrix Effect (%)**
	LDR (μg/mL)	r^2	**LOD** (ng/mL)		**LOQ** (ng/mL)		**Intra-day**	**Inter-day**	**Accuracy (%)**	
Lycopene	0.10-1.50	0.9938	18.88	64.94	62.94	219.79	2.90	10.75	104.41	97.17
β-Carotene	0.01-5.00	0.9978	12.26		40.88					

Sensitivity

LOD and LOQ were also determinated for carotenoids in UV-Vis (Table 4.1). Figure 4.15 shows the comparison for LODs and LOQs determinated for carotenoids by both analytical methods, LL-USAE/UV-Vis and LL-USAE/UHPLC-PDA. It can be observed that UHPLC is able to identify and quantify β-carotene and lycopene at concentrations 1.4 and 5.2 times lower than UV-Vis, with UHPLC demonstrating, as expected, a higher sensitivity.

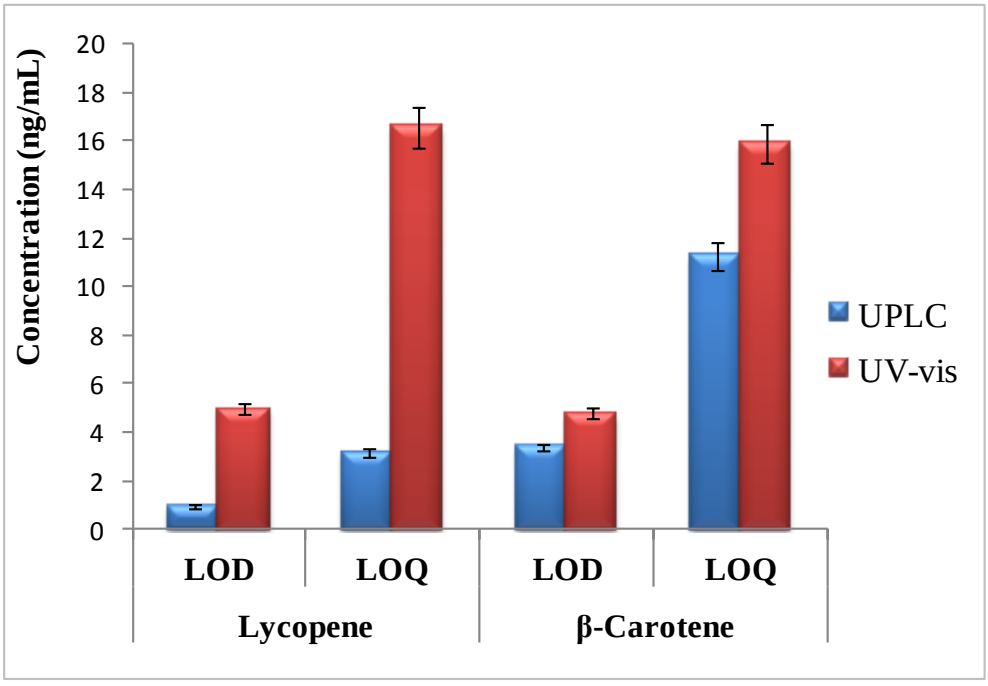


Figure 4.15. LODs and LOQs comparasion among UHPLC and UV-Vis for lycopene and β-carotene.

Precision

For precision assessment, 3 concentrations at LL, ML and HL, (Table A4), were evaluated (*n*=4), and the RSD calculated. Intra-day (repeatability) and inter-day precision (reproducibility) were also calculated. These results are presented in Table 4.1. Precision was assessed with the method of mean, and so, values correspond to total and not individual carotenoids. As expected, repeatability (2.9 %) is lower than reproducibility (10.8 %) and both are under the reference limit of 20% [215, 224].

Accuracy

For accuracy determination, 3 different standard concentrations (*n*=3) were evaluated in standard solutionsand fortified matrix solutions, and Equation 10 applied for recovery percentage. The result obtained, 104.4% (Table 4.1), is within the tolerance range (80 to 120%) [215].

Matrix Effect

Matrix effect percentage was determinated through tomato matrix (without carotenoids) instead of tomato sample, with a result of 97.2%, as seen in Table 4.1. This value is within tolerance range (80 to 120%) [215, 225].

4.3.2. Application of LL-USAE/UV-Vis to Determination of Total Carotenoids

After validation, LL-USAE/UV-Vis was applied to the determination of total carotenoid content in tomato from *gordal* variety in four ripening stages (“immature green”, “full growth”, “breaker” and “ripe”). In addition, the carotenoid content was also determinated in different tomatoes varieties (*gordal*, *regional*, *campari* and *grape*) at ripe stage, and in different commercial processed tomato pastes (ketchup, tomato paste and concentrated tomato paste).

The evaluation of total carotenoid content in four ripening stages of *gordal* tomato, was not an easy task due to the low carotenoid content in the first stages, being difficult to identify the maximum at λ = 450 nm. To overcome this drawback, a second derivative was applied (Figure 4.16),. As a result, we can see an increase of carotenoid content during maturation, reaching its maximum at ripe stage [88].

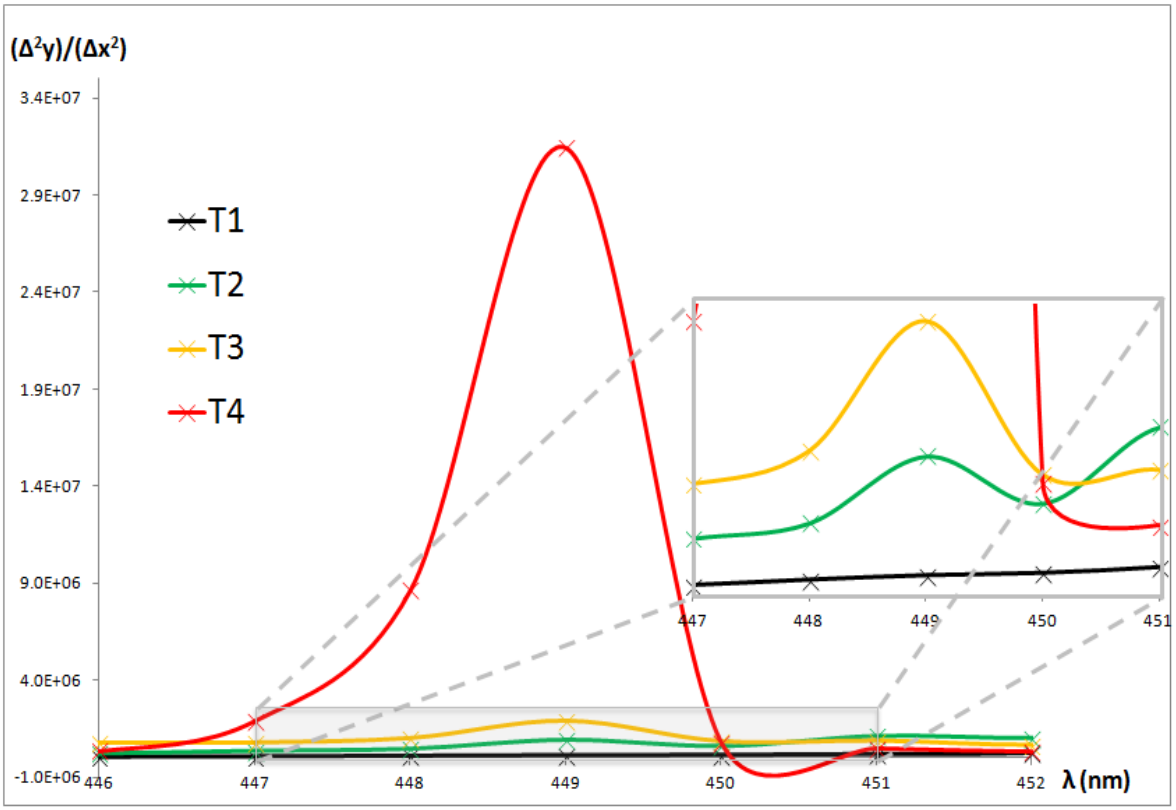


Figure 4.16. Application of second derivative to UV-Vis data obtained for tomato from *gordal* variety at 4 ripening stages, immature green (T1), full mature green (T2), breaker (T3) and ripe (T4) (range 446 – 452 nm).

Among ripe tomatoes, regional tomato presents the highest carotenoid content, followed by campari, gordal and grape (Figure 4.17-A). Different tomato samples from *gordal* variety tomato at ripe stage were collected at different sites from the same tomato plantation. The results revealed similar carotenoid content among the tomato samples from different locations (Figure 4.17-B) with an RSD of 12.1%.

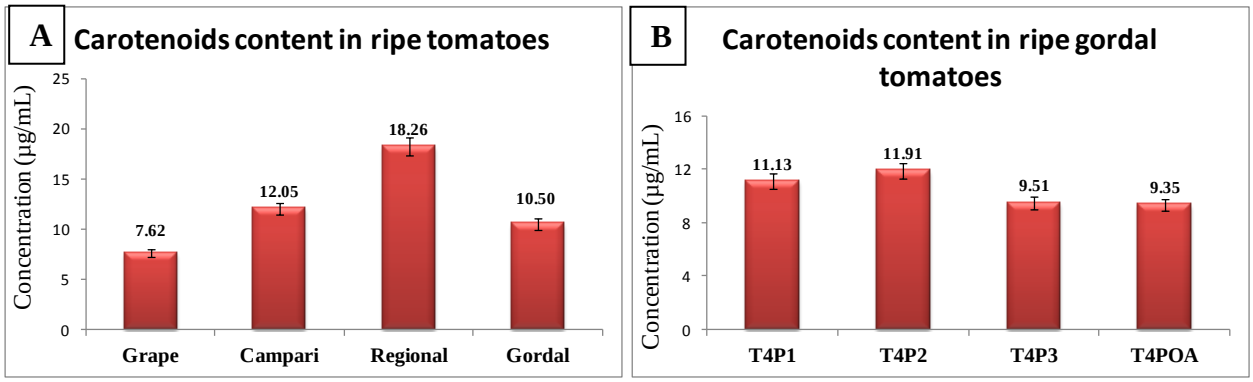


Figure 4.17. Total carotenoids content in ripe tomatoes. **(A)** Carotenoids content in four different varieties, *grape*, *campari*, *regional and gordal*. **(B)** Tomato samples from *gordal* variety collected on different sites from the same tomato plantation.

Ketchup presents the more differentiated results for carotenoid content, with significant differences between the groups of "low cost" (brands Continente, Pingo Doce and Perdiz) and the traditional (Heinz) brands evaluated. The low cost brands analysed present low carotenoids values (between 5.2 and 10.2 µg/mL) when compared with Heinz (25.8 µg/mL). Comparing ketchup with tomato paste, low cost brands present present lower carotenoid content, whereas Heinz presents higher carotenoid content.

Tomato paste (Guloso and Compal) have carotenoids contents (11.6 and 22.1 µg/mL), slightly higher than raw tomato (between 7.6 and 18.3 µg/mL). Concentrated tomato paste (Guloso from can, and Compal and Guloso from tube) presents the highest carotenoid content (between 36.8 and 46.0 µg/mL).

Tomatoes processing increases carotenoids availability, and the same procedure leads to a concentration of the resulting tomato pastes. This concentration occurrence can be seen in the results obtained (Figure 4.18), with all tomatoes foodstuffs presenting higher carotenoid content than raw tomatoes, with the exception of "low cost" ketchup, possibly due to the product dilution.

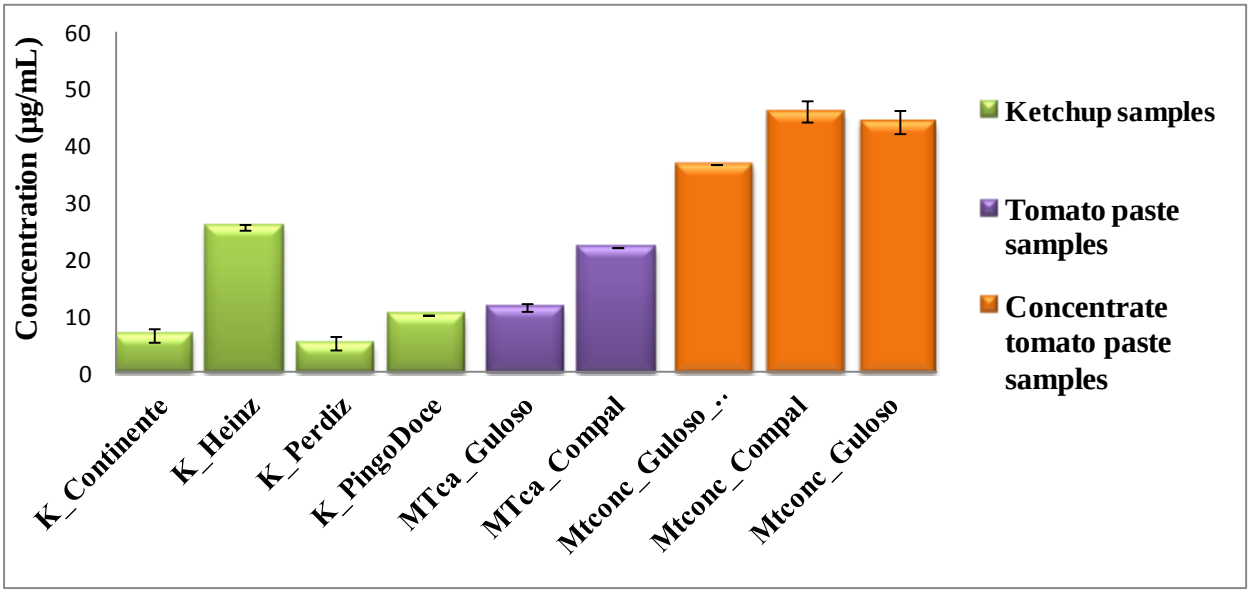


Figure 4.18. Total carotenoids content in processed tomatoes. In **green**, four different samples of ketchup samples: Continente, Heinz, Perdiz and Pingo Doce; In **purple**, two tomato paste samples: Guloso and Compal; In **orange**, three different concentrate tomato paste samples: Guloso from 850g can (Mtconc_Guloso_Lata), Compal (Mtconc_Compal) and Guloso 135g (Mtconc_Guloso).

4.4. Validation and Application of LL-USAE/UHPLC-PDA and LL-USAE/UHPLC-FLR Methods for Lipophilic and Hydrophilic Antioxidants

With the optimized conditions, LL-USAE/UHPLC-PDA and LL-USAE/UHPLC-FLR were submitted to validation and then were applied for determination of lipophilic (β-carotene, lycopene, α- and δ-tocopherol) and hydrophilic (L-AA) antioxidants in ripe tomato from *gordal* variety.

4.4.1. Validation of the LL-USAE/UHPLC-PDA and LL-USAE/UHPLC-FLR Analytical Methods

Validation design for LL-USAE/UHPLC-PDA and LL-USAE/UHPLC-FLR analytical methods were the same as for LL-USAE/UV-Vis: (*i*) selectivity, (*ii*) linearity, (*iii*) sensibility by LODs and LOQs, (*iv*) intra- and inter-day precision, (*v*) accuracy and (*vi*) matrix effect. Method evaluation for carotenoids was performed as described in 4.3.1., using tomato matrix without carotenoids.

Selectivity

The selectivity of the method was assessed by the absence of interfering peaks in the UHPLC-PDA chromatogram obtained at λ = 450 nm for carotenoids and λ = 245 nm for L-AA (Figures 5.19 and 5.20). [201, 207]. It was also observed the absence of interfering peaks in the UHPLC-FLR chromatogram obtained at λ_{Exc} = 296 nm and λ_{Em} = 330 nm for α- and δ-tocopherol (Figure 4.21).

Identification of chromatogram peaks of *gordal* tomato sample was performed through comparison with chromatogram of the standards β-carotene, lycopene, α- and δ-tocopherol, and L-AA (Figures 4.19 to 4.21).

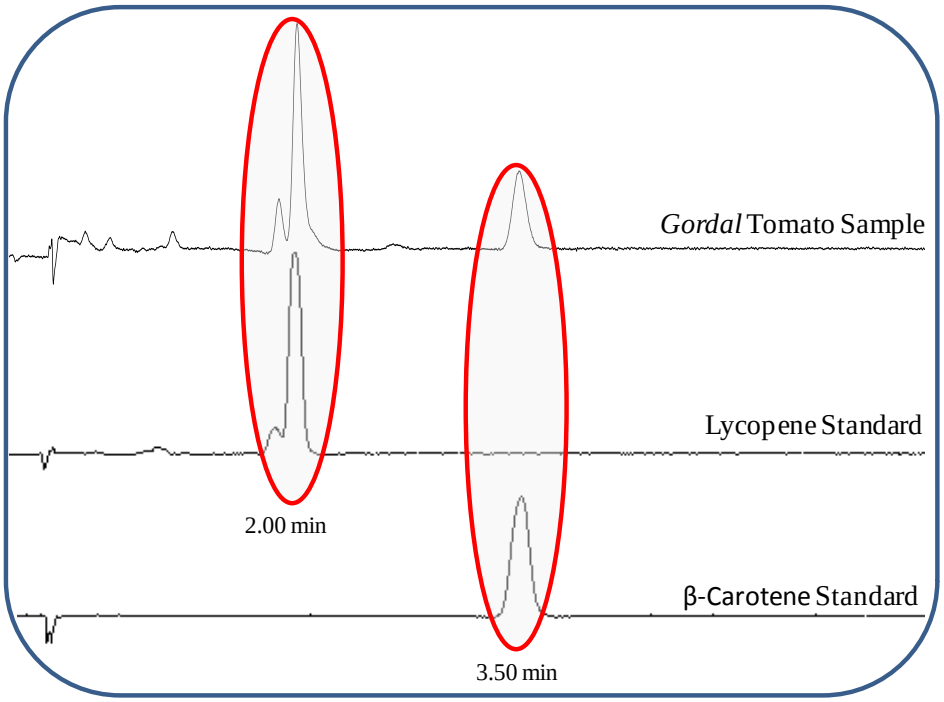


Figure 4.19. UHPLC-PDA chromatograms obtained at λ = 450 nm, for tomato sample, lycopene standard and β-carotene standard.

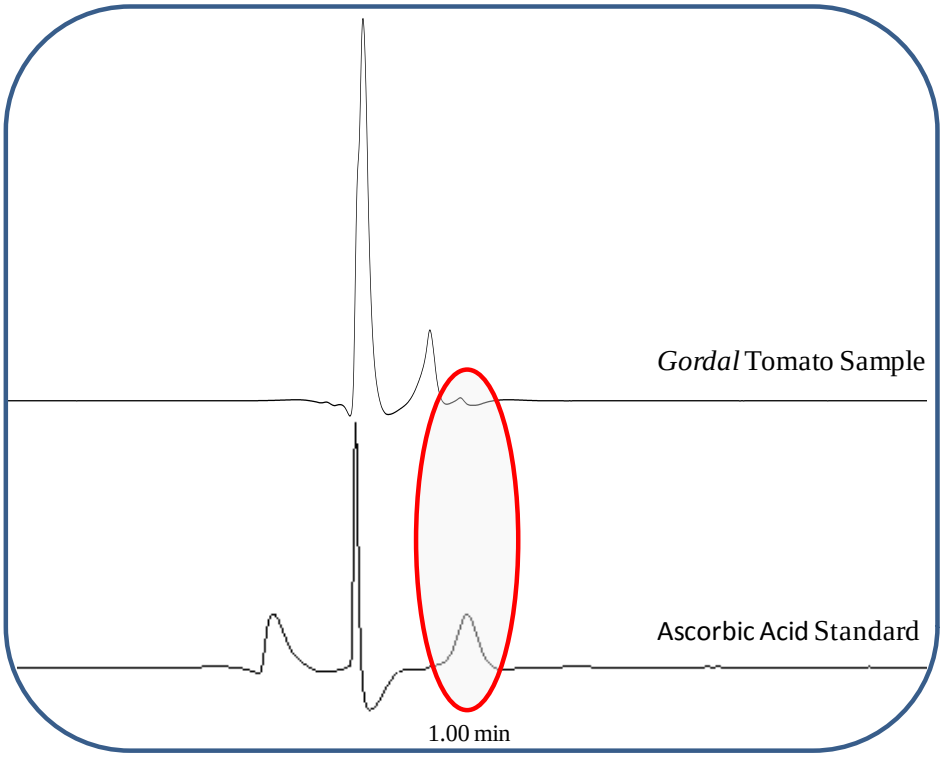


Figure 4.20. Typical UHPLC-PDA chromatograms obtained at λ = 245 nm, for tomato sample and L-AA standard.

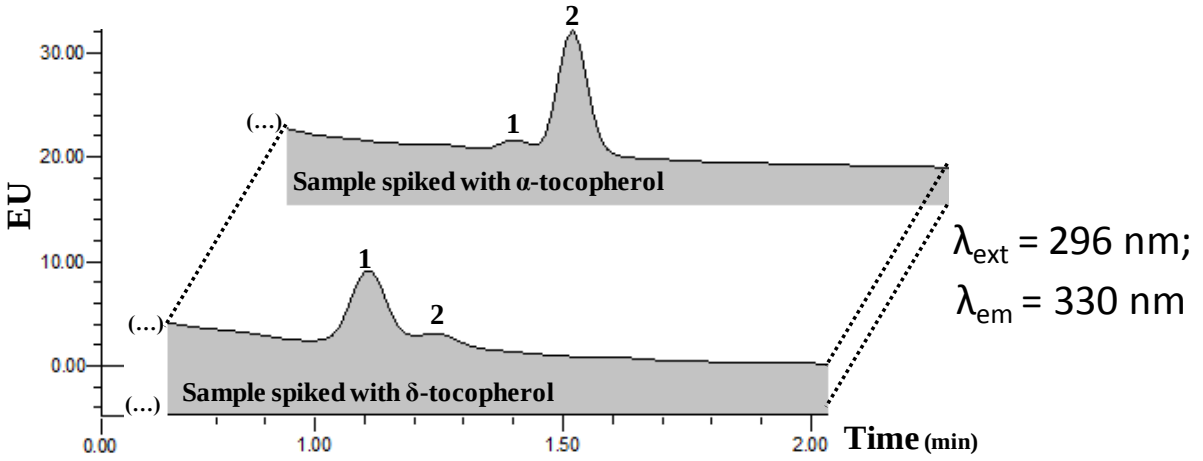


Figure 4.21. UHPLC-FLR chromatograms obtained at λ_{Exc} = 296 nm and λ_{Em} = 330 nm, for tomato sample, δ-tocopherol standard and α-tocopherol standard: 1 - δ-tocopherol; 2 - α-tocopherol..

The difference between PDA and FLR, results from the nature of the electronic transitions involved. Not only there is less substances able to fluorescence phenomena, but also FLR uses a specific wavelength excitation plus a specific wavelength emission. This higher selectivity results in a higher sensitivity, as can be seen for tocopherols. In this case, tocopherols peak areas are more than 100 times higher than carotenoids and L-AA peaks for the same concentrations (Figure 4.22). In Figure 4.21, we can see that the tocopherols peaks are highly specific both in the tocopherols standards and sample solutions analysed by FLR, presenting only the target peaks.

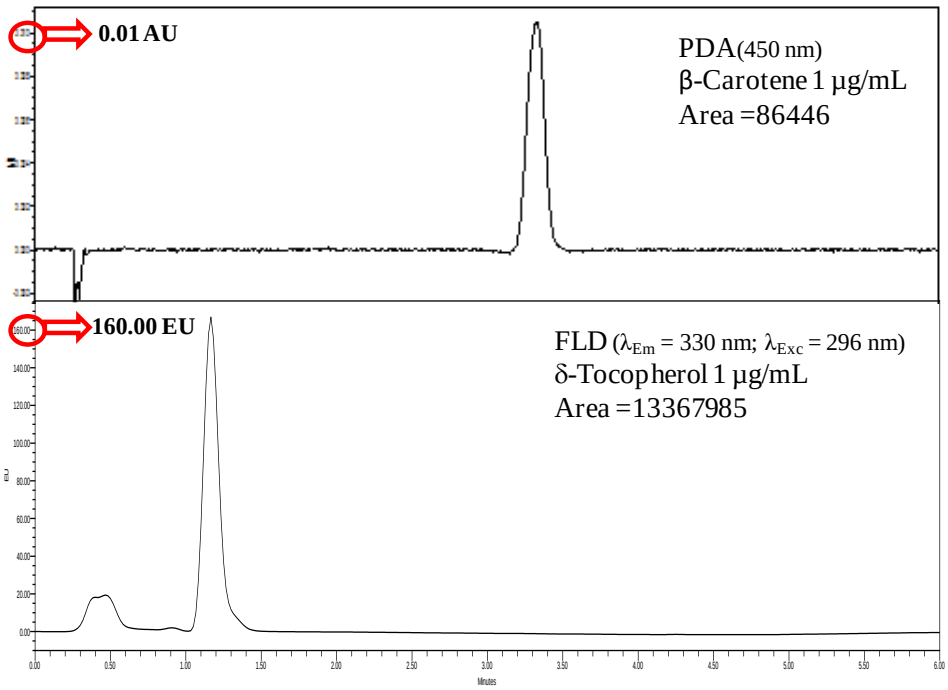


Figure 4.22. Typical chromatograms obtained with UHPLC-PDA for β-carotene, and with UHPLC-FLR for δ-tocopherol standards, with the same concentration (1 µg/mL).

Linearity

Linearity was evaluated by external standard addition method, using the analytes standards (β-carotene, lycopene, δ-tocopherol and α-tocopherol) linear regression (n=3), with 6 different concentrations (Table A5), and applying the least-squares method, to obtain the respective correlation coefficient (r^2) [201, 207]. For each standard, a calibration curve was obtained, as seen in Figure 4.23. From calibration curves, linearity was estimated through determination of correlation coefficient.

The method validation for L-AA, as referred before, was not completed, lacking the extraction and matrix effects of the target analyte. In Figure 4.23, the corresponded linear regression was performed with L-AA in solvent, giving the UHPLC capacity for L-AA determination but not the method LL-USAE/UHPLC-PDA capacity.

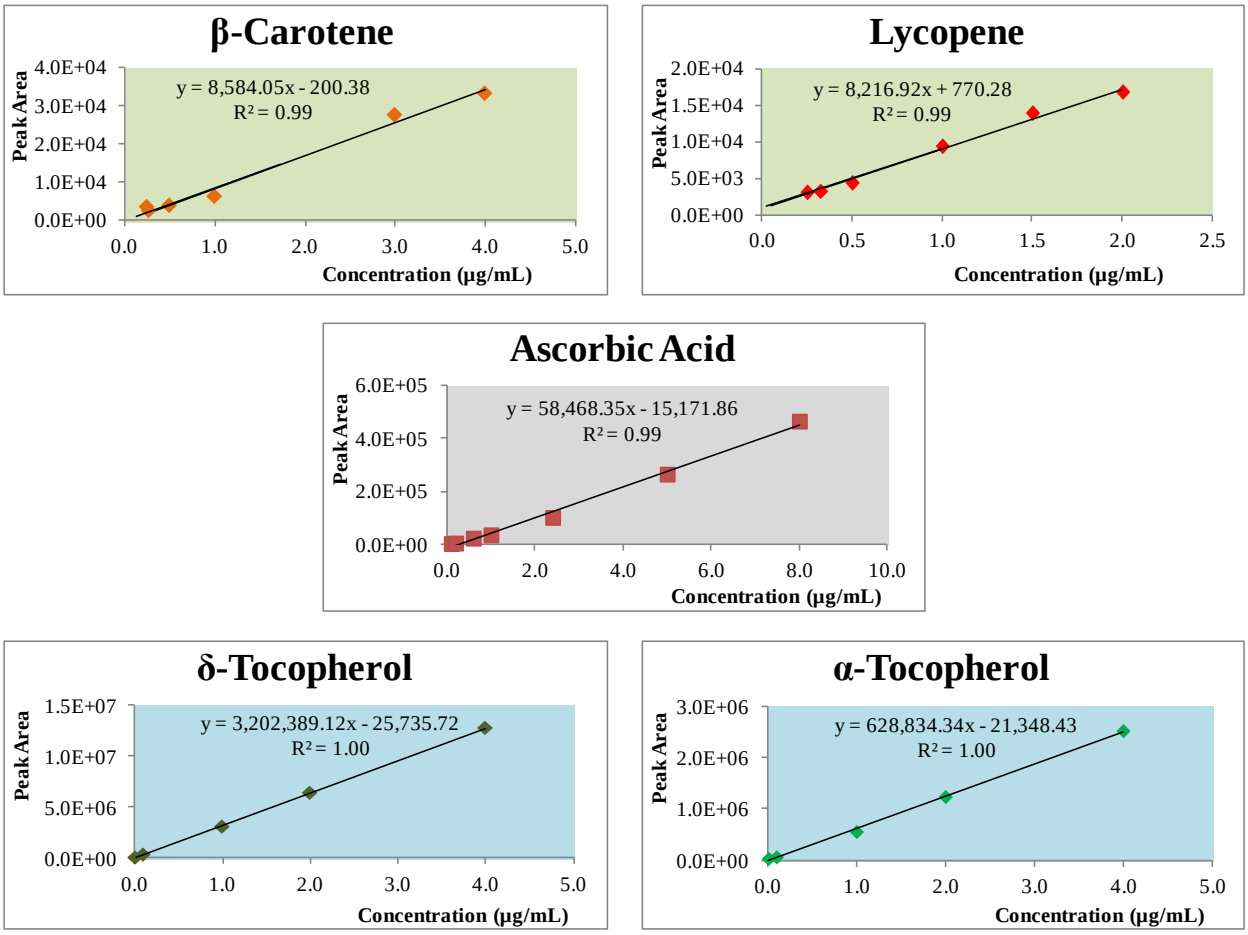


Figure 4.23. Calibration plots for lycopene, β-carotene, L-AA, α-tocopherol and δ-tocopherol. Results obtained by LL-USAE/UHPLC-PDA (carotenoids and L-AA) and LL-USAE/UHPLC-FLR (tocopherols).

Sensitivity

LOD and LOQ are summarized in Table 4.2. All standards present high linearity with correlation coefficient between 0.9854 and 0.9999. As expected, tocopherols present the lower LOD and LOQ, followed by β-carotene with similar values. The concentrations reported in literature for tomato samples [5, 115], but also in human serum studies [110, 206, 222, 223], are 10 times higher than the LOD and LOQ obtained in this work. This means that the UHPLC methodology here described can analyse both tocopherols and carotenoids concentrations about 10 times lower than those commonly analyzed.

Table 4.2. Linearity and sensitivity of the developed methods, LL-USAE/UHPLC-PDA and LL-USAE/UHPLC-FLR. LDR – limit range; r^2 – correlation coefficient; LOD – limits of detection; LOQ – limits of quantification.

				Linearity		Sensitivity	
Analytical Methods	**Antioxidants**	**Retention Time**	**λ (nm)**	**LDR** (μg/mL)	r^2	**LOD** (ng/mL)	**LOQ** (ng/mL)
LL-USAE/UHPLC-PDA	**L-AA***	1.0	245	0.01-8.0	0.9938	4.8	16.1
	Lycopene	2.0	450	0.25-2.0	0.9911	24.0	80.0
	β-Carotene	3.5		0.25-4.0	0.9854	3.0	9.9
LL-USAE/UHPLC-FLR	**δ -Tocopherol**	1.3	λ_{em}=330	0.01-4.0	0.9999	2.2	7.2
	α -Tocopherol	1.5	λ_{exc}=296	0.01-4.0	0.9987	1.7	5.6

* Standard linear regression effectuated in solvent, not by external standard addition

The evaluation of the extraction method for the target analytes, is summarized Figure 4.24. Differences between standard solution direct injection in UHPLC and standard solutions (in solvent) submitted to LL-USAE/UHPLC, varies between 2.9% for lycopene and 20.7% for β-carotene. It was observed that the target analytes β-carotene (-20.8%) and α-tocopherol (-5.1%) analysis are favoured by LL-USAE extraction procedure. Lycopene (2.9%) reveal a slight decrease, being L-AA (19.5%) the more negatively affected by LL-USAE.

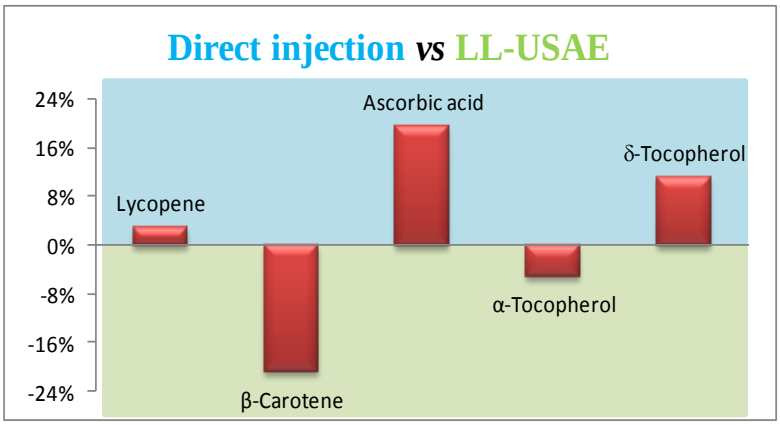


Figure 4.24. Comparison between direct injection and LL-USAE.

Precision

Precision was estimated for carotenoids and tocopherols (Table 4.3.). Repeatability was for all analytes lower than reproducibility, and both under reference limit of 20 % [215, 224], with exception of lycopene that presents reproducibility of 26.5 %, this may be explained by the high sensitivity of lycopene towards light and heat (as room temperature ± 22º C), but also towards water, namely the high humidity levels, typical of Madeira island.

Accuracy

For recovery percentage determination, Equation 9 was applied to tocopherols and Equation 10 for carotenoids. The results obtained (Table 4.3.) are within tolerance range [215], but with α-tocopherol presenting the lowest value, with 80.1 %.

Table 4.3. Precision, recovery and matrix effect of the developed methods, LL-USAE/UHPLC-PDA and –FLR, obtained in the methods validation.

Analyte	Spiking Levels (µg/mL)	Precision (%)		Recovery (%)[c]	Matrix Effect (%)[d]
		Intra-day[a]	Inter-day[b]		
Lycopene	0.6	13.4	16.5	92.8	98.5
	1.0	18.1	31.5	99.4	
	2.0	13.4	31.6	99.2	
	Average	15.0	26.5	97.1	
β-Carotene	0.5	5.8	12.1	118.5	98.6
	1.0	7.0	11.9	107.7	
	3.0	4.4	7.9	98.7	
	Average	5.7	10.6	108.3	
δ-Tocopherol	0.1	3.0	6.4	105.3	96.8
	1.0	2.1	5.8	105.3	
	4.0	2.1	4.9	97.0	
	Average	2.4	5.7	102.5	
α-Tocopherol	0.1	3.0	6.3	80.1	84.9
	1.0	2.2	6.1	79.5	
	4.0	1.9	5.8	85.2	
	Average	2.36	6.07	81.61	

[a] – n = 4 extractions in the same day at different concentrations: LL, ML and HL

[b] – n = 9 extractions in 3 not consecutive days at different concentrations: LL, ML and HL

[c] – Recoveries were calculated as described in section 3.6., with n = 3 at different concentrations: LL, ML and HL

[d] – Matrix effect was calculated as described in section 3.6.

Matrix Effect

Matrix effect percentage was determinated through tomato sample for tocopherols, and through tomato matrix (without carotenoids) for carotenoids. Tha matrix effect was within tolerance range [215, 225], and was low for all analytes presented with exception of α-tocopherol, that present 84.9 % of matrix effect, which can explain the low recovery value for α-tocopherol.

We can conclude from the analysis to validation parameters, that the methods LL-USAE/UHPLC-PDA and –FLR were successfully validated for tocopherols (δ- and α-tocopherol) and carotenoids (lycopenen and β-carotene) determination in tomatoes. The UPLC® breakdown not allowed the full validation of the method LL-USAE/UHPLC-PDA for L-AA, namely the influence of the matrix in the extraction of target analytes.

4.4.2. Application of LL-USAE/UHPLC-PDA and LL-USAE/UHPLC-FLR for Determination of Lipophilic and Hydrophilic Antioxidants

The identification of carotenoids and L-AA was achieved by comparison with their standards, and UV-Vis spectrum. Tocopherols were identified through comparison with standards. Figure 4.25. shows the three chromatograms of *gordal* tomato sample, where it is easily identified our five target analytes. In PDA at λ = 450 nm, we can see highlighted the peaks of carotenoids, first lycopene (retention time around 2 minutes), followed by β-carotene (retention time around 3.5 minutes). Still with PDA, but at λ = 245 nm, we can see highlighted L-AA (retention time around 1 minute) in the second chromatogram. Finally in the third chromatogram we can see highlighted the peaks of δ-tocopherol (retention time around 1.3 minutes) and α-tocopherol (retention time around 1.5 minutes).

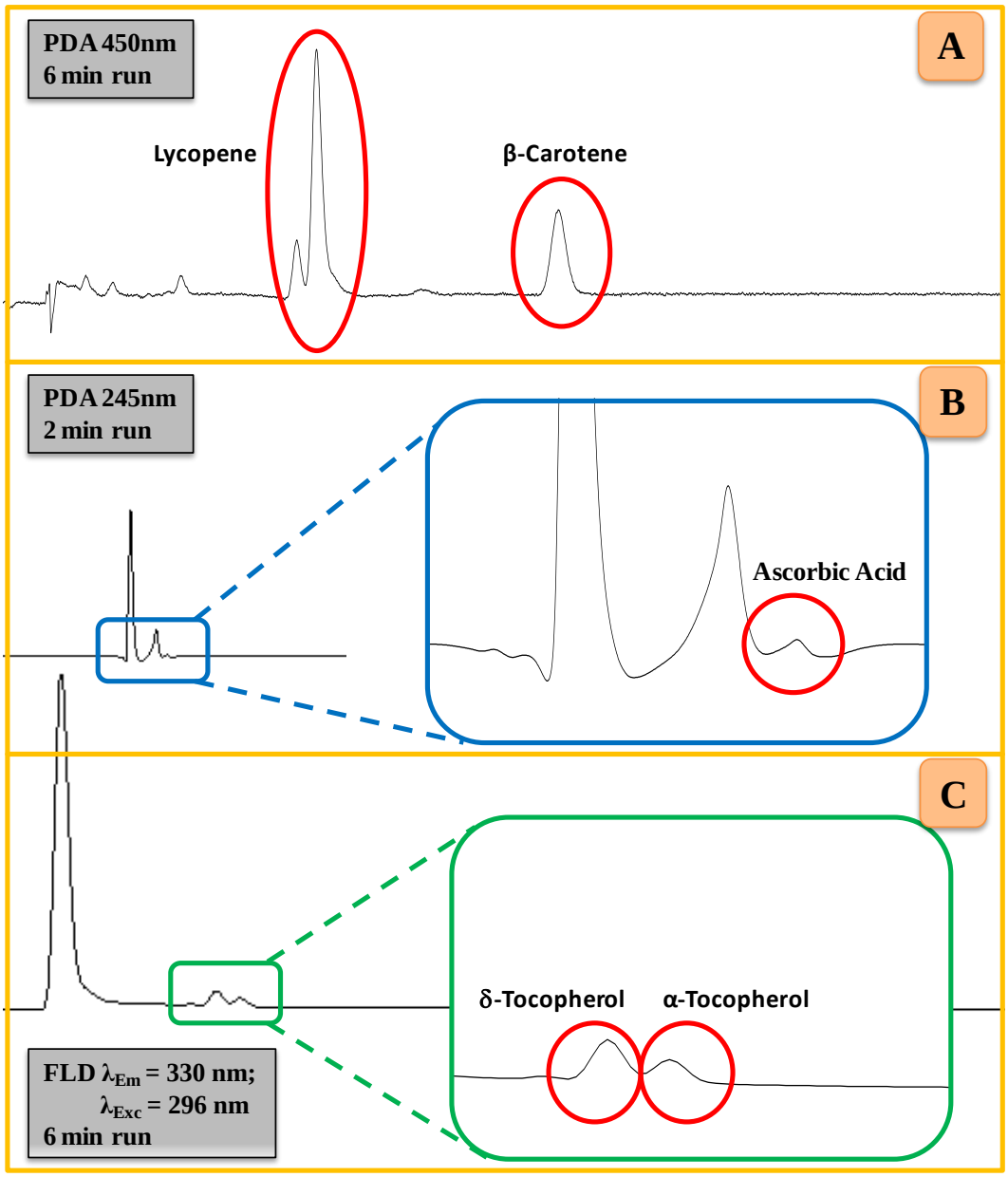


Figure 4.25. Chromatograms, of lipophilic and hydrophilic antioxidants from gordal tomato variety, obtained by: (i) LL-USAE/UHPLC-PDA – (**A)** λ = 450 nm – carotenoids; (**B)** λ = 245 nm – L-AA; (ii) LL-USAE/UHPLC-FLR - **C:** with λ_{Exc} = 296 nm and λ_{Em} = 330 nm – tocopherols.

The content of each antioxidant (tocopherols and carotenoids) found in the ripe tomato samples from *gordal* variety assayed are summarized in Table 4.4., where stands the low level of lycopene when compared with values found in literature for other tomatoes varieties. It was visible in filtration the presence of tomato skin particles, not being possible the recovery of the lycopene (where is found in highest concentration in the tomatoes), what can probably explain the low levels of lycopene. One solution for this problem can be the lyophilization of the sample before being mashed.

The highlight in the results was the unexpected presence and quantification of δ-tocopherol. The results obtained from the application of the methodology to tomatoes suggested that δ-tocopherol was found and quantified for the first time, as far we know.

Table 4.4. Content (μg/mL) of lipophilic (lycopene, β-carotene, δ-tocopherol and α-tocopherol) and hydrophilic (L-AA) antioxidants from tomato gordal variety. Comparison with values reported on literature for other tomato varieties.

	Lycopene	**β-Carotene**	**δ-Tocopherol**	**α-Tocopherol**	**L-AA**
Tomato$_{Gordal}$ (μg/mL)	25.0 ± 2.1	74.9 ± 2.1	7.18 ± 0.03	23.1 ± 3.5	7.8 ± 0.4*
Tomato$_{other\ varieties}$ (μg/mL)[5, 115]	186-1462	11-107	trace amount	11-184	220-2100

* Estimated by comparison with standard (in solvent) linear regression, and from 1 sample only.

CHAPTER V

CONCLUSIONS

In order to determine the lipophilic (lycopene, β-carotene, α-tocopherol and δ-tocopherol) and hydrophilic (L-AA) antioxidants in tomatoes and tomato foodstuff, extraction and analysis methods were develop.

eVol®MEPS extraction procedures optimization was shown not to be suitable for the following UHPLC injection. This was because the evaporation of hexane (the MEPS elution solvent), and subsequent resuspension in ACN/MeOH (2:1; v/v) solution (UHPLC mobile phase), was not reproducible, resulting in the loss of linearity. LL-USAE was selected as an alternative to MEPS extraction, and its optimization was performed successfully. The optimized UHPLC-PDA/FLR resulted in an initial 6 min of running time for lipophilic antioxidants determination (carotenoids and tocopherols), and 2 min for hydrophilic antioxidant determination (L-AA) in ripe tomato from *gordal* variety. UV-Vis was also optimized successfully in order to determine total carotenoids content in tomatoes and tomato foodstuff.

LL-USAE/UHPLC-PDA and -FLR analytical method validation was successfully carried out. LOD and LOQ were more than 10 times lower than antioxidants quantifications found in literature [5, 110, 115, 206, 222], with precision, accuracy and matrix effect presenting results under reference limits. Also the LL-USAE/UV-Vis used for total carotenoid content determination was performed, with LOD and LOQ obtained were more than 10 times lower than the reported in the literature [5, 110, 115, 206, 222].

The results obtained from the application of the methodology to tomatoes suggested that δ-tocopherol was found and quantified for the first time, as far we know. In literature there are no reports about quantification of δ-tocopherol in tomatoes, only trace amounts. LL-USAE-UV-Vis determination of total carotenoids during ripening of tomato from *gordal* variety shows an increase of carotenoid content, reaching its maximum concentration when ripe. A second study was performed with four different tomatoes varieties, at ripe stage, with results showing that *regional* variety presented the highest content of carotenoids, followed by *campari* and *gordal*, and for last *grape*. Processed tomatoes available commercially were also analysed, revelling higher concentration of carotenoids than in raw tomatoes, which probably results from the sample concentration in tomato processing.

TAP was study during ripening of tomato from *gordal* variety through ORAC and TBARS assays. Results showed an increase of antioxidant potential, even with an increase of LP. This antioxidant potential increase can be associated to the increase of antioxidants content, as reported in literature for L-AA, tocopherols and carotenoids [88, 217].

We can conclude, that LL-USAE-UV-Vis is a reliable method for total carotenoids evaluation in tomatoes, presenting LL-USAE/UHPLC-PDA even higher capacity for carotenoids determination. Additionally, it can be also used for others antioxidants determination (L-AA and

tocopherols), even at concentrations 10 times lower than those commonly analyzed in biological samples. This work is laying the foundations for future adaptation of the method (in particular LL-USAE/UHPLC-PDA and -FLR) to biological samples, and monitoring the presence of the antioxidants lycopene, β-carotene, α-tocopherol, δ-tocopherol and L-AA in these matrices. Tomatoes present antioxidant potential with its maximum at ripe stage, suggesting a positive correlation between antioxidants content and antioxidant potential. In order to verify this benefit, further studies will be needed, as example, an antioxidant-rich controlled diet followed for regular serum analysis, in order to follow antioxidants levels in serum, as well as LP biomarkers levels.

Future outlooks

MEPS can be applied only for L-AA, thus avoiding evaporation and resuspension that was the limitation founded in this work. QuEChERS, or derivative μ-QuEChERS can be applied to tocopherols, since exploratory studies (data not shown) reveal a non linear degradation of carotenoids, but for tocopherols the results were promising.

The antioxidants analysed in this work presented relative low values when compared with reports in literature, namely lycopene. A better protection to prevent the degradation of the antioxidants (light, temperature and water sensible) can be optimized. In addition, in sample preparation, the use of liophilization or a powerfull homogenizing device can result in a better homogenization of tomato sample and facilitate antioxidants recovery.

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APPENDIX FIGURES

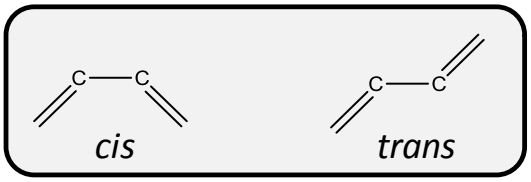

Figure A1. Isomerism around C=C double bonds, *cis / trans* configuration.

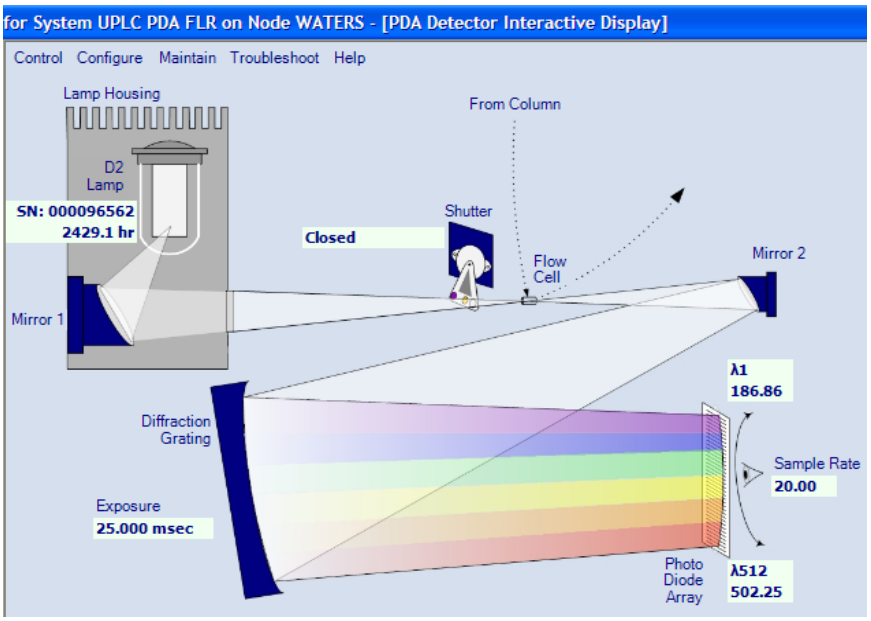

Figure A2. PDA function. Schematic of the WATERS PDA detector interactive display. (Figure withdrawal of Empower software v2.0 from Waters Corporation)

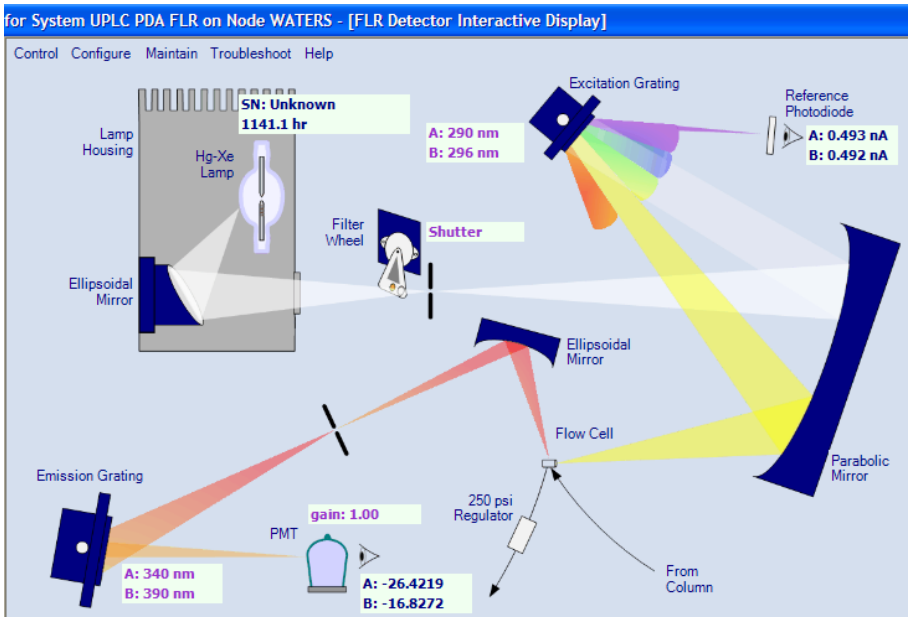

Figure A3. FLR function. Schematic of the WATERS FLR detector interactive display. (Figure withdrawal of Empower software v2.0 from Waters Corporation)

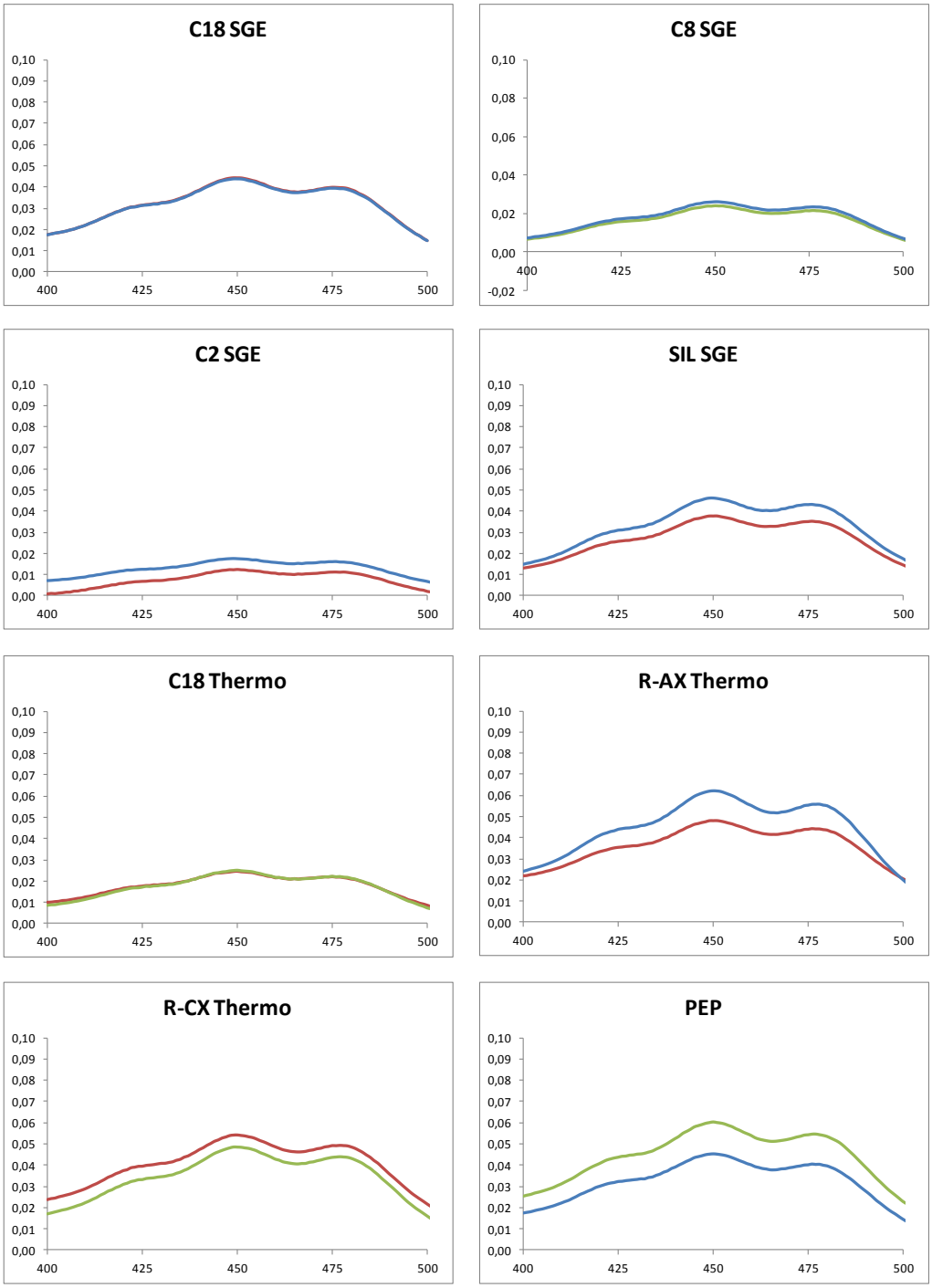

Figure A4. UV-Vis spectrum for carotenoids obtained by MEPS extraction (*n*=2) with different sorbents.

APPENDIX TABLES

Table A1. Physical proprieties of lycopene and β-carotene.

Compound		Lycopene	β-Carotene
CAS number		502-65-8	7235-40-7
Molecular weight (g/mol)		536.87	536.87
Storage conditions		protect from light and O_2; -70º C	protect from light and O_2; -20º C
Physical state at 25ºC		solid	solid
Solubility		insoluble in water, and can be dissolved only in organic solvents and oils	practically insoluble in water, and can be dissolved only in organic solvents and oils
Colour		dark red	deep orange
Melting point (ºC)		172 - 175	176 - 184
UV-Vis	λmax (nm)	472 (457, 485, 519) in hexane	450 (427, 450, 477) in hexane
	Molar extinction coefficient	184900 in hexane	139000 in hexane; 141000 in ethanol
Aromaticity		polyene chain (11 double bounds)	polyene chain (11 double bounds)

Table A2. Physical proprieties of α and δ-tocopherol.

Compound		α/δ-Tocopherol
CAS number		10191-41-0 / 119-13-1
Molecular weight (g/mol)		430.71
Storage conditions		protect from light; 2 - 8º C; air and moist sensitive
Physical state at 25º C		viscous oil
Solubility		Insoluble in water, freely soluble in ethanol, miscible with ether
Colour		dark yellow
Melting point (º C)		Boiling point: 200 - 220º C
UV-Vis	λmax (nm)	292 in absolute ethanol
	Molar extinction coefficient ($M^{-1}cm^{-1}$)	4059 in ethanol
Fluorescence		λ_{Em} 330 nm , λ_{Exc} 296 nm
Aromaticity		C6 aromatic ring,

Table A3. Physical proprieties of L-AA.

Compound		L-L-AA
CAS number		50-81-7
Molecular weight (g/mol)		176.12
Storage conditions		protect from light
Physical state at 25º C		solid
Solubility		water soluble
Colour		light yellow
Melting point (º C)		190 - 194
UV-Vis	λmax (nm)	247 in H_2O 0.1% F.A.
	Molar extinction coefficient ($M^{-1}cm^{-1}$)	8710 in water
Aromaticity		not aromatic

Table A4. Standard concentration used for linear regression, in LL-USAE/UV method validation.

Lycopene	β-Carotene
0.10	0.50
0.40	1.00
0.75	1.50
1.00	2.50
1.50	4.00
2.29	5.00

Table A5. Standard concentration used for linear regression, in LL-UHPLC-PDA/FLR method validation.

Lycopene	L-AA	β-Carotene	α-Tocopherol	δ-Tocopherol
0.25	0.10	0.25	0.01	0.01
0.40	0.60	0.50	0.10	0.10
0.60	1.00	1.00	0.50	0.50
1.00	2.40	2.00	1.00	1.00
1.50	5.00	3.00	2.00	2.00
2.00	8.00	4.00	4.00	4.00

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