Bárbara Vieira

Evaluation of *Sargassum muticum* as a source of vitamin K_1 with anti-inflammatory and vascular protective properties.

UAlg
UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e tecnologia

2022

Bárbara Vieira

Evaluation of *Sargassum muticum* as a source of vitamin K_1 with anti-inflammatory and vascular protective properties.

Master in Molecular and Microbial Biology.

Work under the supervision of:

Assistant Professor FCT-UAlg - Dina Costa Simes

Research Assistant UAlg-CCMAR - Carla Viegas

UAlg
UNIVERSIDADE DO ALGARVE
Faculdade de Ciências e Tecnologia

2022

Declaração de autoria

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída**.**

_____

(Bárbara Vieira)

Agradecimentos

Na etapa final desta jornada, há um número de pessoas a quem não poderia passar sem demonstrar o meu agradecimento, que m ajudaram no decorrer deste projeto e sinto-me bastante sortuda por ter tido esse privilégio.

Em primeiro lugar, queria agradecer à Professora Doutora Dina Simes, posso dizer que recebi imenso da sua parte vindo da sua sensatez e disposição que sempre teve para alargar o meu conhecimento, permitindo-me desenvolver as minhas competências e que realmente fico feliz por a ter tido como mentora e pela oportunidade que me deu de fazer parte deste projeto e pelo privilégio de ter estado num laboratório onde sempre me senti bem-vinda e desfrutei bastante do trabalho que lá desempenhei. O mesmo posso dizer da Doutora Carla Viegas, que graças à sua dedicação, paciência e auxílio mostrou-me que o laboratório é um sítio seguro para cometer erros e mais importante, evoluir a partir dos mesmos. Foi uma honra ter ambas como minhas orientadoras, e queria salientar que vos vejo como um exemplo a seguir.

Queria também agradecer às minhas colegas de laboratório, à Catarina pela bondade e suporte. À *Mestre* Joana, por me ter apoiado incondicionalmente tanto dentro como fora do laboratório e por me ter ensinado aptidões únicas que vou levar para a vida como uma boa "pipetagem inversa". À Inês à melhor parceira do HPLC de 1900 e troca o passo que tinha mais dias maus que bons, contigo aprendi a apreciar as pequenas vitórias. Também queria agradecer à Mélanie, que me ajudou a explorar e nutrir a minha admiração pelas algas.

À minha mãe, por todos os seus sacrifícios que fez para me proporcionar o melhor, ao meu texugo Matilde por ter sido tão compreensiva por não ter tido mais tempo para brincar com ela e por se preocupar tanto com a "tés" da mana. Ao Miguel, sinto-me grata por poder contar com o teu suporte e partilhar dos momentos bons aos maus contigo. À Sá a única pessoa que me tranquiliza relativamente aos receios e obstáculos enfrentados, já há muitos anos. À Paulinha a minha inspiração e orgulho por conseguir enfrentar todos os desafios.

Abstract

Age-related diseases are a health burden for our society, chronic inflammation, cardiovascular calcification, and oxidative stress are the main pillars of age-linked pathologies. So far there is no treatment for the pathologies mentioned, and the search for ways to prevent and alleviate the symptoms is high. Consumers often resort to dietary supplements to add nutrients that might be missing from the diet and benefit health. Natural diet supplement products obtained with eco-friendlier and more sustainable processes have increasing demand in the market.

Recently vitamin K has been considered a potential factor to relieve chronic inflammation and cardiovascular calcification. At the present, the diet supplement of vitamin K_1 (phylloquinone) available on the market is produced synthetically. In this work, the aim was to optimize K_1 extraction from a natural marine source, *Sargassum muticum*, an invasive macroalga present on the Portugal coast and to test the anti-inflammatory and antioxidant properties of the algae extract containing vitamin K_1

In this work, vitamin K_1 was extracted with a sustainable and innovative technique such as Energized dispersive energy extraction (EDGE). Vitamin K_1 was detected and quantified in several n-hexane extracts obtained with EDGE, using different conditions of temperature and initial biomass, and demonstrated that at 50ºC a higher yield of vitamin K_1 was obtained when using higher amounts of initial biomass weight.

The EDGE n-hexane extracts of *S. muticum* containing vitamin K1 were shown to have antioxidative properties by the DPPH assays and anti-inflammatory preventive activity, in the THP-1 macrophage cells inflammatory assay. EDGE *S. muticum* n-hexane extracts have also been shown to improve gamma-carboxylation of vitamin K-dependent proteins in vascular smooth muscle cells stimulated with TNF-α. The results obtained show the potential of *S. muticum* as a biomass source to obtain an extract rich in vitamin K_1 or to purifyvitamin K_1 to produce natural and sustainable diet supplements to help fight, chronic inflammation and oxidative stress.

Keywords words: Vitamin K_1;*Sargassum muticum*; Anti-inflammatory; γ-carboxylation; antioxidant.

Resumo

A inflamação crónica é uma patologia em que há sempre um nível base de inflamação a longo prazo que pode durar meses ou até anos. Esta é a origem de muitas doenças, como aterosclerose, osteoartrite, doenças cardiovasculares, diabetes, etc. As doenças inflamatórias crónicas são cada vez mais preocupantes visto que constituem uma das principais causas de mortalidade. A inflamação crónica é muito comum na população idosa, porque com o envelhecimento há um declínio do sistema imunitário. Neste momento não existe uma cura para a inflamação crónica, o que leva as pessoas a recorrer a anti-inflamatórios para aliviar os sintomas. Contudo a toma de inflamatórios de forma continuada causa efeitos secundários, como por exemplo a irritação gástrica, problemas hemorrágicos e hepáticos. Novas formas de prevenir as doenças crónicas inflamatórias são de necessidade urgente. Atualmente estudos mostram que vitamina K pode ter um papel fundamental em doenças crónicas inflamatórias. Vitamina K é uma família lipofílica cujo todos os compostos têm em comum um anel de naftoquinona (2-metil - 1,4- naftoquinona), e pode ser dividida em vitamina K_1 e em vitamina K_2. Vitamina K_1 também conhecida como filoquinona, é um aceitador de eletrões da fotossíntese, estando presente em plantas, algas e algumas cianobactérias. Esta vitamina tem uma cadeia lateral alifática na posição 3 no anel de naftoquinona. Vitamina K_2 é um grupo de menaquinonas com uma cadeia lateral não saturada de polyprenyl com um número variável de unidades de isopreno, de 4 a 13 unidades (MKn-1). As menaquinonas fazem parte da cadeia respiratória de microrganismos, como bactérias, fungos, etc. Exceto a menaquinona - 4 que é produzida nos humanos a partir da vitamina K_1. As menaquinonas estão presentes em alimentos fermentados e são produzidas na indústria através do processo de fermentação com bactérias da família *Bacillus subtilis*. Neste momento não há nenhum suplemento natural de vitamina K_1 no mercado, apenas sintéticos que têm um baixo rendimento, um alto custo de produção e métodos de produção pouco ambientalmente sustentáveis.

Neste momento recorre-se muito às algas, quer microalgas ou macroalgas para a produção biotecnológica de produtos naturais. Estas possuem bastantes vantagens, como uma taxa de crescimento rápido, uma grande adaptabilidade ambiental e uma produção mais sustentável. *Sargassum muticum* é uma alga invasiva com origem no Japão encontrando-se na costa portuguesa. Num estudo de 1991 esta alga foi descrita como tendo elevadas concentrações de vitamina K_1. Este projeto tem como objetivo otimizar a extração de

vitamina K_1 de *S. muticum* e testar as suas propriedades anti-inflamatórias e antioxidantes, assim como o efeito na γ-carboxilação. Para esse objetivo foram utilizadas técnicas inovadoras para a extração de vitamina K_1 marinha, como *Energized Dispersive Guided Extraction* (EDGE). EDGE é um método que se baseia na extração líquida pressurizada, automática onde se pode escolher facilmente as condições como o solvente, o seu volume final, número de ciclos e a temperatura de extração. Esta extração permite não só utilizar menos solvente como realizar extrações de forma rápida, em apenas alguns minutos. através do EDGE, com n-hexano como solvente de extração, obteve-se um valor máximo de 10.98 μg de vitamina K_1 marinha por grama de biomassa nas condições de três gramas de biomassa inicial, 5 ml de hexano por ciclo, 3 ciclos a 60ºC. No entanto a experiência nestas condições de temperatura e biomassa inicial foi realizada apenas uma vez e terá ser repetida para confirmação. Quando testada a atividade antioxidante com o ensaio 2,2-difenil-1-picrilhidrazil (DPPH), o extrato EDGE nas condições mencionadas anteriormente apresentou 62 ± 2.26 % de inibição a uma concentração de 5 mg/ml. No futuro, o teste antioxidante deveria ser realizado em testes *in vitro* com células, sendo que neste projeto foi apenas utilizado o teste DPPH que é um teste químico que verifica a capacidade do extrato da alga de captação de radicais livres.

Extratos analisados obtido a partir de EDGE nas condições de 1g de biomassa inicial a 50ºC apresentaram atividade anti-inflamatória, a 10 μg/ml diminuíram a concentração de IL-8 no meio em células THP-1 macrófagos estimuladas com LPS. Nas células também se demonstrou que as vitaminas K sintéticas (K_1 e MK_4) têm poder anti-inflamatório, na concentração de 10 μM, e mesmo em concentrações muito elevadas (500 μM) não apresentam toxicidade ou efeitos pró-inflamatórios.

Nas células vasculares lisas do músculo foi realizado um ensaio preliminar inflamatório preventivo, não se observou um efeito anti-inflamatório nas células estimuladas com TNF-α, contudo foi analisado o conteúdo de proteína total e detetados os resíduos Gla (γ-carboxiglutamato) por *Western Blot* com o anticorpo M3B que deteta de forma especifica a presença destes resíduos Gla em várias proteínas nomeadamente as proteínas dependentes da vitamina K (VKDP). No perfil proteico obtido verificou-se que os níveis de γ-carboxilação aumentavam quando as células eram pré-tratadas com vitamina K sintética (K_1 e MK_4) e da mesma forma com os extratos de *S. muticum* obtido com hexano no EDGE (nas condições de 1 grama de biomassa inicial a 50ºC). Contudo, mais ensaios

de imunodeteção serão necessários para identificar as proteínas dependentes de vitamina K no perfil obtido de Western blot com o anticorpo M3B e entender como os extratos e a vitamina K afetam o nível de γ-carboxilação em células em condições inflamatórias.

Os resultados deste projeto são promissores, pois além de mostrar a possibilidade de extração e purificação da vitamina K_1 de forma inovadora e sustentável, também mostram que o extrato obtido, rico em vitamina K_1, tem potencial de servir como um suplemento nutricional com propriedades anti-inflamatórias e antioxidantes. Este trabalho também mostrou que é possível utilizar como biomassa a *Sargassum muticum*, uma alga invasora, para obtenção de vitamina K_1 natural e com um baixo custo, possibilitando o desenvolvimento de um suplemento acessível ao consumidor.

Palavras chave: Vitamina K_1; *Sargassum muticum*, Anti-inflamatória; γ-carboxilação; Antioxidante.

List of tables

List of figures

Abreviations

AI	Adequate intake
BCA	Bicinchoninic acid
BGP	Bone Gla protein
BMP_2	Bone Morphogenetic Protein -2
cGRP	Carboxilated Gla rich protein
CKD	Chronic Kidney Disease
cOC	Carboxilated osteocalcin
CPPs	Circulating calcium particles
DMSO	Dimethyl sulfoxide
DPPH	1,1-diphenyl2-picryl hydrazyl
dpucMGP	Dephosphorylated undercarboxylated Matrix gla protein
DVR	Daily reference values
DXMT	Dexamethasone
EDGE	Energized dispersive guided extraction
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
FPG	Fasting plasma glucose
FSP1	Ferroptosis suppressor protein 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gas6	Growth Arrest Specific 6
GGCX	Gamma-glutamyl carboxylase
GLA	Gamma carboxylated Glutamic acid residues
GLU	Glutamic acid residues
GRP	Gla rich protein
HbA1c	Glycated haemoglobin
IL	Interleucine
LC-ESI-MS/MS	Liquid chromatography electrospray ionization tandem mass spectrometry
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LDL	Low density liproteins
LPS	Liposaccharide
MAE	Microwave-assisted extraction

MGP	Matrix Gla Protein
MK	Menaquinone
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
OC	Osteocalcin
OPN	Osteopontin
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline tween-20
PLE	Pressurized liquid extraction
PMA	Phorbol 12-myristate 13-acetate
PMS	Phenazine methosulphate
PS	Penicillin-streptomycin
RIPA	Radio immunoprecipitation assay
ROS	Reactive oxygen species
RP-HPLC	Reverse phase high pressure liquid chromatography
Runx2	Runt- related transcription factor 2
scCO₂	Supercritical fluid extraction with CO_2
SDS PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SPE	Solid phase extraction
T2DM	Diabetes mellitus type 2
TGF- β	Transforming Growth Factor betta
THP-1 Mac	THP-1 macrophages cells
TNF-α	Tumour necrosis alpha
UAE	Ultrasound-assisted extraction
ucGRP	Undercarboxylated Gla rich protein
ucma	Upper zone of growth plate and cartilage matrix associated
VC	Vascular calcification
VK	Quinone
VKDP	Vitamin K dependent Proteins
VKH2	Hydroquinone
VKO	Quinone epoxide
VSMCs	Vascular smooth muscle cells

1. Introduction

1.1 Vitamin K

1.1.1 Nomenclature and characterisation

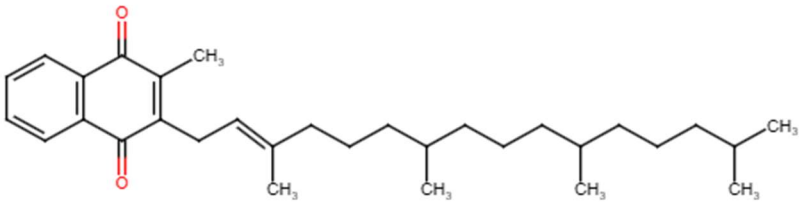
Vitamin K was found in 1929 by the biochemist Henrik Dam during an experiment regarding the chicken's metabolism [1]. This micronutrient was associated with coagulation since the lack of Vitamin K would lead the *in vivo* model (the chicken) to suffer internal haemorrhages [2]. In the next decade, the chemical structure of vitamin K was characterised as a lipophilic vitamin representing a family of naphthoquinones with a 2-methyl - 1,4-naphthoquinone as the common structure [3].

Vitamin K can be divided into two groups depending on the compound's side chain. It can be divided into vitamin K_1, also known as phylloquinone, and vitamin K_2, also known as menaquinones (MKn). Vitamin K_1 contains a phytyl side chain in position 3 of the naphthoquinone ring (2-methyl-3phytyl-1,4-naphthoquinone) (Figure 1) [3-5]. Phylloquinone is involved in the electron transport chain in photosynthesis, functioning as an electron acceptor from chlorophyll-a in photosystem I (PSI) [6]. As a product of oxygenic photosynthesis vitamin K_1 is present in plants, algae, and most cyanobacteria.

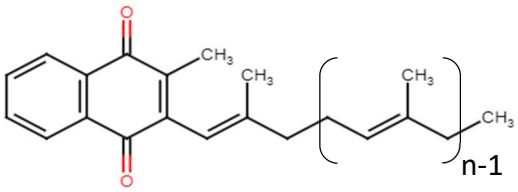
Vitamin K_2 is a group of components with an unsaturated isoprenyl side chain at position 3 of the 2-methyl-1,4 -naphthoquinone ring [7] (Figure 1). This side chain can vary in the number of isoprene units (n, MKn) from 4 to 13, classified as MK_4-MK_{13}. Vitamin K_2 is involved in the electron transport of the respiratory chain of microorganisms such as bacteria, archaea, and fungi, except for MK_4, which is metabolized from phylloquinone in humans [8]. Menadione known as Vitamin K_3 is a pro-vitamin, without a substituent at position 3 (reviewed by 9).

Vitamin K in humans is obtained mainly through our diet, being 90% vitamin K_1. Phylloquinone is found in a western diet in vegetables, oils and margarines. A high source of K_1 are kale, spinach and broccoli with 8.17, 3.87 and 1.56 μg of vitamin K_1 per g, respectively [10]. It has also been shown that even when the vegetables are boiled the concentration of vitamin K is maintained unaltered [11, 12]. Menaquinones are mainly found in dairy and fermented food such as natto (fermented soybeans) that contains 9,36 μg of MK per g [35]. Phylloquinones and menaquinones are very similar in bioactivity, however, there are differences in bioavailability. Phylloquinone is absorbed inefficiently compared to menaquinones, which have also a longer half-life contributing to higher

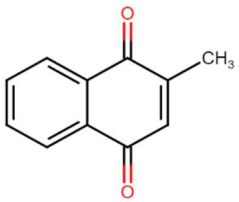
bioavailability. In a study vitamin K_1 and MK_4 were administrated and detected 8-24h after in the plasma, while menaquinones with longer side chains such as MK_7 and MK_9 were detected up to 96h after administration [81,82]. Lipophilic compounds such as vitamin K need to be transported by other proteins to the organ and tissues where they are metabolized. While phylloquinone is transported by lipoproteins rich in triacylglycerols, menaquinones are transported to the liver by the same transport proteins but are distributed by low density lipoproteins (LDL), which is not the case for vitamin K_1 [13]. Tissue distribution of vitamin K also differ between vitamin K_1 and menaquinones. Vitamin K_1 is mainly found in the liver, heart and pancreas, while menaquinones with longer side chains are found mainly in the liver. [14].



Vitamin K_1 (Phylloquinone)



Vitamin K2 (Menaquinone)



Vitamin K3 (Menadione)

Figure 1: Chemical structures of Vitamin K.

1.1.2 Vitamin K dependent proteins

Vitamin K is a co-factor for the enzyme Gamma-Glutamyl Carboxylase (GGCX), which catalyses de post-translation modification of specific glutamic acid residues (Glu) into gamma-carboxyglutamate residues (Gla), in a process designated as γ-carboxylation. This post-translation reaction allows Vitamin K Dependent Proteins (VKDP) to became biologically active [15]. Although in nature Vitamin K exists mainly in an oxidized form (quinone, VK), GGCX uses the reduced form (VKH_2, hydroquinone) as a co-factor. In the organism, VKH_2 is obtained from the reduction of quinone by glutathione and/or NADP(H) epoxide reductases ensuring its continuous recycling. During the process of γ-carboxylation, the hydroquinone is simultaneously converted into the VKO (epoxide) form. This is possible because GGCX recognizes a domain conserved in all VKDP

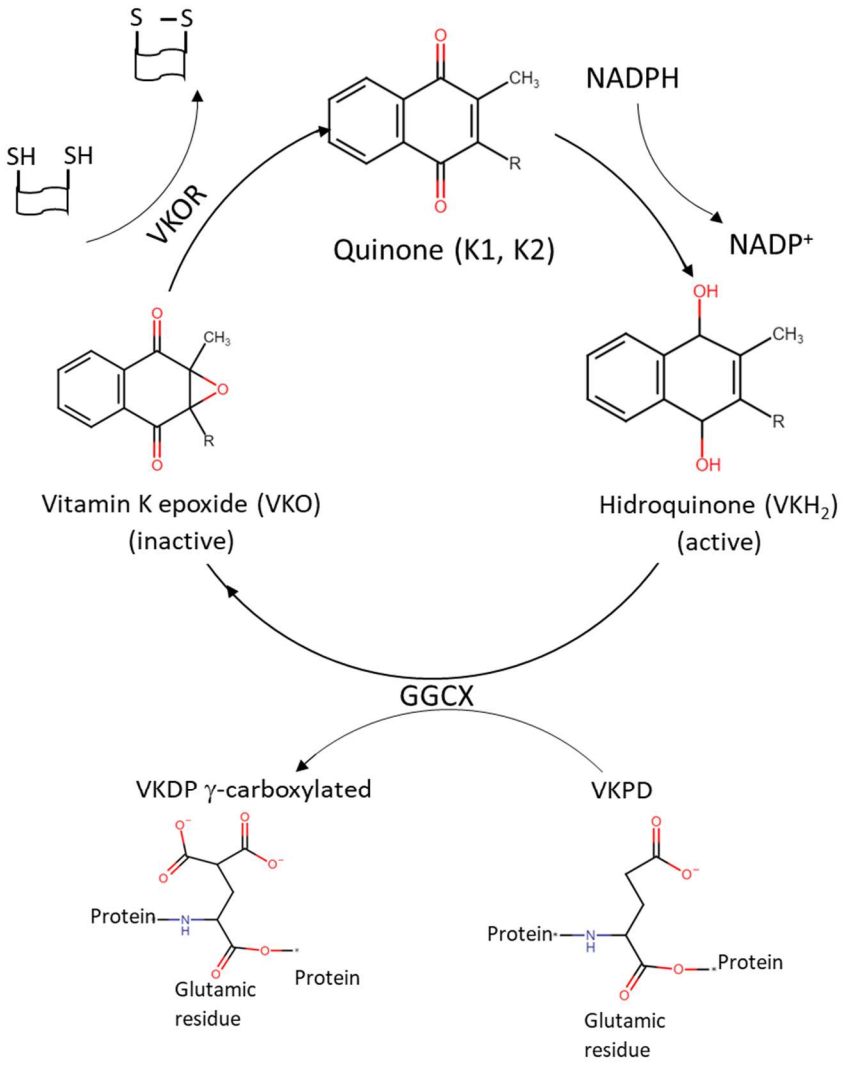


Figure 2: Vitamin K γ-carboxylation cycle. Gamma Glutamyl Carboxylase, GGCX, Vitamin K dependent Protein, VKDP. Vitamin K epoxide reductase, VKOR.

localized in the propeptide, with the exception of matrix gla protein (MGP), where the propeptide sequence is part of the mature protein [16, 17].

The firsts VKDP found were proteins involved in coagulation, such as prothrombin [18, 19], clotting factors FII, FVII, FIX, and FX and anticoagulants such as protein S, protein C, and protein Z [20]. All the proteins mentioned are produced in the liver and help the normal functioning of coagulation; in healthy adults, the level of γ-carboxylation of VKDPs is usually stable, since the liver can efficiently store vitamin K from circulation ensuring the process of protein γ-carboxylation. The Gla residues as part of the Gla domain of coagulation factors mediate the binding of calcium ions and are critical for their interaction with the membrane phospholipids [21]. More proteins dependent on vitamin K have been found in recent years, with diverse functions. These proteins are extra-hepatic and represent functions besides coagulation, such as calcification, either physiological or pathological, inflammation, neuroprotection and many more. Several extra-hepatic vitamin K-dependent proteins are circulating proteins and usually, even in healthy individuals, are not fully γ-carboxylated [22].

1.2.3 Vitamin K dependent Proteins – mineral related Gla proteins

Osteocalcin (OC), also known as Bone Gla Protein (BGP) is a small protein with 3 to 4 Gla residues (depending on the species). The protein found in 1976, is the most abundant non-collagenous protein of the bone being highly involved in physiologic calcification [23, 24]. Physiological calcification is the process that leads to bone and teeth formation, through mineralisation. BGP is produced by osteoblast, and when γ-carboxylated shows high affinity to calcium ions and calcium phosphate mineral in the form of hydroxyapatite. Osteocalcin is thought to be involved in the regulation of bone mineralization and the regulation of osteoblast and osteoclast [25, 26]. However, there is still no agreement on whether osteocalcin affects bone quality and/or quantity. *Karsenty al.et* showed in the knockout BGP mice that bone formation increased the number of osteoclasts [27]. Contrarily, recent studies also with BGP knockout mice, showed that the quantity of the bone was not altered compared with wild-type mice, nevertheless it was observed a disruption in the orientation of the biological apatite crystals in the bone, reducing its strength [28, 29].

A distinct extra-hepatic VKDP is Matrix Gla protein (MGP) with 4 to 5 Gla residues (depending on the specie), MGP suffers several post-translation modifications, such as γ-carboxylation and serine phosphorylation [30]. The protein is found in the bone's extracellular matrix, cartilage, and calcified tissues [31], and is produced by hypertrophic chondrocytes and vascular smooth muscle cells (VSMCs). The secreted protein presents an affinity to calcium phosphate, it is highly involved in calcification, and is considered as one of the main pathological calcification inhibitors [32, 33 - 34]. Gla rich protein (GRP), also known as upper zone growth plate and cartilage matrix associated protein (ucma), is the newest member of the VKDP family, with the highest number of Gla residues, relative to the size of the mature protein. It has 15 Gla residues in humans within a sequence of 74 amino acid residues of the mature protein [35, 36]. This protein shows excellent affinity for calcium ions and calcium phosphate mineral and has been described to have a role as an inhibitor of pathological calcification in the articular and vascular system [37-42]. Recently, many studies also revealed an anti-inflammatory role of GRP independently of its gamma-carboxylation status [40, 43-45].

Other extrahepatic VKDPs such as gas6 and periostin are involved in other cell mechanisms, such as proliferation regulation, hemostasis, neuroprotection, and cardiac development [46-49].

Table 1 Extra-hepatic vitamin K-dependent proteins and their functions.

Proteins	Function	References
Osteocalcin (OC), Bone Gla Protein (BGP)	Regulation of bone formation, glucose metabolism, and testosterone synthesis	[27, 29, 50, 51]
Matrix Gla Protein (MGP)	Inhibition of pathological calcification, bone health regulation	[30, 31, 33]
Gla Rich Protein (GRP) or upper zone growth plate and cartilage matrix associated protein. (UCMA)	Inhibition of pathological calcification, anti-inflammatory	[40-42, 52]
Growth arrest-specific protein-6 (Gas6)	Regulation of proliferation, signal transduction, anti-inflammatory	[47, 53, 54]
Periostin	Regulator of proliferation, differentiation, and bone growth	[55-57]
Proline rich Gla protein-1/2 (PRGP)	Signal transduction	[58]
Transmembrane Gla protein 4 (TGP)	Signal transduction	[59]

1.2 Vitamin K role in age-related diseases

With the advances in the medicine and technology life expectancy is increasing, but unfortunately the health span does not follow this tendency. Aging is a complex process that is a risk factor for numerous diseases [60]. Recently, vitamin K has shown to be beneficial in age-related diseases. In this thesis it will be approached pathologies linked to age-related diseases such osteoporosis, cardiovascular calcification, and chronic inflammation, and how vitamin K and VKDPs can help prevent these pathologies common in ageing population.

1.2.1 Bone health

The bone is formed through physiological calcification and relies on bone cells such as osteoblasts, osteoclasts, and osteocytes. Osteoblasts are responsible for synthesizing extracellular matrix that later will be mineralized. Osteoclasts resorb the bone; they solubilize calcium phosphate mineral phase and break down the extracellular bone matrix. Chondrocytes constitute the cartilage and are fundamental for bone development and repair. Bone maintenance is a balance between bone formation and bone degradation performed by the osteoblasts and the osteoclasts, respectively. When there are alterations in this balance severe pathologies can occur, one of the most common is osteoporosis, a degenerative chronic disease that impacts the skeleton health, that is highly prevalent in the women over 50-year-old and can lead to physical incapacity.

Vitamin K is an essential vitamin in bone health and has been associated with osteoporosis. In a clinical study the consumption of low doses of vitamin K (MK-7) in the concentrations of 180 μg/day for 3 years in postmenopausal women, was shown to prevent osteoporosis with an increase in bone mineralization [61]. *Mandatori* et al. showed in a 2D model obtained from a co-culture of bone fragments and peripheral blood samples, an increase in the mineral matrix deposition and in the osteogenic markers, when the 2D system was treated with MK4 [62]. The vitamin K-dependent protein GRP has also showed to have chondroprotective properties. In a study with GRP knockout mice with a medial meniscus destabilization provoked surgically, the mice developed a severe osteoarthritis phenotype [63].

In most bone health studies, a common circulating biomarker linked to bone health is γ-carboxylated osteocalcin (cOC), that show a positive association between levels of cOC and increased bone metabolism. There are several studies that support a positive

relationship between vitamin K and cOC, where supplementation of vitamin K either K_1 or K_2 increases the levels of circulating cOC. However, studies that rely only on K_1 supplementation are outdated and are in a low quantity compared with studies associated vitamin K_2 [64-69].

1.2.2 Vascular calcification

Calcification can be a pathological process; this happens when soft tissues are mineralized, with deposition of calcium-phosphate mineral, usually due to an unbalance between calcification promoters and calcification inhibitors. When it occurs in the vasculature it leads to several cardiovascular pathologies

Vascular calcification (VC) can be categorized into two groups intimal and medial calcification, according to the local of calcification. Intimal calcification occurs in the intima and it is mainly associated with lipidic deposits (reviewed in reference 70). Intimal calcification is a highly complex inflammatory disease, accompanied high levels of pro-inflammatory cytokines, immune cells infiltration and engulfment of the lipidic deposits [71, 72]. With the progressive accumulation of lipids, mineralization, and infiltration of monocytes/macrophages in the atherosclerotic plaques, there is a reduction of the lumen's diameter, causing a burden to the vessel. Atherosclerotic plaques can become very unstable, and the rupture of the plaques can lead to vessel lumen obstruction and a cardiovascular event (see figure 3). Medial calcification is independent of atherosclerosis since it occurs in the absence of lipids deposition. Calcification occurs in the medial layer mainly constituted by VSMCs, and an extracellular matrix rich in elastin/fibrillin, collagen and proteoglycans. This calcification is more related to diastolic dysfunction also known as stiffness. The Medial calcification is associated with ageing and is highly prevalent in other diseases such as chronic kidney disease (CKD), diabetes mellitus and osteoporosis.

Vascular calcification is a highly complex process, where vascular smooth muscle cells (VSMCs) have a crucial role. For VC to happen VSMC must suffer osteochondrogenic trans differentiation, these cells have a different phenotype expressing different calcification mediators, such as osteocalcin and Runt- related transcription factor 2 (Runx2) [73, 74]. Another key factor, on VC is the VSMCs the decrease in calcification inhibitors production [75].

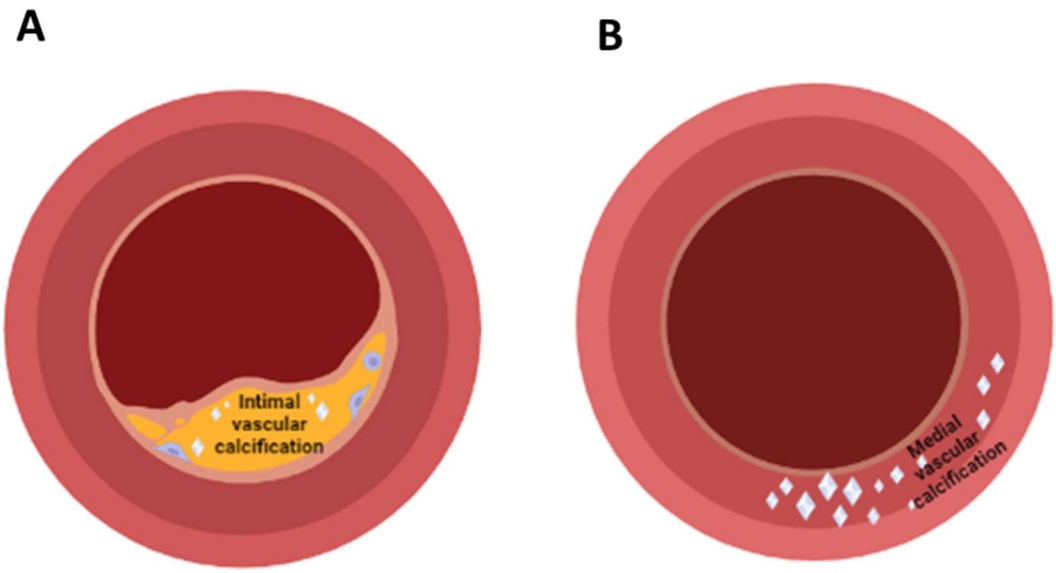


Figure 3: Cardiovascular calcification. (A) intimal calcification. (B) Medial calcification.

In our organism phosphate and calcium are always present, however it does not instantly precipitate, this is mainly due to the presence of calcification inhibitors. One of the inhibitors associated with vitamin K is MGP. *In vitro* assays have shown that MGP accumulates in the extracellular matrix of calcified human and rat arteries [31]. Luo et al. revealed extensive calcification in soft tissues and a late development in knockout mice for MGP compared to the wild-type mice [76]. This process seems to be dependent of γ-carboxylation. In another study, warfarin was administrated to mice and calcification in soft tissues was observed [77]. Additionally, a study with transgenic mice where MGP could not be γ-carboxylated, showed a similar phenotype as the MGP-KO mice [78]. As a result of the importance of the post translation modifications of MGP, dephosphorylated undercarboxylated MGP (dpucMGP) has been widely used in clinical studies, and considered as a biomarker for vitamin K status. High concentrations of dp-ucMGP have been reported to be linked with increased risk of cardiovascular diseases and all-cause mortality, and lower dp-ucMGP levels lead to a lower risk of atrial fibrillation [79, 80].

Matrix Gla Protein has a high affinity for calcium ions and for hydroxyapatite crystals, and function as an inhibitor of pathological calcification. MGP also interacts with other bone proteins such as BMP_2 (Bone Morphogenetic Protein -2) which is an osteogenic

protein that promotes bone formation, and that has also been implicated in vascular calcification [81, 82]. Inhibition of BMP-2 osteogenic differentiation function through BMP-2-MGP interaction is reported to be dependent on the presence of Gla residues, highlighting again the importance of gamma-carboxylation for MGP function.

Gla rich protein is also considered a pathological calcification inhibitor, GRP interacts with calcium ions and calcium phosphate crystals with high affinity and is reported to be able to inhibit the maturation of calcium phosphate crystals, as part of the circulating calciprotein particles (CPPs) [41]. Both forms of GRP, either gamma-carboxylated (cGRP) and uncarboxylated (ucGRP) can interact with calcium phosphate mineral particles and are present in both healthy and pathological tissues. However accumulation of ucGRP has been associated to pathological tissues namely in cancer, osteoarthritis and cardiovascular disease [30, 83 - 85]. A study with VSMCs obtained from GRP knock out mice, exposed to calcifying conditions showed an increase in mineralization and expression of osteochondrogenic markers [42, 86]. The γ-carboxylation status of GRP has been shown to be very important for its function as an inhibitor of ectopic calcification. In an *ex vivo* study with human aortic fragments, tissue mineralization was inhibited by treatments with cGRP but not ucGRP, by decreasing VSMCs osteochondrogenic differentiation [86].

Several clinical studies show that vitamin K might be the key to prevent cardiovascular diseases. Indeed, it was reported that higher intake of Vitamin K_1 reduces cardiovascular disease mortality [87]. Furthermore, menaquinone (MK_4 through MK_{10}) intake was shown to be inversely linked to aortic calcification, nevertheless in the same study it was not found any relationship with the vitamin K_1 intake [88]. Contrarily, one study suggested that high concentrations of vitamin K_1 is related with higher prevalence of coronary artery calcification [89]. However, we need to consider on how the different vitamin K vitamers are transported and stored in the organism. Vitamin K is a lipophilic vitamin, and it is normally stored in the liver. When it is in circulation, phylloquinone is transported by low density lipoproteins (LDL) like triglycerides that are present in the atherosclerotic plaque interfering with phylloquinone quantification[90]. Quantification of vitamin K in clinical studies should consider the low half-life of vitamin K, and be careful when collecting blood samples with particular attention to the time frame after vitamin K_1 consumption.

Vitamin K_1 was shown to decrease the formation of crystals in the kidney of mice, and in human kidney cells (HK2) treated with calcium oxalate monohydrate crystals [91]. Matrix Gla Protein (MGP) expression was quantified both *in vivo* and *in vitro and* demonstrated that when the models were exposed to Vitamin K_1 MGP expression levels increased. Vitamin K_1 is now being seen as a potential therapy or attenuator for kidney failure and transplant patients [92]. Vitamin K seems to affect the kidney despite calcification, since it has a metabolic role. In a randomized clinical trial with a placebo group, patients with diabetes who were insulin-independent received a controlled concentration of MK_7. After 12 weeks, parameters such as fasting plasma glucose (FPG), and glycated haemoglobin (HbA1c) decreased when compared to the placebo group [93]. It was also shown that both menaquinone and phylloquinone intake were inversely associated with diabetes mellitus type 2 (T2DM) incidence [94].

1.2.3 Chronic inflammation

Inflammation is a process to fight any pathogen in the organism with the involvement of immune cells, like macrophages, monocytes and neutrophiles, and the release of factors, such as cytokines. Inflammation is a fundamental protection process, however when dysregulated can lead to chronic inflammation. Chronic inflammation is highly related to aging or age-related diseases, which led to the new concept of "inflammaging". As people get older, there is a decline of the immune system and a stable low level of inflammation considered chronic inflammation [95]. Chronic inflammation is believed to be the pilar of innumerable diseases, such cardiovascular diseases, osteoporosis, diabetes, rheumatoid arthritis, neurodegenerative diseases, cancer, etc. Recently, COVID-19 severity and mortality were associated with *inflammaging* because it increases the inflammatory response and affect primarily the elderly [96, 97].

Chronic inflammation does not have a cure, which leads to overconsumption of anti-inflammatory medicines to alleviate the symptoms. However, long term consumption of anti-inflammatory drugs is harmful, it can lead to gastric irritation and haemorrhage aggravation, highlighting the need for new products to prevent and ease chronic inflammation diseases.

Currently, biomarkers used to analyse *inflammaging* include tumour necrosis factor α- (TNF-α) and several interleukins such IL-1, IL-6 and IL-8; these cytokines are known to be master players in inflammation. Most cytokines studied are pro-inflammatory,

meaning they enhance and induce inflammation. However other cytokines like IL-10 are anti-inflammatory with a role in decreasing/resolving inflammation [98].

I*n vitro* studies have shown that vitamin K reduces pro-inflammatory cytokines in macrophages-like cells and stimulated with lipopolysaccharide (LPS) [99]. Pre-treatments with vitamin K_1 and several menaquinones, MK_4 and MK_7, showed an anti-inflammatory effect in macrophage and lymphocyte cells (by decreasing level of several pro-inflammatory cytokines such TNF-α IL-1β, and IL-6 [99-101]. Although the involvement of vitamin K in COVID-19 is being explored, two different cohort studies reported that low levels of vitamin K are associated with higher mortality risk in COVID-19 [102, 103].

GRP has been shown to have anti-inflammatory properties independent of its γ-carboxylation status, and to reduced pro-inflammatory markers in several different human cells, such as VSMCs, chondrocytes, synoviocytes, and macrophages [43, 45]. THP-1 macrophage cells overexpressing GRP, and induced to inflame with hydroxyapatite (HA) and lipopolysaccharide (LPS), were shown to downregulate pro-inflammatory mediators, demonstrating its anti-inflammatory potential [40].

Overall, many available basic research and clinical information highlights the therapeutic effect that vitamin K dependent proteins can have in inflammation, and recent studies proposed nano-formulations as a way to safely deliver GRP, such as encapsulation with chitosan nanoparticles [45].

1.2.4 Oxidative stress

Oxidation at the cell level is a physiological process where very unstable free radicals or reactive oxygen species (ROS) are formed and can provoke several damages. The human organism is in a balance between oxidation and antioxidation. Antioxidants, also known as free radical scavengers, are capable of downregulate oxidation of a substrate by stabilizing or eliminating ROS. Oxidative stress happens when there is unbalance and overproduction of free radicals or/and suppression of ROS elimination (review in [104]).Oxidative stress is linked with aging and most of age-related diseases, as well as chronic inflammatory diseases, since extended periods of oxidative stress is an inflammatory factor (review in [104]).

Vitamin K is a powerful antioxidant agent, but only in the reduced form (VKH_2), an *in vitro* study showed that hydroquinone is a great lipid peroxidation inhibitor [105]. Additionally, a more recent study analysed the impact of vitamin K (menaquinone 4 and phylloquinone) in lipid peroxidation and ferroptosis. It was found that ferroptosis suppressor protein 1 (FSP1) reduce vitamin K and this will inhibit lipid peroxidation [106]. More interesting, vitamin K_1 and MK_4 were shown to drastically prevent the ROS accumulation and cell death of *in vitro* oligodendrocytes [107, 108]. However, additional studies are required to establish a solid base for vitamin K as an antioxidant and its mechanism of action, particularly in different cells and systems. Antioxidant products are very popular in the market and with the consumers choosing more natural sourced options.

1.3 Vitamin K diet supplementation

1.3.1 Vitamin K adequate consumption

The daily reference values (DRV) and adequate intake (AI) of vitamin K_1 and vitamin K_2 is still not well established, due to insufficient evidence published [109]. There are daily recommended dosage of total vitamin K, not discriminating between phylloquinone and menaquinones, and these recommendations change significantly according to the different authorities and countries. Additionally, some recommendations are not well-defined, not considering age, gender, or body weight. In North America (Canada and United States of America) the recommended daily dose is 90 μg and 120 μg for women and men, respectively [22]. In Europe, the panel Dietetic Products, Nutrition and Allergies published a recommendation of 70 μg of vitamin K for people over 19 years old [110]. Nevertheless, in Great Britain the daily recommendation was considered 1μg/Kg of body weight [111]. Additionally, Poland established 200 μg as maximum daily recommendation for adults, going further to have a daily AI of phylloquinone of 55μg and 65μg for women and men over 16 years-old, respectively [112].

There are scarce studies considering the consumption of vitamin K in different populations. Moreover, these studies are very limited and not comparable due to the lack of standardized question forms, and complete food products composition. Additionally, it requires a lot of effort from the participants, and a significant number of participants.

Despite being difficult to compare these populational studies, they help understand the different diets worldwide. In a recent polish study with 1985 participants (1252 women

and 733 men) was revealed that vegetables, processed meat products, and cheese were responsible for about 70% of the total intake of vitamin K, being around 358.6 μg in women and 331.1 μg in men. Interestingly, this study associated a higher content of diet vitamin K_1 in women and lower vitamin K_2 comparing to men. A higher content of vitamin K_1 was also find in urban habitants compared to rural [113]. A Japanese study that took in consideration the quantification of vitamin K, reported a daily intake of 230 μg of total vitamin K in women between 18-29 years old. In this study phylloquinone contributed with 67.7% of the total vitamin K, mainly obtained from vegetables and algae, while MK_7 contributed with 24.9% of the total content of vitamin K, mainly from legumes including fermented soybean (natto) [12]. In a Netherlands study it was reported that 90% of the vitamin K ingested was in the phylloquinone form, with menaquinone daily intake of about 27.0 μg in women and 30.8 μg in men [114]. Despite the diversity between the population's diets, these population-based studies to estimate the consumption of vitamin K, are based in different detection methods and consequently reference values of vitamin K in food. Vitamin K recommendations were mainly constructed considering the coagulation effects of vitamin K, not including the effects on non-hepatic VKDPs. Even considering vitamin K recycling capacity, most of the recommended dosages might be insufficient to conduct the carboxylation of all VKDPs, particularly non-hepatic VKDPs [22]. This highlights the need for vitamin K supplements to ensure a good health condition. Diet supplements of vitamin K available are considered safe and there is no report of toxicity in the intake of higher doses than recommended [115]. Additionally, the route of vitamin K administration is important, with different vitamin K_1 supplements having a better bioavailability than dietary phylloquinone when given in the same dosage [116]. Another point that affects vitamin K bioactivity is the isomers, which is an important point regarding production of vitamin K supplements. Vitamin K can have stereoisomers, but only the trans isomer offers bioactivity. An *in vivo* study showed that phylloquinone *cis* isomer does not have any biological potential [117]. Additionally, supplementation of vitamin K in healthy subjects is reported not to affect coagulation and to increase γ-carboxylation of extra hepatic VKDPs. Also, higher concentration of phylloquinone was shown to increase osteocalcin γ-carboxylation [67-69, 118].

1.4 Vitamin K extraction

1.4.1 Food matrix

Optimization of vitamin K detection and quantification in foods is still a worth subject to investigate further. Conventional methods rely on an extraction with a solvent similar in polarity to the wanted product (in this case n-hexane), these methods are usually based on solid-liquid, liquid-liquid extraction and Soxhlet. Conventional methods present several disadvantages such as lack of efficiency and low yields, requiring enormous quantities of solvent that can be hazards to the environment, and time consuming, making them a not so sustainable approach. However, vitamin K_1 extraction from food matrix is normally obtained with conventional methods followed by chromatography analysis with reverse phase HPLC. New techniques are being developed to fight these disadvantages to be more efficient, sustainable, and less time-consuming. The latest techniques include microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), enzymatic assisted extraction (EAE), and ultrasound-assisted extraction (UAE). Pressurized liquid and microwave-assisted extraction resort to high temperatures to effectively break the cell wall, and both methods have the advantage of requiring a low quantity of solvent. Pressurized liquid extraction is based on increasing the temperature to enhance the solvents extraction characteristics allowing it to easily penetrates the matrix while remaining in the liquid stage. PLE and MAE may have the disadvantage of using high temperature and damage the wanted compound, however phylloquinone extraction has been shown to not be affected by high temperatures [11, 12]. The ultrasound-assisted extraction principle is to form intracellular bubbles that will collapse and release the components within the cells to the solvent. Table 2 presents several quantities of phylloquinone extracted from food matrixes, and it is possible to observe that depending on the extraction method the phylloquinone quantification changes.

In the literature, phylloquinone quantification is found mostly in vegetables, and this might be because detection and quantification of vitamin K_1 in rich fat foods is more difficult that in vegetables, due to high content of triglycerides that are co-extracted with phylloquinone. To overcome this problem lipase is often used, however it can be time-consuming and unspecific [119]. In a study where extraction, lipase treatment and quantification of vitamin K (phylloquinone and menaquinone) in foods like hazelnut, cheese, broccoli, and pork were optimized, it was reported that the precision of quantification is still low [119]. However, in another study, extraction, and quantification

of vitamin K_1 in oil rich food was optimized using ultrasound-assisted extraction (UAE), solid phase extraction (SPE) combined with liquid chromatography tandem mass spectrometry (LC-MS/MS), and it was achieved recoveries between 80.9% and 119.1%. The validation of the assay was done using oilseeds of rapeseed, soybean, and peanut [120].

1.4.2 Algae

Vitamin K has been also extracted from algae, which have been reported to have a high content of phylloquinone. However, the diversity of algae from which vitamin K has been extracted is limited. Microalgae from the phylum cyanobacteria *Anabaena cylindrica* was reported to have a vitamin K content of 200.25 μg/g, and this was extracted with bead glass extraction and n-hexane as the solvent. With the conventional method of liquid-liquid n-hexane, extraction of *Tetraselmis suecica* (phylum Chlorophyta) was reported to have 28 μg of phylloquinone per gram of dry matter [121, 122]. In a recent study, a mix of 90% *Nannochloropsis oculate* (CCMP525) and 10*% Tetraselmis chui*, (PLY429) extracted with n-hexane and quantified by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) was reported to have 11.8 μg of vitamin K_1 per gram of dry weight [123].

Vitamin K_1 was also extracted from macroalgae, especially from edible species. In a Japanese study, *Undaria pinnatifida* (wakame) extracted with n-hexane was reported to contain 12.9 μg of vitamin K_1 per g (of dry matter) [102]. So far, the highest concentration of vitamin K_1 per gram of dry matter reported in an alga was from the macroalgae *Sargassum muticum,* containing 750 μg per gram of dry matter. However, this was reported in 1991, and it is difficult to reproduce the extraction method due to the lack of detailed methodology [12, 121].

It is worth to highlight that vitamin K extraction techniques from algae need to be improved, since the conventional method, such as solid-liquid extraction, mainly with n-hexane are the most used and the diversity of algae studied is very scarce.

Table 2 Vitamin K_1 content of several vegetables and legumes, obtained with different extraction methods.

Food	Vitamin K_1 µg/ g sample	Method	Detection and quantification method	reference
Vegetables and legumes				
Spinach (Raw)	4.496	Soxhlet extraction with n-hexane	RP HPLC	[124]
	3.87	n-hexane extraction	RP HPLC	[10]
	2.629	n-hexane extraction	(LCMS/MS)	[125]
	3.75	Organic solvent extraction	RP HPLC	[11]
Cooked Spinach	2.62	Organic solvent extraction	RP HPLC	[11]
Kale (raw)	8.17	n-hexane extraction	RP HPLC	[10]
	5.251	Soxhlet extraction with n-hexane	RP HPLC	[124]
	2.80	Organic solvent extraction	RP HPLC	[11]
	1.285	n-exane extraction	(LCMS/MS)	[125]
Cooked Kale	2.69	Organic solvent extraction	RP HPLC	[11]
Broccoli (Raw)	1.825	Soxhlet extraction with n-hexane	RP HPLC	[124]
	2.79	Organic solvent extraction	RP HPLC	[11]
	1.56	n-hexane extraction	RP HPLC	[10]
	0.679	n-hexane extraction	(LCMS/MS)	[125]
Cooked Broccoli	2.67	Organic solvent extraction	RP HPLC	[11]
Celery (Raw)	0.507	Soxhlet extraction with n-hexane	RP HPLC	[124]
	0.173	Organic solvent extraction	RP HPLC	[11]
Cooked Celery	3.09	Organic solvent extraction	RP HPLC	[11]
Soybeans	0.128	Enzyme extraction	RP HPLC	[126]
	0.279	Direct solvent extraction	RP HPLC	
	0.190	Soxhlet extraction	RP HPLC	

1.5 Vitamin K production

Vitamin K supplements are available and used in many industry sectors, such as animal feed, food, pharmaceutical and cosmetics. Production of synthetic vitamin K can be used in the pharmaceutical industry, however the synthetic via has many drawbacks. Chemical production of vitamin K in either form is cost-ineffective, hazardous for the environment and for the consumers, due to menadione traces [127, 128] .

1.5.1 Vitamin K_2

The chemical and biochemical production of menaquinones is more updated and studied than vitamin K_1. The chemical synthesis of menaquinones has a very low yield. Vitamin K_2 production is a known subject of biotechnology research. A study showed that it was possible to produce the active form of MK_7 in large production scale from menadione, and with 99.9% of purity, which was safe to be a food and pharmaceutical product [129]. Since this is a growing industry, the goal is to optimize the scale up industry level, and in has been shown that with biofilms reactors and glucose fermentation might be used for large scale production of MK_7[130]. Despite the advances in the chemical synthesis of menaquinones, the natural production of vitamin K_2 is more studied and used in the industry. Vitamin K_2 is mostly obtained by fermentation, a common process in the food industry to obtain the most varied products such yogurt, cheese, milk and beverages such as beer, wine, and cider and much more. To obtain vitamin K_2, species such as *Bacillus subtilis* are commonly used, since this microorganism is widely used in the fermentation industry. MK_7 is the menaquinone most produced by this method, and in the industry in general. Indeed, there are 168 strains with the ability to produce MK_7 with high yield [131]

1.5.2 Vitamin K_1

Vitamin K_1 production for pharmaceutical purposes is produced by chemical synthesis, in a process that remains unaltered since it was first synthetized in 1939 [4]. The process consists in condensing menadione with phytol to obtain vitamin K_1. However, this method is still ineffective because although the biologically active trans I-vitamin K_1 isomer is formed in higher percentage the inactive Z (cis) isomer is also obtained as a product [132]. The added problem with obtaining vitamin K_1 from menadione, is that it is an arduous process to reduce the menadione traces, and menadione was forbidden in the pharmaceutical market in 1963 and cosmetics in 2009, since it represents a risk of

toxicity for humans [133 – 135]. Nevertheless, it is still possible to administrate food products with menadione traces for animal feeding, which is why maybe the highest percentage of vitamin K_1 production is still for animal feed. Nowadays there is still no supplement available in the market of vitamin K_1 produced by the natural biological via, despite the consumers demanding for more natural and sustainable products. Algae are a new source of food products, pharmaceutical and cosmetics and their place in the market is rising. Since vitamin K_1 can be found in photosynthetic organisms, we propose a new strategy to obtain natural phylloquinone from algae. Algae are already highly used in biotechnology and a source of many products in diet supplements, since they present advantages such as sustainable production, high growth rates, and environmental adaptability.

1.6 Potential of *Sargassum muticum* as biomass for vitamin K extraction and purification

Sargassum muticum is a brown alga belonging to the Phaeophyceae class, first described in 1907 by Yendo, native from China and Japan coastlands, and part of the Japanese diet. These macroalgae are highly invasive (See figure 4). In 1944 *S. muticum* was first found in Vancouver Island, British Columbia, and in north America disseminating along the north American coast. In the 1970´s was first found in Southeast England, and from there was able to reach several European countries. The invasion of the Portuguese coast by *Sargassum muticum* was first reported in 1989 [136]. These algae can reach up to 8 meters long and can endure a wide range of temperature (0–28°C), high irradiance and desiccation, which highly contribute to the invasion capacity [137, 138].

Figure 4: Demonstration of Sargassum muticum invasion. Orange invaded locals, blue native localization.

Invasive species have a negative impact on the native environment, with harmful effects on the native species. Interestingly, there is a "invasion paradox'' suggesting that larger scale environment with higher species richness promote invasions, while smaller scale native species richness resist invasions [139]. Nevertheless, studies report that *Sargassum muticum* promoted the decrease of native seaweed such as *Bifurcaria bifurcate* [140, 141], and a decrease in native genotypes [142, 143]. With *Sargassum muticum* invasion there is a competition for light. Due to the *S. muticum* morphology it causes shading, greatly impacting other macroalgae and invertebrate animals, such crustaceans and molluscs.

The invasive species also have a big impact in the economy, due to their adaptability and difficult to prevent further invasions. In 2018 it was estimated that *S. muticum* biomass was around 20 million tonnes, with a severe impact in activities involving tourism and fishing.

There are several ways to mitigate invasive species such as the physical removal that is more efficient and with low cost, but with high demands of manual effort, the chemical and biological removal with many disadvantages such as hazard for the environment and human health. The biological method consisting of introduction another invasive organism to fight the invader can lead to an uncontrolled situation. A recent study proposed that the best way to mitigate the impact of invasive species was to promote public awareness to the biotechnological potential of the specie and as a commercial asset [144]. Curiously, invasive seaweeds, including *Sargassum muticum* were predicted to represent $21,955.6 billion as natural resources in the marine derived drug market [145]. Algae are currently the key to natural products in the pharmaceutical, food and cosmetic industry. In fact, in 2019 algae market was valued at $592.0 Million and it is estimated to be $967.3 in 2027. However, the pharmaceutical industry is still the topmost contributors to environmental pollution, due to carbon emissions. This project is focused on finding the most sustainable way to obtain a marine derived product rich in vitamin K_1 from the biomass of the invasive algae *Sargassum muticum*.

Polysaccharides are a large family of carbohydrates that include alginates, agar, carrageenan, fucoidan and many more, which are responsible for the bioactivities of many algae products in the market. Fucoidan is a popular component that is highly study in brown algae including *Sargassum muticum,* presenting antioxidant and anti-inflammatory properties. A study with hot water extraction of fucoidan from *Sargassum muticum*

demonstrated antioxidant activity in a 1,1-diphenyl2-picryl hydrazyl (DDPH) assay, although lower than the antioxidant activity of vitamin C [146]. However, when extracted with ultrasound assisted, fucoidan from *Sargassum muticum* showed a similar antioxidant activity than the known antioxidant Trolox [147] .

Carotenoids are a group of pigments considered as pro-vitamin A, and present antioxidant activity (reviewed in [148, 149]. These pigments are present in *Sargassum muticum,* and their extraction has been the focus of improvement for more efficient and sustainable methods. Carotenoids are usually extracted with ethanol, but this methodology can have several disadvantages such the contamination by chlorophylls. New methods such non-ionic surfactants have been shown to be more specific, however it still does not have higher yield than the conventional method [150]. In another study, extraction with tween 20 demonstrated to be a more efficient way to extract carotenoids than the conventional method [151].

The alginate is a polysaccharide used in the industry as an emulsifying product. Methods to optimize and obtain alginate are still being studied, but alginate extracted with ethanol from *Sargassum muticum* has been shown to have antioxidant [152], and anti-tumoral activities when extracted with non-conventional methods such as ultrasound assisted extraction [153].

Sargassum muticum was suggested as potential biomass extract for the production of a skincare product that can help prevent sun damage and acne [154]. Indeed, a Portuguese study analysed several fractions of *S. muticum* (such as aqueous, ethyl and acetate and water insoluble fractions) and reported an antioxidant and photoprotective effect in most fractions, by decreasing ROS production in fibroblast cells (3T3) exposed to UV. Additionally, these fractions presented anti-inflammatory properties, by decreasing TNF-α and IL-6 in mouse macrophage cells (RAW 264.7), and skin protection by decreasing enzymatic activity of enzymes related to skin aging such as collagenase, hyaluronidase and tyrosinase. Interestingly, the less polar fraction of the algae showed a 35% growth inhibition of *Cutibacterium acnes*, a bacterium responsible for acne [154].

S. muticum has been shown to contain several components with antioxidant effect [146], which have been mainly described as flavonoids, carotenoids and polyphenols. Nevertheless, the total polar lipids extract seems to also have an antioxidant activity [155, 156]. *Sargassum muticum* was also reported to have antitumoral potential. In 2018, a

study showed that an aqueous fraction of the macroalga presented promising cytotoxicity against human colon cancer cells (HCT 116 cells) without having harmful effect on normal cells [157]. Additionally, it was suggested as a neuroprotector since it prevented the dopamine neurotoxicity in human neuroblastoma cells (SH-SY5Y) [158].

2. Aim and objectives

The aim of this work was to optimize vitamin K_1 extraction from *Sargassum muticum* using Energized Dispersive guided extraction, an innovative and sustainable method and further establish a method for its purification and quantification by RP-HPLC. Also, the goal was to test the antioxidant properties of *S. muticum* vitamin K_1 rich extracts and their anti-inflammatory protective effect in human macrophages-like and vascular smooth muscle cells (VSMCs), comparatively to commercially available vitamin K_1 and MK_4. Furthermore, the aim was to also study the effect of *S. muticum* extracts on Vitamin K dependent proteins γ-carboxylation on inflamed VSMCs.

Material and Methods

3.1 Vitamin K separation and quantification by RP-HPLC quantification.

3.1.1 Reverse phase High-Performance Liquid Chromatography RP-HPLC

HPLC is a chromatography method that allows the physical separation, quantification or identification of analytes included in a mixture sample using a column chromatography containing the stationary solid phase and a mobile liquid phase, also called eluent, running through the system under high pressure at a specific flow rate. In this project, we will use a LC reversed-phase alkyl bonded stationary phase with an octadecyl carbon chain (C18)-bonded silica column. In RP-HPLC separation of the sample, components will depend on their specific affinity between the stationary C18 hydrophobic and mobile phases. In this RP-HPLC separation method using a polar mobile phase and a non-polar hydrophobic stationary phase, the analytes with more affinity to the mobile phase will be eluted first and the non-polar components with a higher affinity to the stationary phase will elute with higher retention times.

The RP-HPLC analysis was performed on a Jasco HPLC apparatus equipped with a binary pump system (880-PU Jasco master pump, 880-PU Jasco slave pump, Tokyo, Japan), a UV/VIS detector (875-UV/Vis, Jasco Corporation, Tokyo, Japan) and a fluorescence detector (Jasco FP-4025), a sample collector (Amersham Biosciences), monitorization was performed using the Clarity program (version 3.06.589 by DataApex). The chromatography conditions, including RP-HPLC column, mobile phase composition, post-column derivatization and elution monitorization used in this project, were based on previously published methods designed to separate vitamin K_1 from different plants, foods and microalgae [122]. In brief, the mobile phase was constituted composed by anhydrous zinc chloride 1.37g/L (Sigma Aldrich), 0.41g/L anhydrous sodium acetate (Sigma Aldrich) and 0.30g/L of glacial acetic acid (Sigma Aldrich), dissolved in 90% (v/v) methanol (HPLC graded, ≥ 99.8% purity, Fischer chemicals) and 10% (v/v) of dichloromethane (HPLC graded, ≥ 99.8% purity, Fischer chemicals). A fresh mobile phase solution was prepared every day.

As the stationary phase, a reverse C18 analytical column (3 μm particle size, 12 nm pore size, 250 mm× 2.6 I.D mm, YMC, Europe GMBH), and guard column (3 μm particle size, 12 nm pore size , 10 mm× 4.0 I.D mm, YMC, Europe GMBH) were used for

separation, and a post-column (30 mm x 4.6 I.D mm, Norleq) packed with zinc powder (> 63 µm, Sigma-Aldrich) was assembled after the column C18 YMC column and used to reduce vitamin K, into Vitamin K hydroquinone (VKH2) (Figure 5). Elution monitorization was performed by A280 nm (UV detector, Jasco) and fluorescence emission at 430 nm (Jasco). The reduced vitamin K forms can be monitored using a fluorescence detector with an excitation wavelength of 243 nm, and emission wavelength of 430 nm (Jasco).

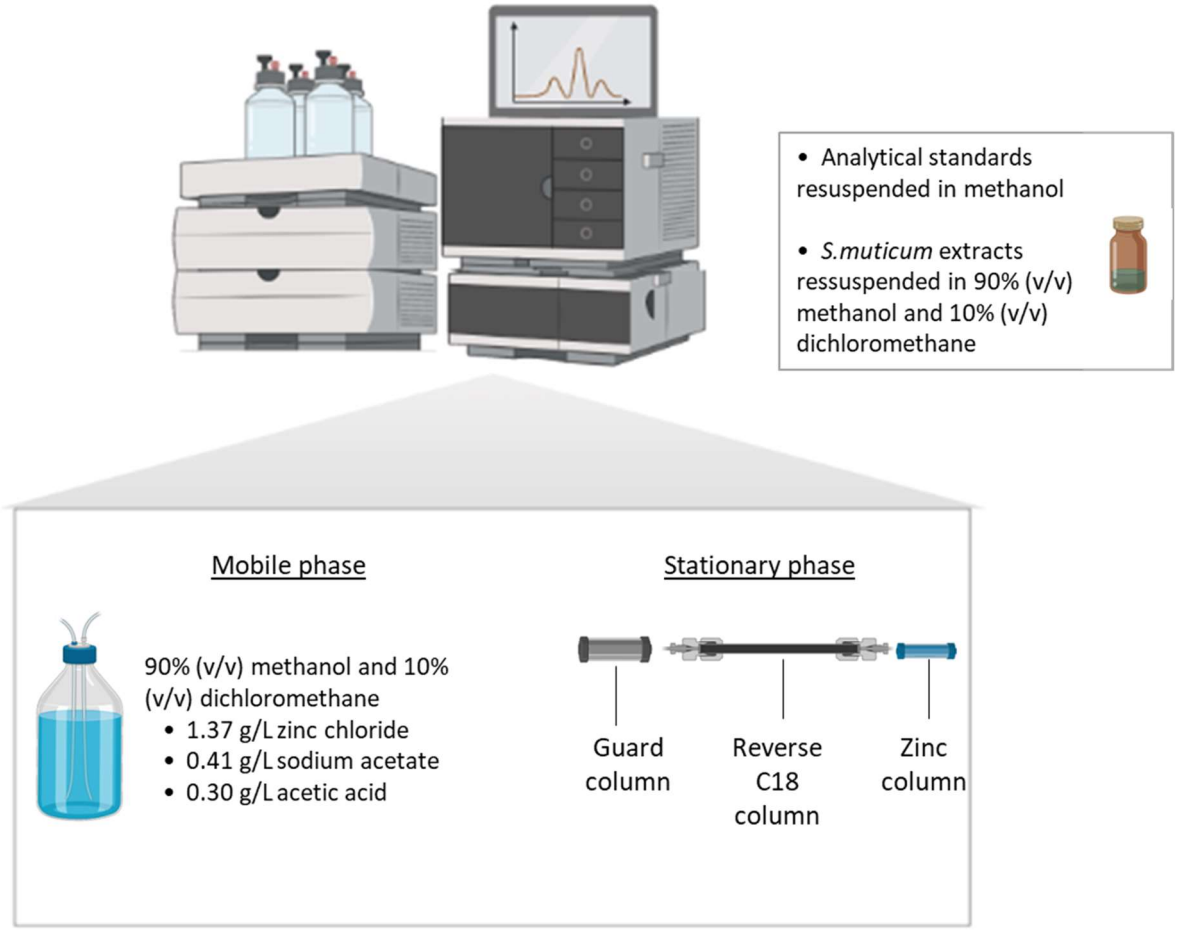


Figure 5: Vitamin K detection and purification by RP-HPLC method.

3.1.2 Preparation of RP-HPLC analytical standard solutions

Stock solutions containing 100 µg/mL of vitamin K_1 (Sigma-Aldrich, ref 47774) and MK_4 (Sigma-Aldrich, ref 47774) in methanol were prepared in amber vials and stored at -20ºC, always protected from the light. The working standard solutions were prepared by dilution of the standard stock solutions of vitamin K_1 and MK_4 with methanol.

3.1.3 Vitamin K_1 and MK_4 quantification

To quantify vitamin K_1 and MK_4 the same RP-HPLC optimized conditions described before were used and calibration curves were obtained. To quantify K_1 or MK_4 sample calibration curves were performed, using the standards fluorometric chromatograms and plotting the area of the peak (mV/s) *versus* the total amount of vitamin K standard injected (μg), followed by performing a simple linear regression analysis. A calibration curve was performed on the same day the chromatographic separation and quantification of the sample extracts were performed.

3.1.4 Sample preparation for RP-HPLC

Extract samples were resuspended in 90% (v/v) methanol and 10% (v/v) dichloromethane and filtrated with a PTFE filter (2μm, 13mm, Branchia), before injecting into the RP-HPLC.

3.2 Collection and preparation of *S muticum* biomass

Sargassum muticum was collected at the end of May 2021 in Viana do Castelo, (Figure 6), Portugal and directly cleaned from artefacts which are not part of the specimen (shells, animals, or other species) and rinsed with salt water to remove sand or debris. The algae were transported at 4ºC within the same day to the CCMAR laboratory where it was further rinsed with filtered salt water followed by distilled water. The algae were then frozen and freeze-dried until complete dryness. Once dried, *S. muticum* was ground to powder using a food grinder (Braun). The powder obtained was further ground in a ball mill (PM 100 Retsch GmbH, Germany) with 3 cycles of 5 minutes (at 450 rotation per minute (rpm) (intermittently for 30 seconds), between each cycle was a pause of 2 minutes to not overheat the product.

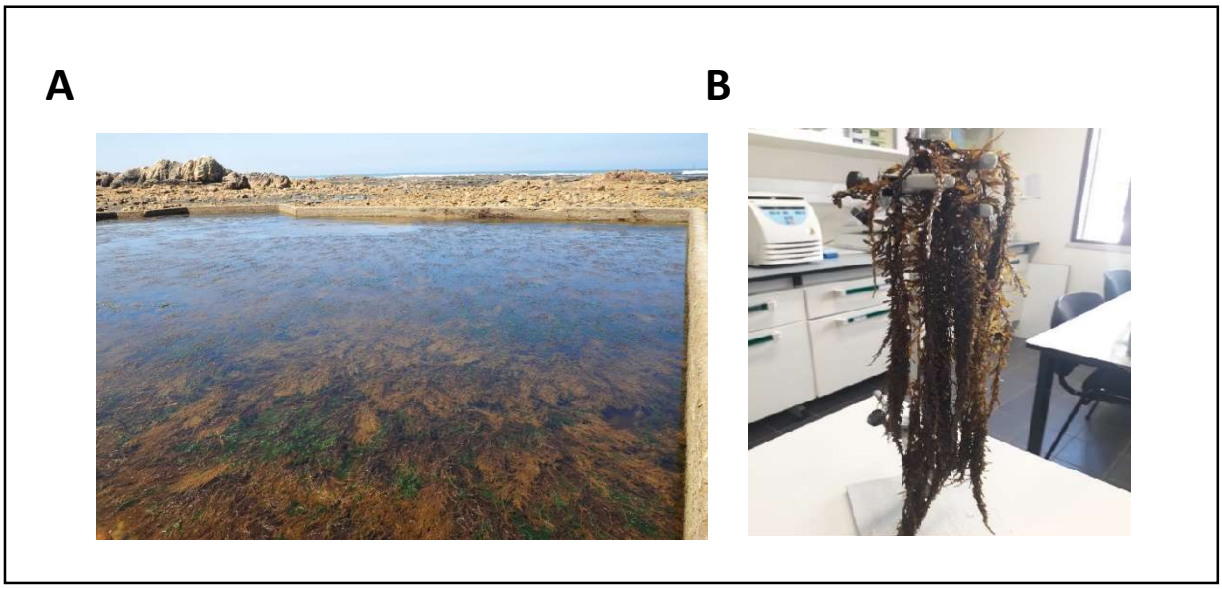


Figure 6: Sargassum muticum. (A) Local of Sargassum muticum collection. (B) Sargassum muticum.

3.3 Preparation of *Sargassum muticum* extracts

3.3.1 Energized Dispersive Guided Extraction

This method is based on pressurized liquid extraction and dispersive solid phase extraction. Energized Dispersive Guided Extraction (EDGE) is an automatic extractor (C.E.M Corporation, 2017), that heats the chamber walls where the sample is found while increasing the pressure and heating the solvent. In this method, the user can set the temperature, the volume of solvent used (maximum of 40 mL total), the cycle time and the number of cycles (maximum of 5 cycles). However, it is impossible to set the pressure since this depends on the solvent and temperature used. In this extractor, the extraction vessel is designated Q-cup, a metal recipient with 2 detachable parts where in between the user should place a set of filters The filters set, designed for the extractor (S1 Q-disc, C.E.M Corporation), consist of two cellulose filters and one glass filter and a glass collection vial is placed next to each Q-cup in the rack. For the *S. muticum* EDGE extraction with n-hexane, different amounts of dried biomass were tested (0.25; 1; 2; 3 grams) and added MK_4 dissolved in n-hexane, directly in the alga powder prior extraction (5 μg per gram of alga biomass), except for the extracts that were further used for the bioactivity assays. Extraction was performed in 3 cycles, each with 10 minutes using 5 ml of n-hexane per cycle at different temperatures as shown in table 3. After EDGE all the extracts were filtered (PTFE, 0.45 μm 25 mm, Branchia) and allowed to evaporate under a gentle nitrogen flux, at room temperature in a brown flask, protected from the

light (procedure summarized in figure 7). The dried extracts were weighted (MSA36S-000-DH, Sartorius) and the extract yield was calculated with the formula (1).

$$Extracted\ yield\ (\%) = \left(\frac{Dried\ extract\ (g)}{Initial\ biomass\ (g)}\right) \times 100 \quad (1)$$

The dried extract was resuspended in 90% (v/v) methanol and 10% (v/v) dichloromethane and stored in the freezer.

To further explore the loss of vitamin K in the EDGE method and in the following sample treatment (Figure 7), a mixture of the analytical standards of vitamin K_1 (20 μg) and MK_4 (10 μg), dissolved in n-hexane, were added directly into the Q-cup and proceeded with the extraction at 50ºC and sample treatment as described previously. The samples were compared with another experiment, where the same quantity of vitamin K were dissolved in 15 mL (final volume of EDGE) and treated as post- extraction samples (Figure 7 B)

Table 3 Sargassum muticum extraction with n-hexane using Energized Dispersive Guided Extraction

Solvent	Biomass (g)	Temperature (ºC)
n-hexane	0.25	50
	1	50
	2	50
	2	60
	3	60

3.4 Vitamin K stability with temperature.

To evaluate the stability of vitamin K with temperature an experiment was performed with a solution of 10 μg of each analytical standard, vitamin K_1 and MK_4, mixed in 90% (v/v) methanol and 10% (v/v) dichloromethane and exposed to different temperatures of 50ºC and approximately of 22ºC considered room temperature (RT) for 30 minutes. The samples during the experiments were always protected from light. Vitamin K recovery was calculated by comparing the concentration of each vitamin K in each sample using by RP-HPLC as previously described in 3.1.3.

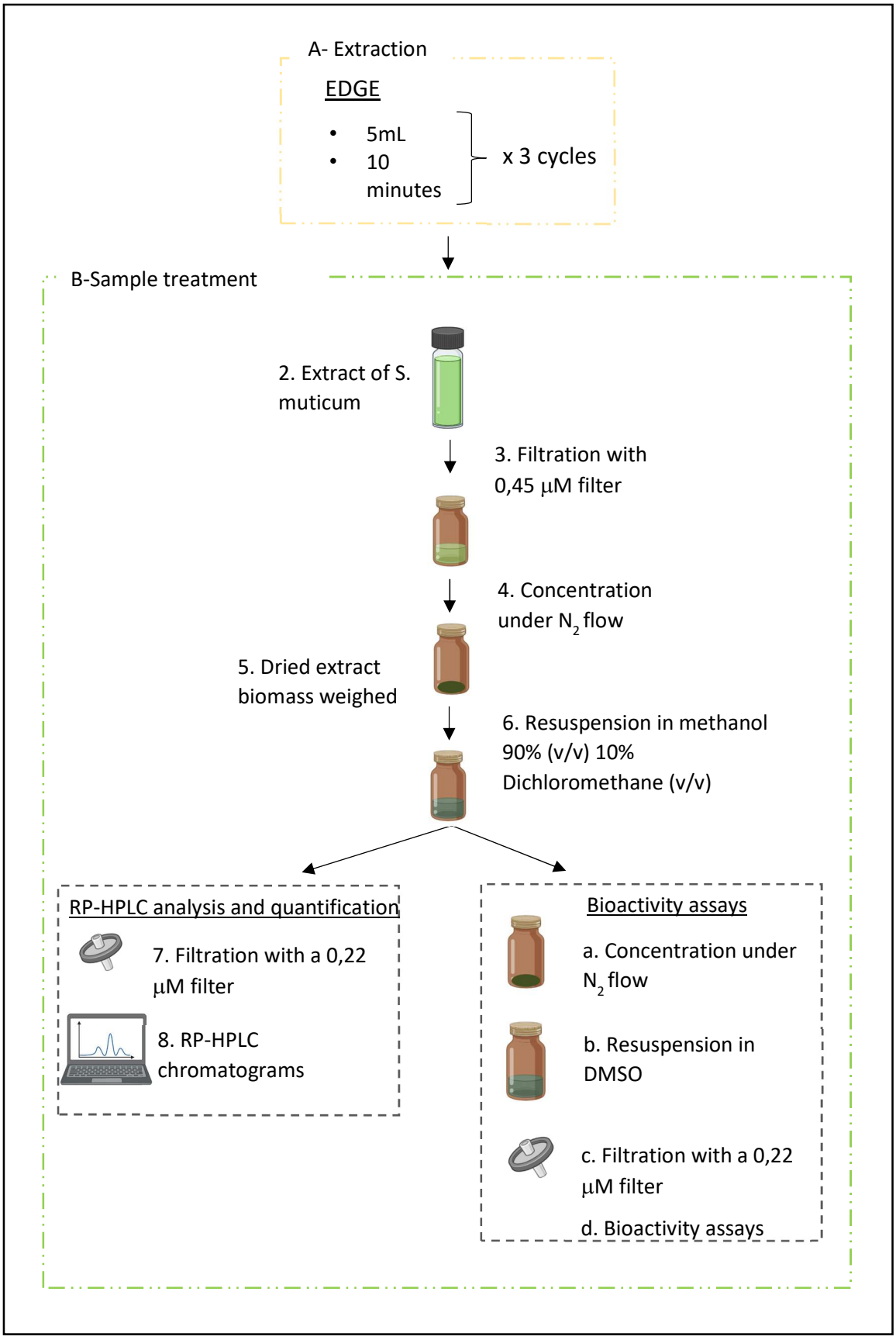


Figure 7: Schematic representation of procedures for Vitamin K_1 extraction (A), and post-extraction treatment purification quantification and bioactivity assays (B)

3.6 Antioxidant and anti-inflammatory bioactivities of *Sargassum muticum* n-hexane extracts and synthetic vitamin K_1 and MK_4

To evaluate the bioactivities, the extracts obtained in the conditions EDGE extraction with n-hexane (initial biomass of 1 gram at 50ºC (3 independent experiments); 3 grams of initial biomass at 60ºC), were concentrated and resuspended in DMSO to a final concentration of 20 mg/ml. Commercially available synthetic vitamin K_1(Sigma-Aldrich) and MK_4 (Sigma-Aldrich) were resuspended in ethanol to a final concentration of 100 mM and 25 mM, respectively. These procedures were performed under aseptic conditions.

3.6.1 Antioxidant bioactivity – 2-Diphenyl-1-picrylhydrazyl DPPH

2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable radical molecule soluble in methanol with a characteristic maximum of absorbance around 517 nm. DPPH radical (DPPH •) in the presence of antioxidant compounds donates an electron or hydrogen atom and converts into a more stable molecule. When DPPH • is reduced it changes colour from purple to yellow. This colour change allows to determine the antioxidant activity by spectrophotometry. DPPH assay was performed following the method described in Moreno et al. (2016) [159]. Briefly, *S. muticum* extracts obtained from EDGE under the conditions of 3g of initial biomass and 60ºC (at concentrations of 5.0; 2.5; 1.25; 0.625 and 0.313 mg/ml) and DPPH solution (200 µL, 120 Mm) were mixed in 96-well microplates and incubated for 30 min in the dark at RT. Acid gallic (2 mg/mL) was used as a positive control. A microplate reader spectrophotometer was used to determine the absorbance at 517 nm (BioTek 4 Synergy Multi-Detection, Agilent, Santa Clara, CA, USA). The scavenging activity was calculated comparing with a blank containing DMSO and the extracts analysed. The scavenging activity was measured by equation (2).

$$Scaveging\ activity\ (\%) = \left(\frac{Abs\ colour\ control - Abs\ extract}{Abs\ colour\ control}\right) \times 100 \quad (2)$$

Three independent experiments were performed with 6 technical replicates each.

3.6.2 Anti-inflammatory activity

3.6.2.1 Cell culture and maintenance

The monocytic cell line THP-1 was cultured according to ATCC recommendations [160] in RPMI 1640 media containing 1% L-glutamine (Gibco), and supplemented with 10% heat-inactivated Foetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). The cell culture was maintained at 37ºC in a 5% CO2 humidified atmosphere in complete RPMI media, with media change every 2-3 days, with a maximum density of $1.0x10^6$ cells/mL. Differentiation into THP-1 macrophages cells (THP-1 Mac) was achieved by culturing $1.0x10^5$ cells/well in 200 µL of complete RPMI in a 96 well plate, supplemented with 25 ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 48 hours. Primary human vascular smooth muscle cells (VSMCs) obtained from aorta tissue explants as described [161], were a kind gift from Dr Leon Schurgers (CARIM, University of Maastricht, The Netherlands). VSMCs between passages 12 and 14 were maintained in M199 (Gibco) media supplemented with 10% FBS and 1% PS, at 37ºC in a 5% CO2 humidified atmosphere, with media change every 2-3 days. Confluent VSMCs were washed 2 times with phosphate-buffered saline solution - PBS1x (1.28mM of KH_2PO_4, 8.09mM $NA2HPO_4.2H_2O$, 0.14M NaCl, 2.68mM KCl) and released from the T75 tissue culture flask using 2 mL of trypsin-EDTA solution (137 mM of sodium chloride, 2,7 mM of potassium chloride, 15,8 mM sodium phosphate dibasic, 1,23 mM of potassium dihydrogen phosphate, 1,1 mM of ethylenediaminetetraacetic acid and 0,2% of trypsin in double-distilled water) and incubation at 37ºC and 5% CO_2 for 5 min. Cell detachment was followed by observation in an inverted microscope (Motic AE 31), and once completed, trypsin activity was inhibited by FBS contained in cell culture media. Cell suspensions were recovered by centrifugation at 800 x *g* for 5 min at room temperature, resuspended in complete M199 media and plated in 96 well plates (200 µL/well).

3.6.2.2 Cell viability

The effect of *S. muticum* extracts, and commercially available purified vitamin K1 and MK4, on THP-1 Mac cell viability was determined using the MTS method (Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay, Promega, USA).This assay is based on a novel tetrazolium compound (MTS) [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)], which is reduced by dehydrogenase enzymes present in metabolically active cells, forming a aqueous, soluble

formazan. The quantity of formazan product is determined by measuring absorbance at 490 nm, which is directly proportional to the number of living cells in culture.

After seeding and differentiation of THP-1 Mac cells in 96 well plates as described above, the media were removed and replaced with 200 µL of new complete media containing the different testing conditions in triplicates. Vitamin K, both K_1 and MK_4 were tested at 10, 50 and 500 µM. Three *Sargassum muticum* extracts derived from 3 independent experiments of EDGE using the same condition (1 gram of initial biomass, 50ºC) were tested at concentrations of 10 and 100 µg/mL. The vehicles ethanol and DMSO were tested at the highest concentrations used for vitamin K treatments (2% v/v) and *S. muticum* extracts (0.5% v/v), respectively. Control THP-1 Mac cells were only treated with complete RPMI media. Treatments were performed for 48 hours in regular cell culture conditions.

After the incubation period, the media were removed and replaced by 100 µL of new complete RPMI media containing 20 µL MTS solution (95,2% MTS+ 4.8% phenazine methosulphate - PMS). The cells were incubated for one hour for colour development (conversion of MTS into soluble formazan), and the absorbance was recorded at 490nm in a synergy neo-2 multi-mode microplate reader (Biotek). The control cells were considered to have 100% of viability, and the viability of the cells under treatment conditions was deduced through the formula (3).

$$Viability\% = \left(\frac{Obtained\ absorbance}{Control\ absorbance\ mean}\right) \times 100 \qquad (3)$$

3.6.2.3 Inflammatory assays

The potential anti-inflammatory preventive effect of vitamin K and *S. muticum* extracts was tested in THP-1 Mac cells and in VSMCs.

THP-1 Mac cells and VSMCs plated and cultured as described in section 3.6.2.1, were pre-treated for 24 hours with the different testing conditions. In both cell lines, vitamin K1 and MK4 were tested at concentrations of 10, 50 and 500 µM. The vehicle ethanol was tested at a concentration of 2% (v/v). *Sargassum muticum* extracts EDGE 50ºC, 1 gram were tested at concentrations of 10 and 100µg/mL. In the THP-1 Mac cells 3 extracts (Extracts 1-3) were tested, while in VSMCs 2 extracts (Extracts 1 and 2) were

analysed. The extracts vehicle DMSO was tested at concentration of 0.5% (v/v) in both cell lines. Following the 24h pre-treatment, inflammation was stimulated with 100 ng/mL of lipopolysaccharide (LPS) in THP-1 Mac cells, and with 20 ng/mL tumour necrosis alpha (TNF-α) in VSMC, during additional 24 h. In both THP1 Mac and VSMCs, non-treated cells (cell culture media only) were used as negative control to inflammation, and cells treated only with LPS and TNF-α as positive controls to THP-1 Mac and VSMCs inflammation stimulation, respectively. Dexamethasone (DXMT), a known anti-inflammatory compound, was used as a positive anti-inflammatory control in THP-1 Mac cells at 2μM. Every condition was performed in four replicates.

Conditioned media from THP-1 Mac and VSMCs experiments were collected and centrifuged at 16.000 xg, for 20 minutes at 4ºC to remove cell debris, and the supernatant was collected and stored at -80ºC until further use. VSMCs were washed 2 times with PBS1x and stored at -80ºC for total protein extraction.

3.6.2.4 Enzyme-linked immunosorbent assay (ELISA)

Levels of IL-8 in the conditioned media were measured following the manufacturer's protocol of the commercially available ELISA Kit (PeproTech). Briefly, 100 μL of capture antibody (0.50 μg/ml diluted in PBS1x) were added to flat bottom high specificity 96 well plates (Thermofisher) and incubated overnight (ON) at room temperature (RT). On the next day, the plate was washed 4 times with 300 μL wash buffer (0.05% Tween-20 in PBS 1x) and blocked with 300 μl block buffer (1% BSA in PBS1x) for 1 hour at RT. After washing 4 times as described, 100 μL of conditioned media samples and the IL-8 standards ranging from 1 to 0,015 ng/ml were added and incubated for 2h at RT. After washing, 100 μL of capture antibody (0.5 μg/ml diluted in 0.05% Tween-20, 0.1% BSA in PBS1x) were added and incubated for 2h at RT. After washing, 100 μL of Avidin Horseradish Peroxidase (HRP) conjugated (diluted 1:2000 in 0.05% Tween-20, 0.1% BSA in PBS1x) were added and incubated for 20 min. After the washing cycles, it 100 μL of azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma) were added, and the absorbance was recorded at 450 nm every 5 min during 1h total, in a synergy neo-2 multi-mode microplate reader (Biotek), with wavelength correction at 650nm. A calibration curve was deduced from the standard dilutions of IL-8, and the concentrations of IL-8 in the conditioned media samples were obtained from the equation $y = ax + b$ where y is the absorbance and x the concentration of IL-8 in the sample (ng/ml).

3.6.2.5 Protein extraction from VSMCs

The cells were thawed on ice and 20 µL of Radio Immunoprecipitation Assay (RIPA) buffer (50mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1%SDS) were added to each well. The cells of the 4 replicates per condition were scrapped and recovered in one Eppendorf tube. Protein extraction was completed by one cycle of cells freezing at -80ºC followed by unfreezing, and incubation for 1h on ice. Total protein extracts were recovered in the supernatant after centrifugation at for 20 minutes at 13000 rpm at 4ºC.

3.6.2.6 Vascular smooth vascular cells total protein quantification

Total protein quantification was performed by a micro bicinchoninic acid (BCA) kit (Thermo Scientific). This technique is based on the reduction of copper and the chelation of two BCA molecules with one cuprous ion (Cu^{+1}). Cu^{+2} is reduced to Cu^{+1} by protein present in an alkaline medium, and when the complex BCA and cuprous ion is formed there is a purple-coloured reaction that can be measured at 562 nm. This reaction is linear with the increase of protein in the sample [162, 163]. Standards in known concentrations of bovine serum albumin (BSA) (1.25 to 100 µg/mL) were added to a low binding 96 well plate (Greiner Bio-One GmbH) with a final volume of 100 µl in PBS 1x. Samples were diluted in 1:20 in PBS 1x and added 100 µL to each well. A volume of 100 µL of working reagent constituted by a cupric sulphate solution, bicinchoninic acid and a tartrate-carbonate buffer, was added to each well and the plate was incubated for two hours at 37ºC. After the incubation, the absorbance was read at 562nm. The calibration curve was used to calculate the total protein quantity present in the VSMCs protein extracts. Twenty micrograms of total protein of each condition were aliquoted, frozen and lyophilized.

3.6.2.7 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis - SDS page

Lyophilized total protein samples were resuspended in 15 µl of loading buffer (Invitrogen NuPAGE) and DTT (0.5 M Sigma) in a ratio 7:5. The samples were incubated at 95ºC for 10 minutes and loaded in a NuPAGE™ 4–12% gradient polyacrylamide Bis-Tris precast gel (Invitrogen) in running buffer (MES 50mM, Trizma Base 50mM, SDS1mM, EDTA 0.1%). Additionally, 8 µL of Precision Plus Protein™ Dual Xtra (Bio-Rad) were

loaded as a molecular weight marker. The samples were run at 180 V in the electrophoresis apparatus (Invitrogen) for approximately 1 h.

3.6.2.8 Western Blot

After the SDS-PAGE, the gel was blotted into a nitrocellulose membrane (0.2 μm, Bio-Rad) and transferred for 2 h at 0.08A constant (Bio-Rad). The membrane was blocked with 5% BSA in PBST (Tween-20 1% (v/v) in PBS1x) for 3 hours at room temperature with agitation. The membrane was washed with PBST solution and incubated ON with the primary monoclonal antibody M3B (America Diagnostica Inc., Stamford, CT. USA) at a concentration of 2.5 μg/mL. This antibody has been validated previously for the detection of gamma-carboxylated glutamic acid residues (Gla) [37]. The membrane was washed, and the secondary Horseradish peroxidase-conjugated anti-mouse IgG antibody was added in a dilution of 1:100000 (Sigma) for 1 hour at RT with agitation. The membrane was washed in PBST, and the results were visualized using a chemiluminescence enhancer solution (Western Lightning pro-ECL PerkinElmer) using an ImageQuant apparatus (GE Healthcare). The membrane was washed and stripped with Stripping Buffer (62.5 nM Tris-HCL, pH 6.8; 2% SDS and 100 mM β-mercaptoethanol) for 30 min at 50ºC. Reprobing was performed with the primary polyclonal antibody for the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:500) (Santa Cruz Biotechnology) of approximately 37kDa, and the anti-rabbit IgG-HRP (1:70000) secondary antibody (Sigma), following the previously described procedure. Semi-quantification of γ-carboxylation levels was performed through densitometry and normalization to GAPDH, using Image Lab software.

3.7 Statistical analysis

Statistical analysis was performed with PRISM software (GraphPad Prism, GraphPad Software, La Jolla, CA, USA). Data are presented as mean ± standard deviation (SD). For more than two groups significance was calculated using ordinary one-way ANOVA with comparison between groups by Dunnett test. Statistical significance was defined as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

Results

4.1 Establishment of an analytical method for the quantification of vitamin K_1 and MK4 by RP-HPLC

To analyse and quantify the amount of vitamin K_1 in the different extracts and MK_4 in the spiked samples obtained from *S. muticum*, a RP-HPLC calibration method was developed using commercially available purified standard analytical vitamers of K_1 and MK_4.

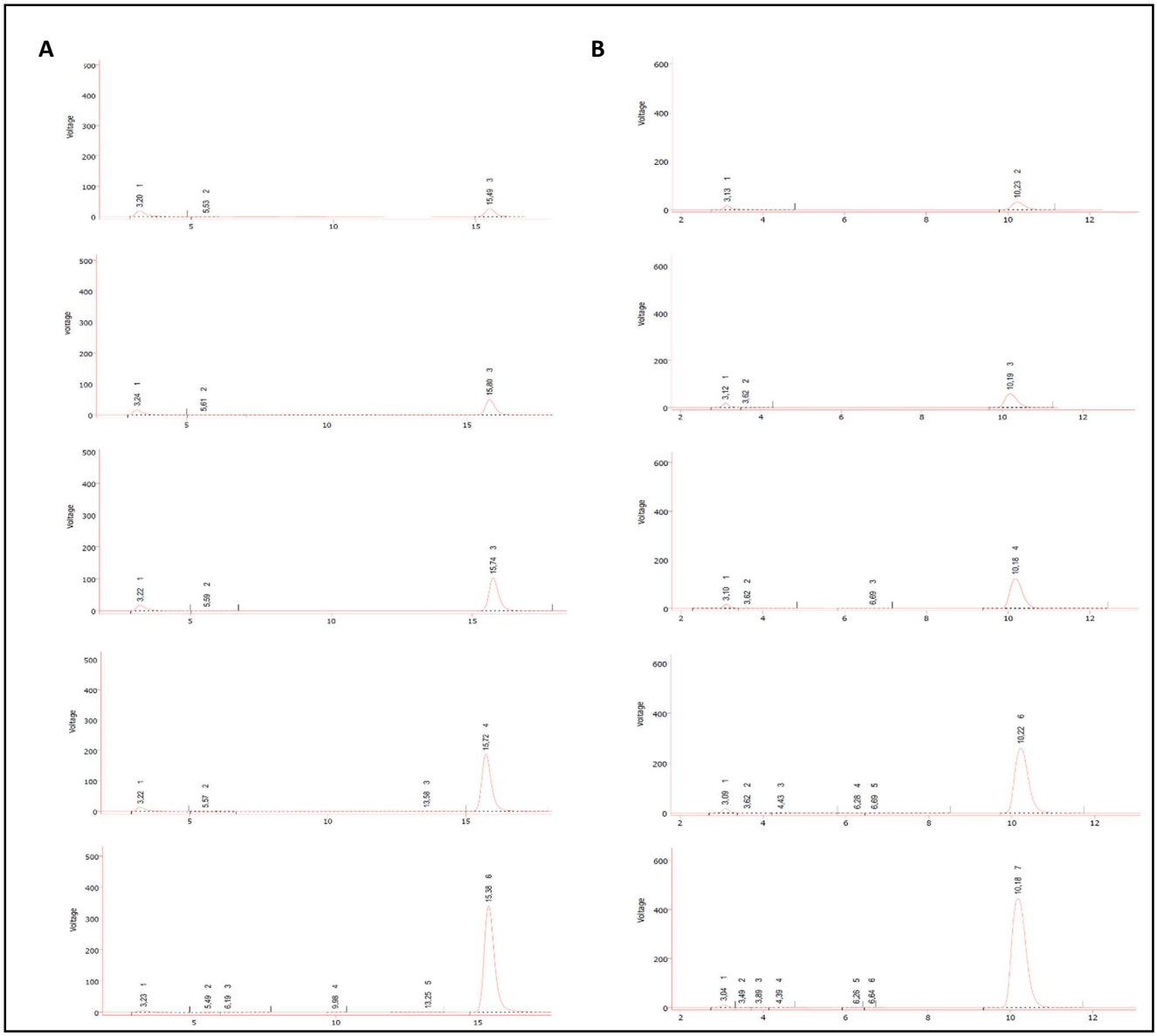


Figure 8: Chromatograms from RP-HPLC fluorometric analysis (ex 243 nm/em 430 nm) of vitamin K_1 (A) and MK4 (B) analytical standards. The chromatograms were obtained under RP-HPLC conditions as described under material and methods 3.1. Concentrations of K_1 and MK4 were 0.125, 0.25, 0.5, 1 and 2 μg with a flow rate of 1ml/min and injection volume of 0.5mL.

Chromatograms from fluorometric analysis, obtained for a sample containing both vitamin K_1 and MK_4 standards (4 and 2 µg each), shown well separated retention times for K_1 (11 min) and MK_4 (17 min) (Figure 9 C). Based on this, several calibration curves of K_1 and MK_4 standards with different quantities were performed (0.125 – 8 µg). Calibration curves for K_1 and MK_4 were obtained by simple regression analysis using concentration *vs* peak area (Figure 8) with a R^2 mean of 0.97 ± 0.04 and 0.98 ± 0.02, respectively (Figure 9).

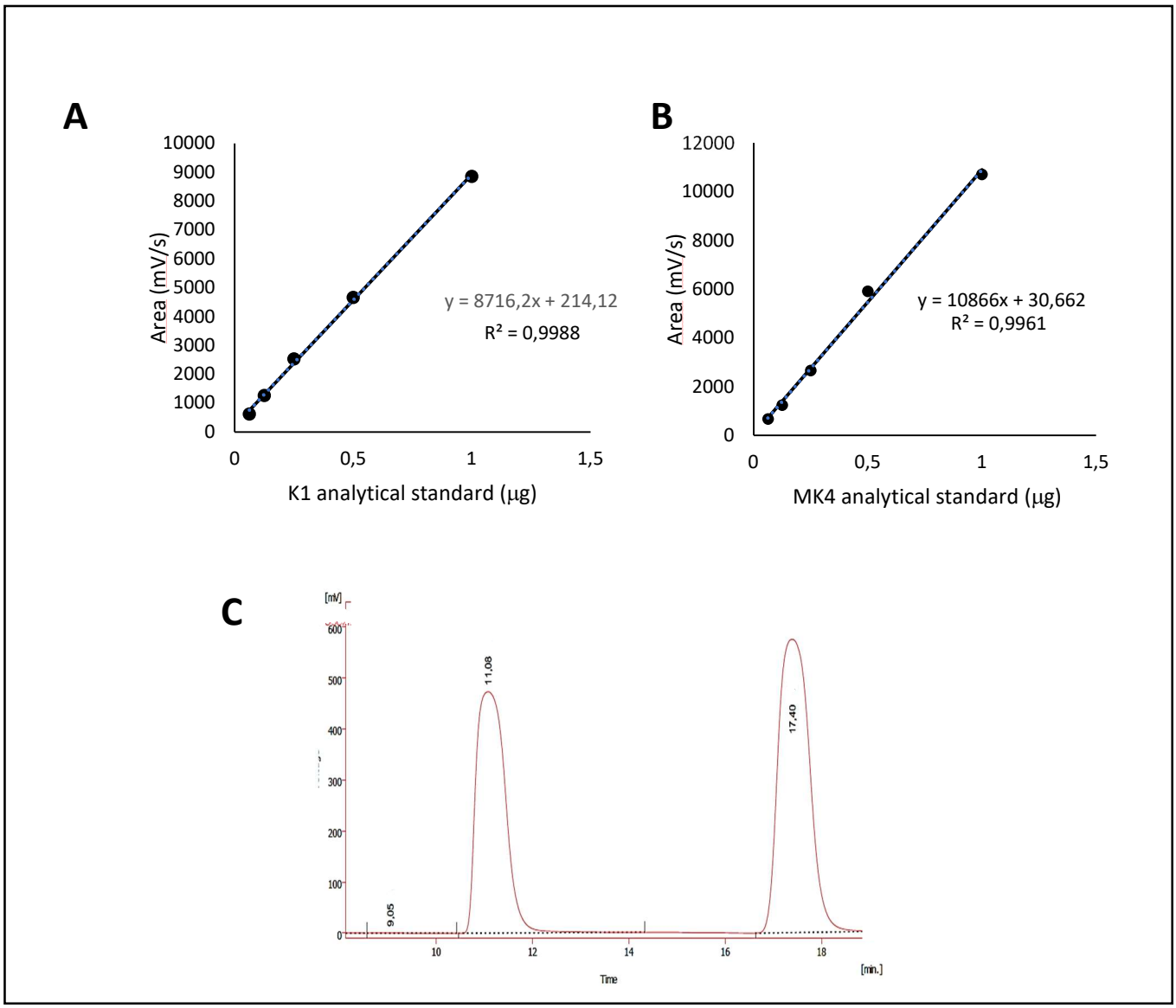


Figure 9. Example of calibrations curves obtained from chromatographic fluorometric analysis (ex 243 nm/em 430 nm) of different concentrations of MK4 and K1 standards (0.0625-1 µg/ml) using RP-HPLC with a flow of 1ml/min, and a 0.5 mL loop, as described in the Materials and Methods Section 3.1. (C) chromatogram from fluorometric analysis of sample containing 2 µg /ml of MK-4 and 4 µg /ml K1 analytical standard. The retention times were 11.06 and 17.40 min for MK4 and K1 respectively.

Table 4: RP-HPLC chromatographic fluorometric analysis of vitamin K_1 standard.

Loop (ml)	K_1 (µg)	Peak Area (mV/s)	Standard deviation (%)
0.5	2,000	11592,13	± 11,66
	1,000	11532,98	± 59,60
	0,500	4377,48	± 28,46
	0,250	2402,38	± 35,81
	0,125	955,23	± 28,72
1	8,000	30770,66	± 12,79
	4,000	18680,39	± 36,13
	2,000	11488,69	± 35,83
	1,000	6292,61	± 33,97
	0,500	3283,70	± 36,36
	0,250	1458,31	± 40,15

Table 5: RP-HPLC chromatographic fluorometric analysis of vitamin MK4 standard.

Loop (ml)	MK4 (µg)	Peak Area (mV/s)	Standard deviation (%)
0.5	2,000	15724,50	± 0,10
	1,000	9157,20	± 24,03
	0,500	4055,07	± 33,20
	0,250	1555,02	± 29,14
	0,125	1295,10	± 5,70

4.2 Comparison of different extraction conditions for isolation of vitamin K_1 from *S. muticum*

Different conditions were tested to evaluate and optimize the extraction yield of vitamin K_1 from *S. muticum* biomass powder using the EDGE extraction method with n-hexane, including the variation of temperature and initial biomass weight. The EDGE method at an extraction temperature of 50ºC, resulted in a decrease in the extract yield with increasing initial biomass weight, with the highest extract yield of 2,01 ± 0.59% (Table 6) using 0.25 g of initial biomass (Figure 10 A). A preliminary experiment was also performed using an extraction temperature of 60ºC, and the same tendency was observed,

with a slight decrease in extract yield when biomass was increased from 2g to 3g (Table 6 and Figure 10A).

Table 6: Yield of the biomass extracted and concentration of the marine vitamin K_1 in S. muticum using different conditions of EDGE extraction with n-hexane

Extraction method	Temperature (ºC)	Initial biomass (g)	Extract yield (%)	Extract yield independent experiments (n)	Marine K_1 extracted (µg/g dry weight)	Marine K_1 quantification analysis independent experiments (n)
EDGE	50	0.25	2.014 ± 0.51	n = 3	2.197 ±0.448	n = 3
	50	1.00	0.763 ± 0.06	n = 6	3.401 ± 0.62	n = 2
	50	2.00	0.651 ± 0.02	n = 12	4.317 ± 0.43	n = 3
	60	2.00	0.747 ± 0.06	n = 5	4.347	n = 1
	60	3.00	0.631 ± 0.01	n = 5	10.982	n = 1

In all the chromatograms of extracts obtained with monitorization at 280 nm and fluorometric detection (ex 243 nm/em 430 nm) it was demonstrated that it was possible to separate the marine vitamin K_1 as a separated peak with retention time of 17 minutes, approximately, from the other alga eluted components, as shown in Figure 11.

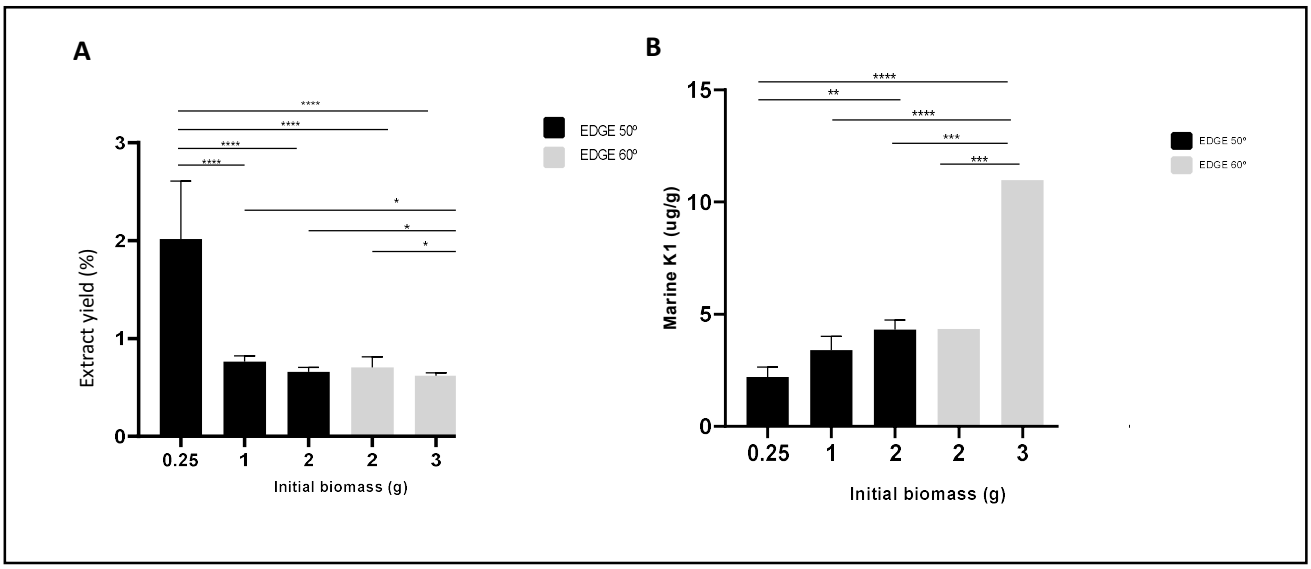


Figure 10. Extract yield and vitamin K1 obtained from Sargassum muticum using different EDGE extraction conditions with n-hexane. (A) Extract yield (%) extract obtained from EDGE with n-hexane in different conditions of initial biomass and temperature Data representative of at least three independent experiments (n≥3). (B) Quantification of marine K1 isolated from EDGE n-hexane extracts (different conditions of initial biomass and temperature), Data representative of n=1-3 (see table 6). Statistical significance was defined as p < 0.05, p < 0.01 (**) and p<0.0001 (****).

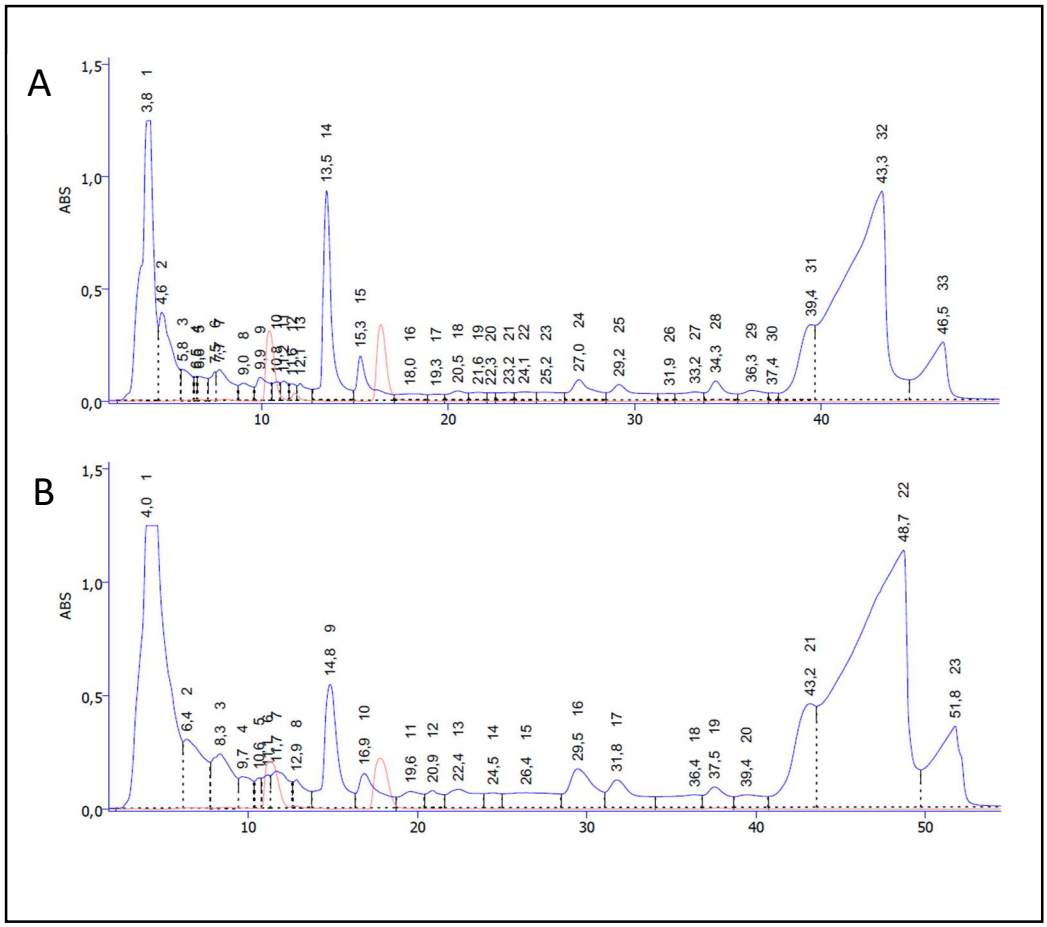


Figure 11: Sargassum muticum extracts chromatograms profiles. (A) Chromatogram representative of S. muticum n-hexane extract of 2 grams biomass, 50℃. (B)Chromatogram representative of S. muticum extract of 2 grams biomass, at 60℃ , spiked with MK4 (retention time ≈10 minutes) . Y axis represents absorbance m(v) and x-axis Red line is the fluorescence absorbance (ex 243nm/em 430 nm) , blue line represents absorbance at 280nm. RP-HLPC conditions as described under 3.1. Flow rate 1mL/min injection volume 0.5 ml loop

After analysis of the results obtained with EDGE at 50ºC of extraction temperature we could conclude that the extract yield increases with the decrease of initial biomass weight, while the concentration of vitamin K_1 extracted and quantified by RP-HPLC increases with the increment of algae biomass weigh. Importantly, these results demonstrate that vitamin K_1 can be separated and purified using the implemented method described in figure 7.

4.3 Evaluation of the efficiency of the EDGE method with n-hexane to extract vitamin K from *S. muticum* biomass

To study the efficiency of extraction of vitamin K from *S.muticum* biomass using the EDGE method with n-hexane as solvent, experiments were conducted in the same conditions previously described for vitamin K_1 extraction, spiked with MK_4 (5µg g_{-1} dry biomass) by adding directly a solution of MK_4 dissolved in n-hexane to the alga biomass prior to the extraction. The results of MK_4 quantitation in the final extract represent the percentage of spiked MK_4 recovery obtained for each condition, ranged from 38.4 ± 5.4% for 1g initial biomass to 64,3 ± 1.3 % for 2g initial biomass at 50ºC (Figure 12). Temperature appears to not effect on MK_4 recovery since at 60ºC with 2 g of initial biomass, the percentage of recovery is similar to that at 50ºC using the same 2 g of initial biomass. Slightly higher recovery was obtained at 60ºC with 3g biomass (Figure 12). However, the experiments at 60ºC were only performed once and need additional repetitions to clearly evaluate the effect of temperature in the % of MK4 recovery.

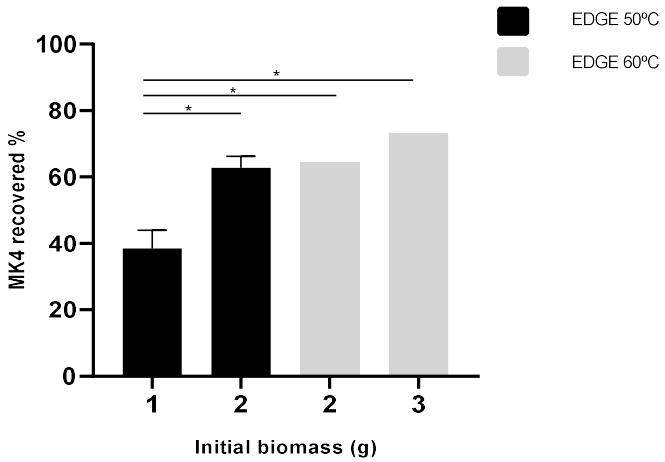


Figure 12 Spiked MK4 (%) recovery of EDGE different conditions of temperature 50ºC (black), 60 ºC (grey) and initial biomass weight (1, 2 and 3g). Analysis obtained from RP-HPLC as described in the method section (3.1) Data represented was obtained from n=2 (EDGE 50º,1g) and from n=3 (EDGE 50º,2g), conditions EDGE 60º were obtained from n=1, Statistical significance was defined as p < 0.05 (*)

To further explore the efficiency of vitamin K recovery in the EDGE method and sample treatment post-extraction, two different experiments were performed: 1) vitamin K_1 and MK_4 followed the complete EDGE extraction method at a 50ºC (see detailed conditions in table 4) and the post sample treatment (Figure 7A and B); 2) vitamin K_1 and MK_4 were only submitted to the post sample treatment (see figure 7B).The results showed that post sample treatment had a low recovery yield of vitamin K with only 44.83% and 42.5 % of the initial K_1 and MK_4, respectively (Figure 13). Recovery yield of samples submitted to

EDGE and post sample treatment (See figure 7A and B) was 49.95% and 53.16% for K_1 and MK_4, respectively (Figure 13). These results show that low recovery yield of vitamin K_1 and MK_4, analysed with RP-HPLC, of samples extracted with EDGE and exposed to post extraction procedure, is mainly due the post-extraction treatment (Figure 13).

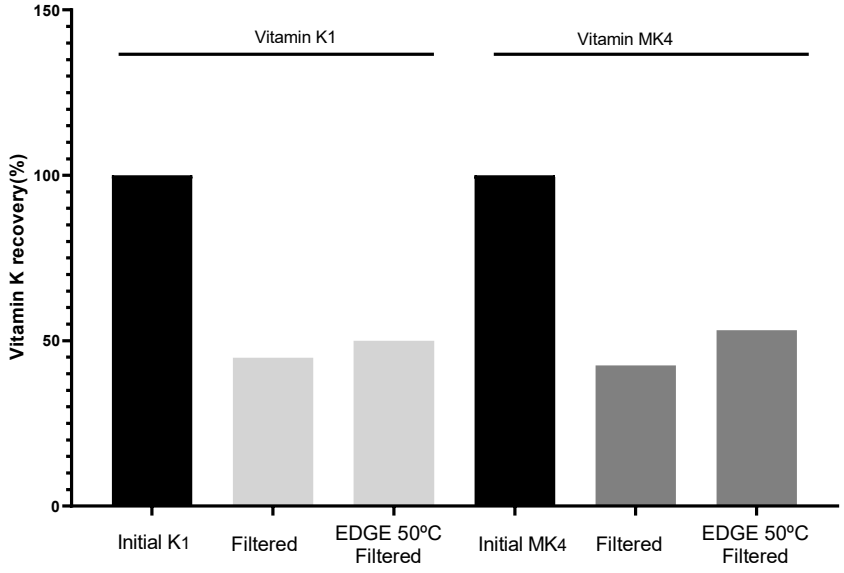


Figure 13: Analytical standards of vitamin K1 and MK4 were separated and quantified by RP-HPLC after EDGE (50ºC) and sample filtration and treatment method as described in the section 3.1. Data is representative of one independent experiment (n=1).

4.4 Study of the effect of temperature on vitamin K_1 and MK_4

Analytical standards of vitamin K_1 and MK_4 exposed to temperature of 50 ºC for 30 minutes showed to have a decrease in RP-HPLC quantification, when compared to vitamin K exposed to room temperature (22ºC) (Figure 14). Vitamin K_1 exposed to 50ºC had a significant decrease of 63.4% when RP-HPLC quantified, while MK_4 decreased

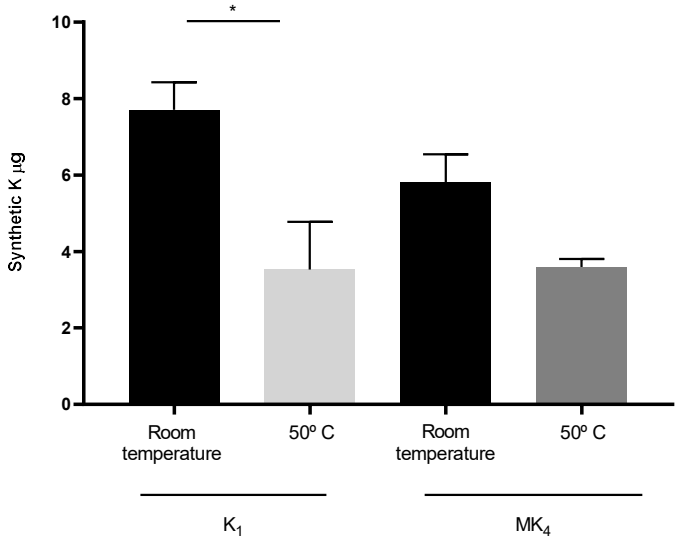


Figure 14: Analysis of the effect of temperature on vitamin K1 and MK4 stability. (A) Ten µg of analytical standards of vitamin K1 and MK4 were exposed to room temperature (± 22ºC) and 50ºC for 30 minutes and analysed by RP-HPLC. Data represented was obtained from n=3, statistical significance was defined as p≤0.05 (*)

vitamin K exposed to room temperature (22ºC) (Figure 14). Vitamin K_1 exposed to 50ºC had a significant decrease of 63.4% when RP-HPLC quantified, while MK_4 decreased 40.8% (Figure 14).

To further explore the effect of temperature on vitamin K's stability, it was analysed the chromatographic profile of a mixture with known concentrations of analytical standards vitamin K_1 and MK_4 exposed to 50ºC and room temperature (22ºC) for 30 minutes. RP-HPLC chromatographic profiles did not show any signs of degradation of the samples exposed to 50ºC when compared to the chromatographic profile of a sample exposed to room temperature (± 22ºC). (Figure 15 A, 15B). The peaks observed in the beginning are part of the analytical vitamin K_1 and MK_4 (data not shown), since the standards used do not have a purity of 100%.

The results indicate that analytical samples quantified by RP-HPLC have a decrease in concentration when exposed to 50ºC comparatively with samples incubated at room temperature (Figure 14), however they do not present any signs of degradation (Figure 15).

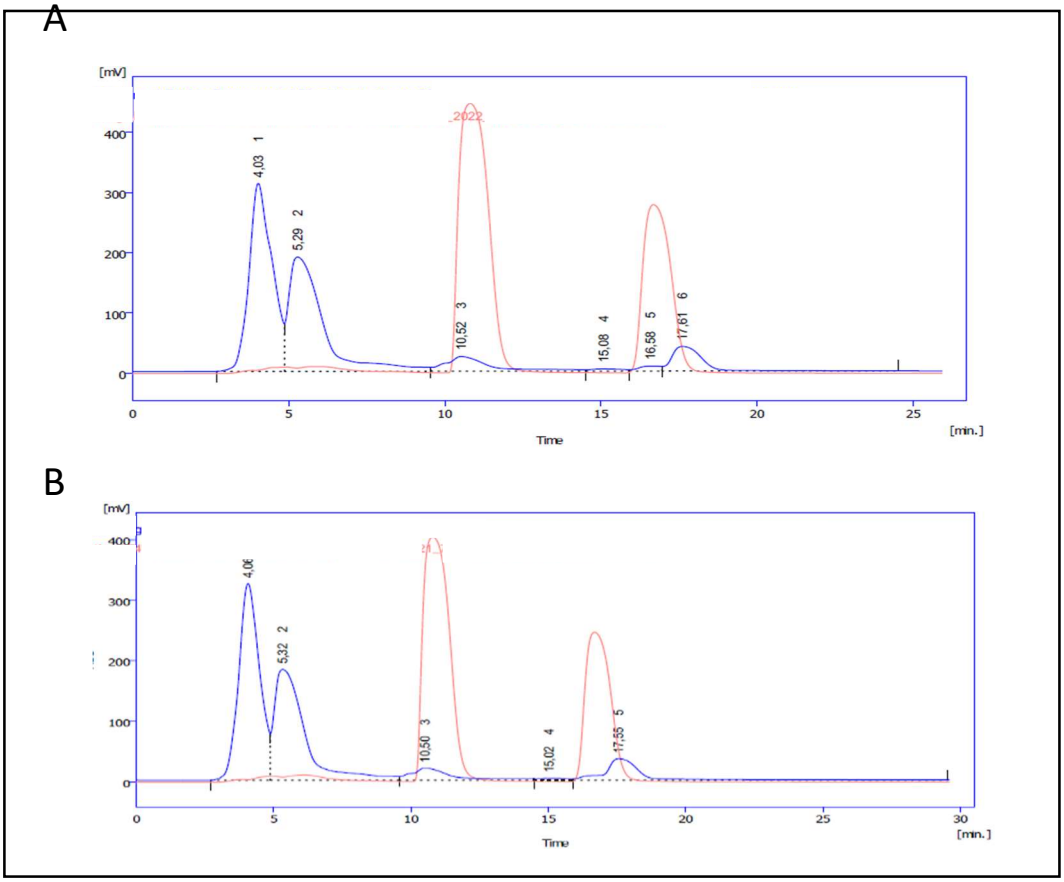


Figure 15. Chromatograms profiles representative of the effect of temperature on vitamin K1 and MK4. (A)-RP-HPLC Chromatogram of a sample containing analytical MK4 and K1 at room temperature for 30 min. (B) RP-HPLC chromatogram of analytical MK4 and K1 exposed to 50℃ for 30 minutes Red line is the fluorescence absorbance (excitation 243 nm/emission 430 nm), blue line absorbance at 280nm. All samples were injected in a volume of 0.5 ml loop

4.5 Sargassum muticum extracts bioactivity

4.5.1 Antioxidant bioactivity of S.muticum extracts

The antioxidant bioactivity of the *S. muticum* EDGE n-hexane extract (60 ºC, 3 g biomass) was evaluated by the DPPH assay using different concentrations of the extract (1.25 - 5 mg/mL). This was a preliminary experience since only one extract was tested. The results obtained showed that the extract presented high DPPH scavenging activity with highest

scavenging activity at a concentration of 5 mg/mL (62 ± 2.36%) (Table 7). In the lowest concentrations tested (0.625 and 0.313 mg/mL) no scavenging activity was detected.

Table 7: Scavenging activity of Sargassum muticum EDGE n-hexane extracts obtained with DPPH assay. Data represented was obtained from n=3.

Extract	Extract concentration (mg/mL)	% Scavenging activity	Standard Deviation (%)
EDGE 60º (3g)	5	62	± 2.26
	2.5	20	± 6.07
	1.25	12	± 4.62

4.5.2 Cytotoxicity and anti-inflammatory activity of purified vitamin K_1, MK4 and Sargassum muticum extracts in THP-1 macrophage (THP-1 Mac) cells

Prior to explore the anti-inflammatory effect of analytical vitamin K standards and *S. muticum* extracts, a cell viability experiment was performed to evaluate possible compounds toxicity. Cell proliferation assays using the MTS method showed that synthetic vitamin K (phylloquinone and menaquinone 4) at the concentrations of 10, 50 and 500 μM did not present a statistically significant effect in THP-1 Mac cells viability, nor the vehicle ethanol at 2% (v/v) (Figure 16 A). Also, the *Sargassum muticum* extracts, obtained by EDGE using 1g of initial biomass at 50ºC, did not present any decrease in THP-1 Mac cells viability at the concentrations of 10, and 100 μg/ml, neither the vehicle DMSO at 0.5% (v/v). Indeed, the results indicate a dose-dependent increase in cell viability in all extracts. Extract 3 at 10 μg/ml showed the lowest cell viability of 87.3 ± 4.1 % (Figure 16 B).

The potential preventive anti-inflammatory activity of synthetic vitamin K_1 and MK_4, and *S. muticum* extracts, was evaluated by measuring the levels of the pro-inflammatory cytokine IL-8 accumulated in the cell culture media of THP-1 Mac cells, pre-treated with the purified compounds and extracts for 24 h, followed by inflammation stimulation with LPS for additional 24 h (Figure 17 A and 17 B). Increased IL-8 levels in THP-1 Mac cells treated with LPS, when compared to untreated control cells, showed a pro-inflammatory response to LPS, while cells pre-treated with the positive anti-inflammatory dexamethasone (DXMT) showed a decrease in IL-8 levels when compared with LPS-treated cells (Figure 17 A and 17 B). Cells pre-treated with vitamin K_1 at the

concentrations of 10 and 50µM showed a significant decrease in the IL-8 levels, relative to the cells only stimulated with LPS (Figure 17 A). Pre-treatments with MK4 only showed decreased IL-8 levels in THP-1 Mac cells at a concentration of 10µM (Figure 17 A). Cells pre-treated with *S. muticum* extracts 1 and 2 showed a significant decrease in IL-8 levels at 10 µg/mL when compared with the cells only stimulated with LPS (Figure 17 B). In addition, *S. muticum* extract 2 at 100 µg/mL present the highest decrease in IL-8 levels. Pre-treatments with extract 3 did not show any significant decrease on IL-8 levels at any of the concentrations tested (Figure 17 B). These results indicate an anti-inflammatory effect on THP-1 LPS-stimulated cells pretreated with a nalytical vitamin K_1 and MK4 as well the *S.muticum* extrats

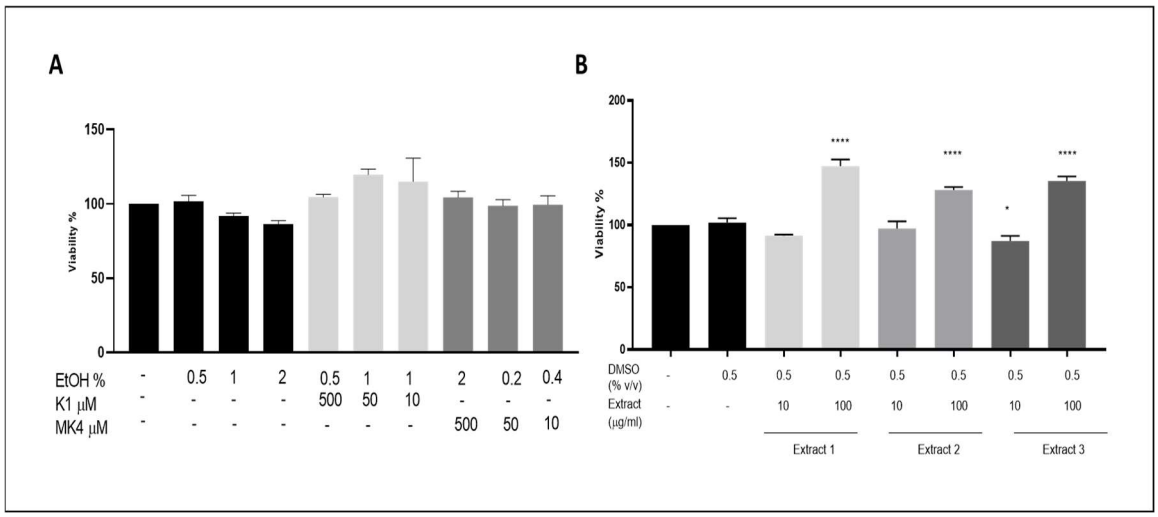


Figure 16: Toxicity effect of commercially available Vitamin K1 and MK4, and Sargassum muticum EDGE extracts (1g, 50℃) on THP-1 macrophages (THP-1 Mac) cells. (A) THP-1 Mac cell viability when exposed to vitamin K1 and MK4 (10, 50, 500 µM) for 48hours. (B) THP-1 Mac cell viability when exposed to extracts 1-3 (10 and 100 µg/ml) for 48 hours. Ethanol (A) and DMSO (B) were tested in LPS-stimulated cell as control for the vehicles. Data representative of one independent experiment and presented as mean ± SD of triplicates. Statistical significance was defined as $p < 0.05$, $p <= 0.01$ (**) and $p<0.0001$ (****).

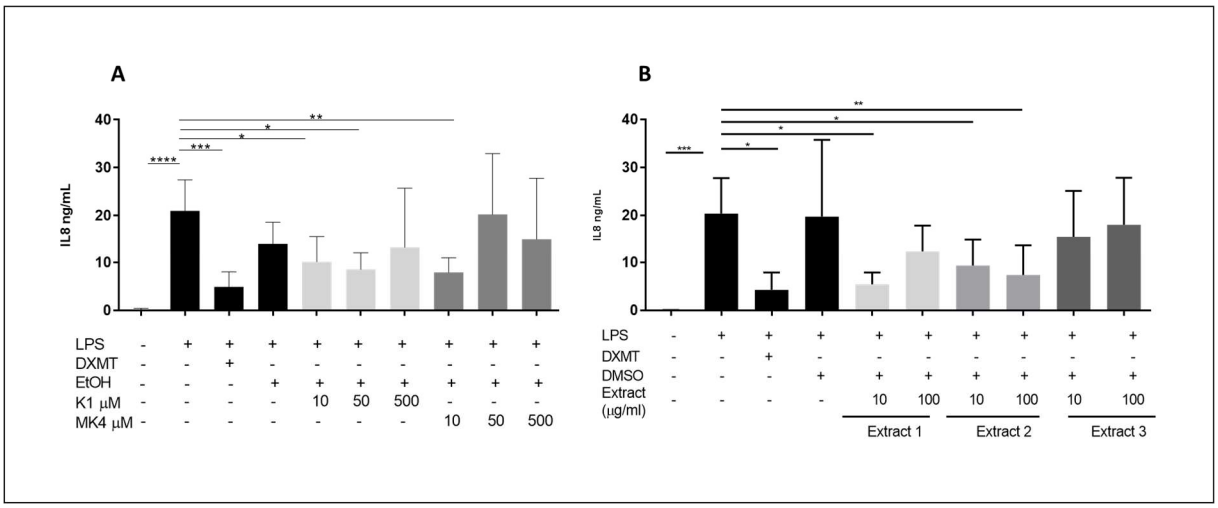


Figure 17: Anti-inflammatory effect of Vitamin K_1 and MK_4 (A), and Sargassum muticum extracts (B) on THP-1 macrophages (THP-1 Mac) cells. Evaluation of the inflammatory marker IL-8 was performed by ELISA in the cell culture media of THP-1 Mac treated for 24 h with K1 and MK4 (10, 50, 500 µM) (A) and with the S. muticum EDGE extracts (B) (10, and 100 µg/ml), and then stimulated with LPS (100 ng/mL) for further 24 h.Dexametasone (DXMT) (2 µM) was used as a positive anti-inflammatory control, ethanol (A) and DMSO (B) were tested in LPS-stimulated cell as control for the vehicles with and non-stimulated cells were used as controls for LPS stimulation. Data are representative of 3 and 2 (A, and B respectively) independent experiment and presented as mean ± SD of triplicates. Statistical significance was defined as $p < 0.05$, $p < 0.01$ (**) and $p<0.0001$ (****).

4.5.3 Anti-inflammatory activity of purified vitamin K_1, MK4 and Sargassum muticum extracts in vascular smooth muscle cells (VSMCs)

The preventive anti-inflammatory activity of commercially available vitamin K_1 and MK4, and EDGE *S. muticum* extracts was also explored on VSMCs inflamed with TNF-α. In these preliminary experiments, levels of IL-8 were unaffected by pre-treatments with either vitamin K_1 or MK4 at all concentrations tested, when compared to TNF-α treated cells (Figure 18 A). Similarly, VSMC cells pre-treated with the *S. muticum* extracts did not present any significant effect on IL-8 levels. Interestingly, the extract 2 showed a dose-dependent tendency, where higher concentrations had lower levels of IL-8 (Figure 18 B).

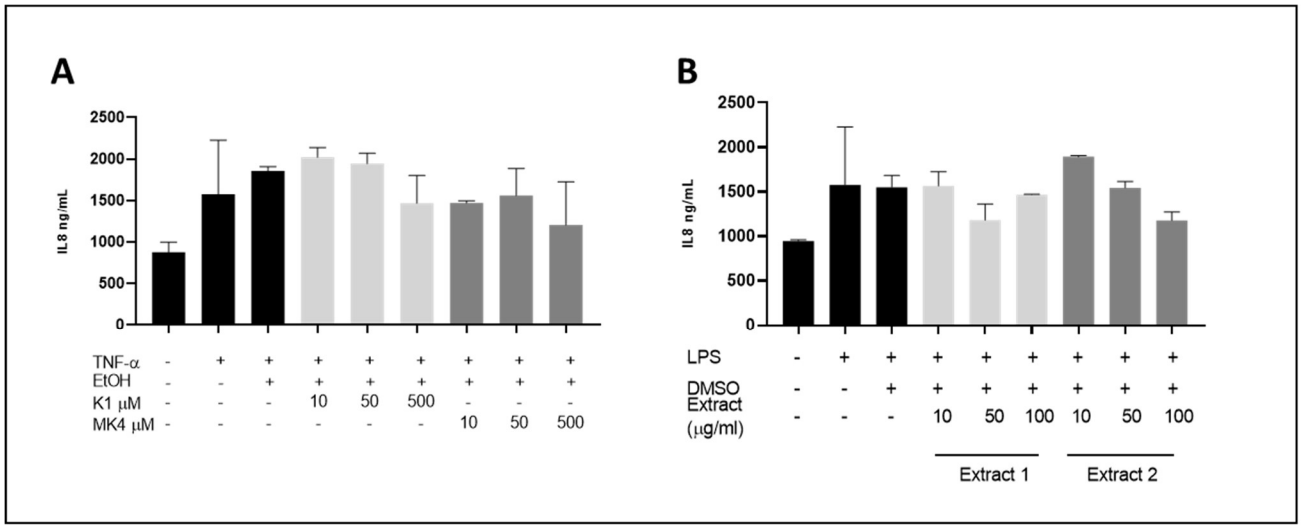


Figure 18. IL-8 levels in the conditioned media of VSMCs pre-treated with vitamin K1 and MK4 at the concentrations 10,50 and 500 μM (A) and Sargassum muticum extracts obtained from EDGE (1gram, 50ºC) at the concentrations 10, 50 and 100 μg/ml) (B), for 24 hours and exposed to 20 ng/mL TNF-α for another 24h. IL-8 concentration on the conditioned media was measured by ELISA. Data are representative of two (A) and three (B) e independent experiment and presented as mean ± SD of quadruplicates.

4.5.3 Effect of vitamin K_1, menaquinone 4 and S. muticum extracts on the gamma-carboxylation profile of vascular smooth muscle cells

It is known that a major function of vitamin K in VSMCs is the gamma-carboxylation of target VKDPs, but a relationship between VKDPs gamma-carboxylation and inflammation is mostly unknown in these cells. To explore a possible relationship between the effect of vitamin K and *S. muticum* - vitamin K_1 containing extracts on VKDPs gamma-carboxylation during inflammation, an overall profile of gamma-carboxylation was explored in VSMCs pre-treated with both vitamin K_1 and MK4, and *S. muticum* extracts for 24 h, followed by inflammation stimulation with TNF-α for additional 24h, using the M3B monoclonal antibody specific for Gla residues.

Western blot analyses of VSMCs total protein extracts with the M3B monoclonal antibody showed a multiple band detection pattern, most probably reflecting the different VKDPs known to be present in VSMCs [32, 164]. Due to the complexity of the profile obtained from M3B Western Blot (Figure 19 A), only the bands that presented higher M3B signal intensity (highlighted in green, red and blue in Figure 19 A) were analysed for semi-quantification using GAPDH (Figure 19 B) normalization. Relative quantification normalized to GAPDH of the band highlighted in red (Figure 19 A) showed decreased gamma-carboxylation in cells treated with TNF-α when compared to the control cells (Figure 19 C), while similar gamma-carboxylation levels were found between control and TNF-α treated cells for the two other bands analysed, highlighted in

blue and green (Figure 18 D, E, respectively). Pre-treatments of VSMCs with vitamin K_1 and MK_4 at concentrations of 10 μM and 50 μM, indicate increased gamma-carboxylation of bands highlighted in red and blue, relatively to the control and TNF-α treated cells (Figure 19 C, 19 D) Similar increased gamma-carboxylation levels were also found for these red and blue bands when VSMCs are pre-treated with *S. muticum* extracts (Figure 19 C, 19 D), clearly suggesting the presence of active vitamin K_1 in these extracts. However, the band highlighted in green (Figure 19 A) showed a very different gamma-carboxylation profile where only MK4 at 50 μg/ml increased γ-carboxylation when compared to both control and TNF-α treated cells, while none of the other conditions seemed to affect the γ-carboxylation levels (Figure 19 E). These preliminary results indicate that inflammation might not affect all VKDPs gamma-carboxylation similarly, and additional experiments are required to further elucidate the identity and specific response of the VKDPs involved in this process.

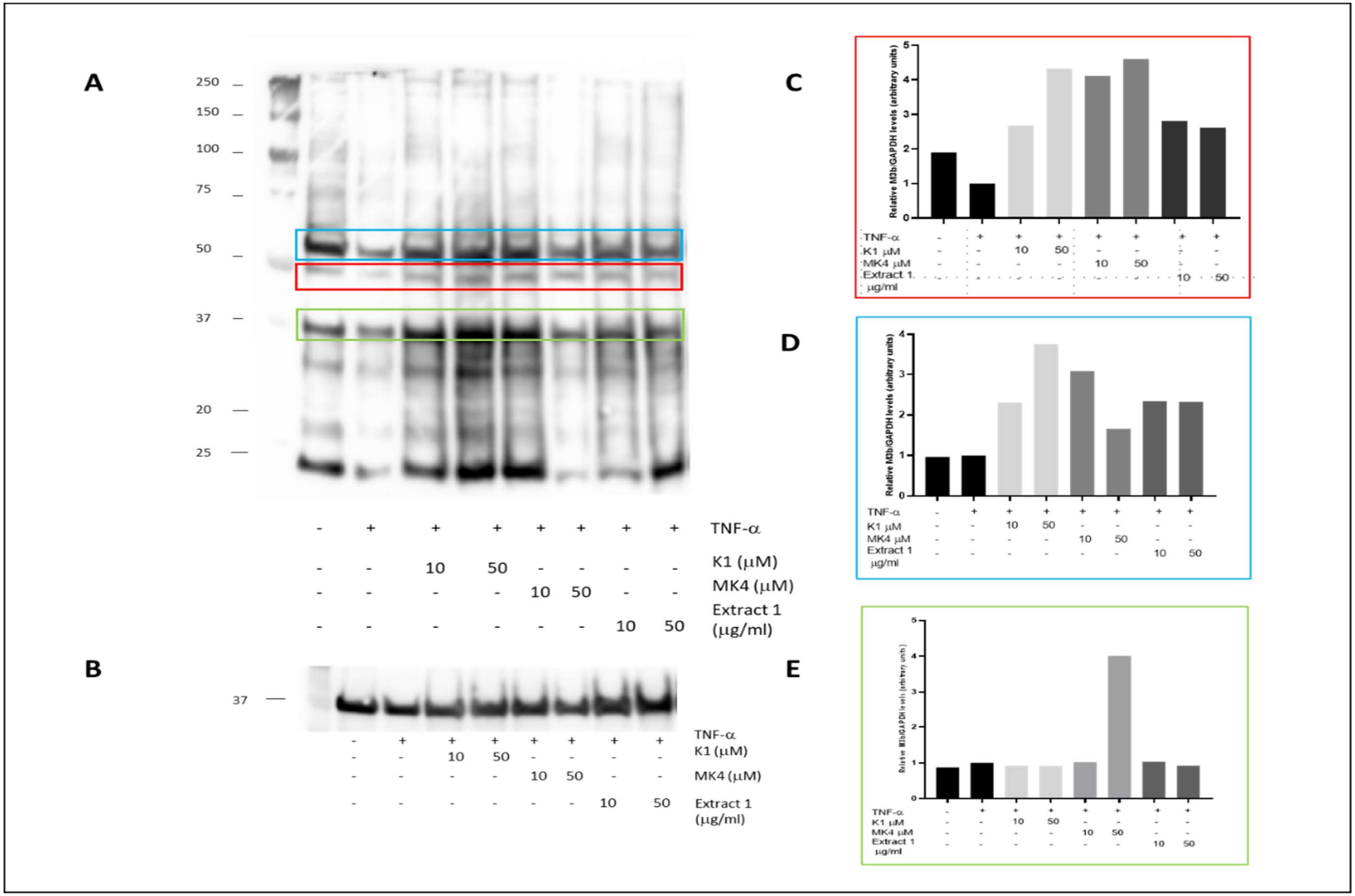


Figure 19: Gamma carboxylation pattern of VSMCs pre-treated with vitamin K1 and MK4, and S. muticum extracts, and under inflammation stimulation. VSMCs were exposed for 24 hours to Vitamin K1 and MK4 at the concentrations of 10 and 50 μM, and Sargassum muticum EDGE (1g, 50ºC) in the concentrations of 10 and 50 μg/ml, followed by inflammation stimulation with 20 ng/mL TNF-α for another 24 hours. Twenty micrograms of total protein cell extracts were analysed by western blot to detect Gla residues using the M3B monoclonal antibody (A), and GAPDH protein (B). (C-E) Relative protein levels normalized to GAPDH of bands highlighted in red (C), blue (D) and green (E) boxes in panel A using LabImage software and presented as arbitrary units. Data are representative of one independent experiment .

5. Discussion

This project demonstrated that it is possible to extract and purify marine vitamin K_1 from the invasive alga *Sargassum muticum* using Energized dispersive guided extraction (EDGE) combined with RP-HPLC. To improve vitamin K_1 extraction efficiency by EDGE, parameters such as initial alga biomass and temperature, were optimized. Based on the results obtained at 50ºC it was possible to conclude that, when increasing the initial alga biomass while maintaining the solvent final volume and temperature, the extract yield decreased, having the highest yield extraction (2.014%) with 0.25 g of initial biomass. It was also shown that the quantity of marine vitamin K_1 extracted increased with the initial biomass with 4.317 μg/g K_1 at the condition of 2 grams biomass and 50ºC, and with of 10.982 μg/g K_1 at the conditions of 3g initial biomass and 60ºC of extraction temperature. Nevertheless, to further explore and confirm these results other conditions of biomass and temperature should be tested. Importantly at least two more independent experiments should be performed to confirm the results obtained with EDGE 60ºC 2 and 3 grams of biomass. Furthermore, parameter such as biomass:solvent ratio, should be taken in consideration and optimized in the near future.

When analysing in more detail the efficiency of the extraction pipeline used in this project (Figure 7), we could conclude that the process involves a considerable loss of vitamin K_1 that is more pronounced in the post sample treatment namely in the filtration, concentration, and resuspension of the extract (Figure 13). In this part of the process (Figure 7B) we obtained 50% and 53.2% recovery efficiency, for vitamin K_1 and MK_4 respectively showing that other extraction methods should be tested to overcome this situation and improve the recovery of vitamin K_1 from *S. muticum*. In a recent study, extraction of vitamin K_1 using ultrasound-assisted extraction (UAE) combined with solid-phase extraction (SPE) showed a minimum recovery yield of 80.9% [120]. Furthermore in another vitamin K_1 extraction study, accelerated solvent extraction method showed a recovery yield in a range of 90-120% [165]. However, it is important to consider, that in both studies mentioned vitamin K_1 was extracted from a food matrix, and not from a macroalgae.

One alternative extraction method that could be tested is supercritical fluid extraction with CO_2. This method focusses on the critical point of the matter, the critical point of a

substance. This process is considered very safe, and sustainable due to recycling properties of CO_2 and it is already used by the food and pharmaceutical industry. Supercritical fluid with CO_2 has been shown to be a great technique to extract vitamin E, a hydrophobic vitamer with a similar polarity to vitamin K [166]. Another point, worth mentioning is that the concentration of Vitamin K_1 extracted in this project was 4.317 μg/g at the condition of 2 g at 50ºC, and 10.982 μg/g at the condition of 3 g at 60ºC. Even considering the low recovery yield of the method employed, is still very different from the reported 750 μ/g. of vitamin K_1 extracted from *Sargassum muticum*. Nevertheless, in this particular study, the authors did not describe the extraction methods.

To further track the losses of vitamin K during the extraction process we performed an experiment where the MK_4 analytical standard was added to the *S. muticum* biomass before extraction with EDGE and n-hexane. This helped us to obtain the percentage of recovery of the spiked vitamin MK_4. It is important to highlight that this does not reflect entirely the efficiency of the extraction itself, since the added standard MK_4 was already resuspended in the extraction solvent (n-hexane), while with vitamin K_1 we aim to extract is inside the cells, requiring cell wall rupture and extraction with the solvent. The results of quantification of MK_4 in the spiked samples showed that MK_4 recovery increased with higher initial biomass weight, in agreement with the data obtained for the vitamin K_1 yield extraction.

Increasing the extraction temperatures from 50º to 60º C did not appear to influence the extract yield and the vitamin K_1 isolated, however this experiment was performed only once and more independent experiments at the conditions of 60ºC should be performed to have significant data. In addition, in the experience performed to evaluate the vitamin K_1 stability with temperature, the results shown that there was no change in the chromatographic profile when vitamin K_1 was subject to 50ºC for 30 min in comparison with room temperature. These results are in concordance with previously studies showing that boiled food (100ºC) had the same amount of vitamin K_1 when compared with the fresh food [11, 12]. Additionally, in another study, extraction of vitamin K_1 using ultrasound assisted extraction with a wide range of tested temperatures (20, 35, 50 and 65ºC), showed that with the increase of temperature from 20 to 50ºC, vitamin K_1 extraction rate would increase slightly with the highest extraction yield at 50ºC , and a significant decrease at the temperature 65ºC[120].

Vitamin K has been reported to have antioxidant activity in its reduced form (hydroquinone, VKH_2) [105, 106] . In this project the *S. muticum* extract obtained by EDGE with n- hexane at 60ºC, was evaluated using the DPPH assay and the results shown a maximum scavenging activity of around 62% at 5 mg/ml concentration. In fact, the extract samples tested for antioxidant activity contained vitamin K_1, however, most likely in the quinone oxidized form since the samples were always protected from light but exposed to air during sample handling and preparation. These is only a preliminary result in terms of antioxidant potential of the tested extract and further studies should be performed namely using the ABTS,ORAC,CUPRAC assays (review in 167). Moreover, the RP-HPLC profile of the DPPH analysed extract, showed several peaks and fractions with green/orange colour, leading us to conclude that other compounds with antioxidant activity, e.g carotenoids or chlorophylls, might be present and be responsible for the antioxidant activity obtained with the DPPH assay [146, 168].

In vitro cell antioxidant assays should also be employed since they can evaluate the uptake of vitamin K_1 by the cells as well as the antioxidant activity of the extracts or compounds. This can be achieved using the cellular antioxidant activity assay (CAA) which is a method based on fluorescent dichlorofluorescein (DCF), a probe that is easily trapped within cells and easily oxidated. The technique is based on measuring the ability to prevent the formation of DCF [169, 170]. Lipid peroxidation activity can also be evaluated using cell-containing *in vitro* models [171] . These two cell-based assays are standardized procedures available in the market for measuring antioxidant activity in different cell lines and should be also used to test the antioxidant bioactivity of the *S. muticum* extracts in the future.

In this work it was demonstrated that commercially available vitamins K_1 and MK_4 have an anti-inflammatory protective effect on inflamed macrophage-like cells. In THP-1 macrophages cells, vitamin K_1 at 10 μM and 50 μM and MK_4 at 10 μM decreased the LPS-induced levels of IL-8. This goes according to previous studies. Ohsaki et al. reported the suppression of the of LPS-induced IL-6 and TNF-α, at gene and protein levels, in human and mice macrophage-like cells treated with vitamin K_1 and MK_4 [99]. However, in the Ohsaki et al experiments the cells were stimulated with 1 μg/ml of LPS, which can be considered very high doses of LPS [172], and not mimicking a low graded inflammation. In our studies aiming to evaluate the preventive action of vitamin K in chronic low grade inflammatory processes associated to chronic inflammatory diseases,

lower concentrations of LPS (100 ng/ml) were used. In a more recent study, the impact of vitamin K as an anti-inflammatory agent was evaluated using lower concentrations of LPS [100]. The results showed a decrease in TNF-α, as well IL-1 α and β expression when THP-1 macrophages cells stimulated with 100 ng/ml LPS were pre-treated with natural MK_7 produced by fermentation at 10 and 100 μM [100].

In this thesis, *Sargassum muticum* extracts showed anti-inflammatory activity. At the concentration of 10 μg/ml, two extracts repressed the levels of LPS-induced IL-8. The anti-inflammatory effect shown in this work goes accordingly to another study with mouse macrophage cells, where several *S.muticum* fractions were studied, and more interestingly the n-hexane fraction showed the most promising results, decreasing the levels of IL-6 and IL-1β, and NOS production on LPS stimulated cells [173].

In this work, the anti-inflammatory effect of Vitamin K_1, MK_4 and *S.muticum* extracts was only analysed by measuring levels of one cytokine, IL-8. To have a bigger perspective on the inflammatory response, more cytokines such IL-6 and TNF-α should be studied in the near future. In the prospects, it would be also interesting to analyse extracts with a higher content of marine phylloquinone.

To further explore the anti-inflammatory effect of vitamin K_1, MK4 and *S.muticum* extracts, preventive inflammatory assays were performed in VSMCs, since these cells are exposed to inflammatory conditions in a scenario of many chronic inflammatory diseases. Inflammatory assays with TNF-α stimulated VSMCs did not show any significant effect on IL-8 levels when exposed to vitamin K (K_1 and MK_4) or *Sargassum muticum* extracts. However, it was only performed one experiment with triplicates, and additional independent experiments are required to fully determine the potential preventive effect of vitamin K and S. *muticum* extracts on VCMCs inflammatory processes. This issue is particularly relevant since vascular calcification is known to function with inflammation in a pathological cycle fuelling each other. Although the role of vitamin K in VSMCs has been mostly focused on its function through the gamma-carboxylation of VKDPs with calcification inhibitory roles, such as MGP and GRP [77 , 86] and the anti-inflammatory effect of vitamin K in macrophage cells has been suggested as gamma-carboxylation independent [99], gamma-carboxylated MGP and GRP have been also identified in macrophage cells [40]. In this context, it is interesting to study how inflammation affects VKDPs gamma-carboxylation in VSMCs, and the effect of vitamin K supplementation,

to give further insights into vitamin K mode of action in vascular inflammation. To this aim, a pilot experiment was performed to evaluate the γ-carboxylation levels on TNF-α stimulated VSMCs cells pre-treated with vitamin K_1, MK_4 and *S.muticum* extracts, by Western Blot with the M3B antibody for specific immunodetection of Gla-residues [37]. The Western Blot detection of the Gla residues showed a profile with different molecular weight bands, which very likely reflects the several γ-carboxylated VKDPs. The different bands had different γ-carboxylation profiles, showing different responses to both TNF-α stimulation, and treatments with vitamin K and *S. muticum* extracts. Some bands were shown to have decreased gamma-carboxylation in response to inflammation stimulation with TNF-a, while others were not responsive. However, the majority of the bands presented increased γ-carboxylation in VSMCs pre-treated with synthetic vitamin K_1 and MK_4, and *S.muticum* extracts. These results suggest that VKDPs might be involved in the inflammatory process of VSMCs, although with different responses in terms of gamma-carboxylation dependent function.

These results also show the potential of marine-derived vitamin K_1 ability to increase the γ-carboxylation of VKDP in VSMCs under situation of inflammation. The study of γ-carboxylation only by M3B detection is very limited. In the future, specific VKDPs should be detected and analysed individually, to explore further the involvement of each VKDP in VSMCs inflammation and the role of gamma-carboxylation in this process. Interestingly, the gamma-carboxylation of one particular band appears to be responsive only to MK_4 suggesting a differential effect of the different vitamin K forms on specific VKDPs and highlighting further the complexity of the involvement of different VKDPs in TNF-α stimulated VSMCs. Additionally, M3B immunodetection only provided a comparatively quantification of γ-carboxylation, it is not possible to know by the Western blot method if the proteins are fully or partially γ-carboxylated. Despite the γ-carboxylation assay limitations, it was shown impressive preliminary results, that demonstrates that natural vitamin K_1 from *S. muticum* has the potential to increase vitamin K dependent proteins γ-carboxylation.

6. Conclusion

Overall, this work showed that the concentration of vitamin K_1 extracted was highly dependent on the extractions conditions when extracting with *Energized Dispersive Guided Extraction* system, where vitamin K_1 extracted increased with the initial alga biomass increment. Furthermore, this work demonstrated that n-hexane extracts of *Sargassum muticum* obtained from EDGE as well as synthetic vitamins K_1 and MK_4 have anti-inflammatory properties in human immune cells THP-1 macrophages stimulated with LPS. Furthermore, this thesis demonstrated that *S. muticum* extracts increased γ-carboxylation in TNF-α stimulated Vascular Smooth Muscle cells. This work also showed EDGE extracts great antioxidative activity in a DPPH assay. With these results we showed that *S. muticum* has the potential to be the source for a natural diet supplement rich in vitamin K_1 with anti-inflammatory and antioxidant properties. The use of *S. muticum*, an invasive algae, as a biomass source to produce diet supplement is a two-folded opportunity where, it is possible to create a low cost product and yet help mitigating the invasion of the species.

7. References

1. H, D., *Cholesterinstoffwechsel in Hühnereiern und Hühnchen. .* Biochem Z, 1929. **215: 475–492**

2. Dam, H., *The Antihæmorrhagic Vitamin of the Chick.: Occurrence And Chemical Nature.* Nature, 1935. **135**(3417): p. 652-653.
3. Dam, H. and F. Schønheyder, *The occurrence and chemical nature of vitamin K.* Biochem J, 1936. **30**(5): p. 897-901.
4. Binkley, S.B., et al., *THE ISOLATION OF VITAMIN K1.* Journal of Biological Chemistry, 1939. **130**(1): p. 219-234.
5. MacCorquodale, D.W., et al., *ON THE CONSTITUTION OF VITAMIN K1.* Journal of the American Chemical Society, 1939. **61**(7): p. 1928-1929.
6. Biggins, J., N.A. Tanguay, and H.A. Frank, *Electron transfer reactions in photosystem I following vitamin K1 depletion by ultraviolet irradiation.* FEBS Lett, 1989. **250**(2): p. 271-4.
7. Binkley, S.B., et al., *DERIVATIVES OF VITAMINS K1 AND K2.* Journal of the American Chemical Society, 1939. **61**(6): p. 1612-1613.
8. Nakagawa, K., et al., *Identification of UBIAD1 as a novel human menaquinone-4 biosynthetic enzyme.* Nature, 2010. **468**(7320): p. 117-21.
9. Hassan, G.S., *Menadione.* Profiles Drug Subst Excip Relat Methodol, 2013. **38**: p. 227-313.
10. Schurgers, L.J. and C. Vermeer, *Determination of phylloquinone and menaquinones in food. Effect of food matrix on circulating vitamin K concentrations.* Haemostasis, 2000. **30**(6): p. 298-307.
11. Faria, S.A.S.C., et al., *Vitamin K: Content in food consumed in São Paulo, Brazil.* Brazilian Journal of Pharmaceutical Sciences, 2017. **53**(2).
12. Kamao, M., et al., *Vitamin K content of foods and dietary vitamin K intake in Japanese young women.* J Nutr Sci Vitaminol (Tokyo), 2007. **53**(6): p. 464-70.
13. Schurgers, L.J. and C. Vermeer, *Differential lipoprotein transport pathways of K-vitamins in healthy subjects.* Biochim Biophys Acta, 2002. **1570**(1): p. 27-32.
14. Thijssen, H.H. and M.J. Drittij-Reijnders, *Vitamin K status in human tissues: tissue-specific accumulation of phylloquinone and menaquinone-4.* Br J Nutr, 1996. **75**(1): p. 121-7.
15. Furie, B. and B.C. Furie, *Molecular Basis of Vitamin K-Dependent γ-Carboxylation.* Blood, 1990. **75**(9): p. 1753-1762.
16. Pan, L.C. and P.A. Price, *The propeptide of rat bone gamma-carboxyglutamic acid protein shares homology with other vitamin K-dependent protein precursors.* Proceedings of the National Academy of Sciences, 1985. **82**(18): p. 6109-6113.
17. Price, P.A., J.D. Fraser, and G. Metz-Virca, *Molecular cloning of matrix Gla protein: implications for substrate recognition by the vitamin K-dependent gamma-carboxylase.* Proceedings of the National Academy of Sciences, 1987. **84**(23): p. 8335-8339.
18. Nelsestuen, G.L. and J.W. Suttie, *Mode of action of vitamin K. Calcium binding properties of bovine prothrombin.* Biochemistry, 1972. **11**(26): p. 4961-4.
19. *Vitamin K and prothrombin structure.* Nutr Rev, 1974. **32**(9): p. 279-81.
20. Girolami, A., et al., *Vitamin K-Dependent Coagulation Factors That May be Responsible for Both Bleeding and Thrombosis (FII, FVII, and FIX).* Clin Appl Thromb Hemost, 2018. **24**(9_suppl): p. 42s-47s.

21. Norström, E. and G. Escolar, *CHAPTER 36 - Natural anticoagulants and thrombophilia*, in *Blood and Bone Marrow Pathology (Second Edition)*, A. Porwit, J. McCullough, and W.N. Erber, Editors. 2011, Churchill Livingstone: Edinburgh. p. 583-595.
22. Trumbo, P., et al., *Dietary reference intakes: vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc.* J Am Diet Assoc, 2001. **101**(3): p. 294-301.
23. Price, P.A., et al., *Characterization of a gamma-carboxyglutamic acid-containing protein from bone.* Proceedings of the National Academy of Sciences of the United States of America, 1976. **73**(5): p. 1447-1451.
24. Price, P.A., J.W. Poser, and N. Raman, *Primary structure of the gamma-carboxyglutamic acid-containing protein from bovine bone.* Proceedings of the National Academy of Sciences of the United States of America, 1976. **73**(10): p. 3374-3375.
25. Willems, B.A., et al., *The realm of vitamin K dependent proteins: shifting from coagulation toward calcification.* Mol Nutr Food Res, 2014. **58**(8): p. 1620-35.
26. Hoang, Q.Q., et al., *Bone recognition mechanism of porcine osteocalcin from crystal structure.* Nature, 2003. **425**(6961): p. 977-80.
27. Ducy, P., et al., *Increased bone formation in osteocalcin-deficient mice.* Nature, 1996. **382**(6590): p. 448-452.
28. Lambert, L.J., et al., *Increased trabecular bone and improved biomechanics in an osteocalcin-null rat model created by CRISPR/Cas9 technology.* Dis Model Mech, 2016. **9**(10): p. 1169-1179.
29. Moriishi, T., et al., *Osteocalcin is necessary for the alignment of apatite crystallites, but not glucose metabolism, testosterone synthesis, or muscle mass.* PLOS Genetics, 2020. **16**(5): p. e1008586.
30. Schurgers, L.J., et al., *Post-translational modifications regulate matrix Gla protein function: importance for inhibition of vascular smooth muscle cell calcification.* J Thromb Haemost, 2007. **5**(12): p. 2503-11.
31. Spronk, H.M., et al., *Matrix Gla protein accumulates at the border of regions of calcification and normal tissue in the media of the arterial vessel wall.* Biochem Biophys Res Commun, 2001. **289**(2): p. 485-90.
32. Schurgers, L.J., et al., *Novel conformation-specific antibodies against matrix γ-carboxyglutamic acid (Gla) protein: Undercarboitylated matrix Gla protein as marker for vascular calcification.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2005. **25**(8): p. 1629-1633.
33. Schurgers, L.J., E.C. Cranenburg, and C. Vermeer, *Matrix Gla-protein: the calcification inhibitor in need of vitamin K.* Thromb Haemost, 2008. **100**(4): p. 593-603.
34. Price, P.A., M.R. Urist, and Y. Otawara, *Matrix Gla protein, a new gamma-carboxyglutamic acid-containing protein which is associated with the organic matrix of bone.* Biochem Biophys Res Commun, 1983. **117**(3): p. 765-71.
35. Viegas, C.S., et al., *Gla-rich protein (GRP), a new vitamin K-dependent protein identified from sturgeon cartilage and highly conserved in vertebrates.* J Biol Chem, 2008. **283**(52): p. 36655-64.
36. Cancela, M.L., N. Conceição, and V. Laizé, *Gla-rich protein, a new player in tissue calcification?* Advances in nutrition (Bethesda, Md.), 2012. **3**(2): p. 174-181.
37. Viegas, C.S.B., et al., *Gla-rich protein is a novel vitamin K-dependent protein present in serum that accumulates at sites of pathological calcifications.* The American journal of pathology, 2009. **175**(6): p. 2288-2298.
38. Viegas, C.S.B., et al., *Expression of Gla-rich protein (GRP) in newly developed cartilage-derived cell cultures from sturgeon (Acipenser naccarii).* Journal of Applied Ichthyology, 2010. **26**(2): p. 214-218.

39. Viegas, C.S.B., et al., *Gla-rich protein acts as a calcification inhibitor in the human cardiovascular system.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2016. **35**(2): p. 399-408.
40. Viegas, C.S.B., et al., *Gla-rich protein function as an anti-inflammatory agent in monocytes/macrophages: Implications for calcification-related chronic inflammatory diseases.* PLoS ONE, 2017. **12**(5).
41. Viegas, C.S.B., et al., *Chronic kidney disease circulating calciprotein particles and extracellular vesicles promote vascular calcification: A role for GRP (Gla-Rich Protein).* Arteriosclerosis, Thrombosis, and Vascular Biology, 2018. **38**(3): p. 575-587.
42. Willems, B.A., et al., *Ucma/GRP inhibits phosphate-induced vascular smooth muscle cell calcification via SMAD-dependent BMP signalling.* Scientific Reports, 2018. **8**(1).
43. Cavaco, S., et al., *Gla-rich protein is involved in the cross-talk between calcification and inflammation in osteoarthritis.* Cell Mol Life Sci, 2016. **73**(5): p. 1051-65.
44. Viegas, C., et al., *The interplay between mineral metabolism, vascular calcification and inflammation in Chronic Kidney Disease (CKD): Challenging old concepts with new facts.* Aging, 2019. **11**(12): p. 4274-4299.
45. *Unknown article.*
46. Manfioletti, G., et al., *The protein encoded by a growth arrest-specific gene (gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade.* Mol Cell Biol, 1993. **13**(8): p. 4976-85.
47. Goudarzi, S., S.E. Gilchrist, and S. Hafizi, *Gas6 Induces Myelination through Anti-Inflammatory IL-10 and TGF-β Upregulation in White Matter and Glia.* Cells, 2020. **9**(8).
48. Tutusaus, A., et al., *Role of Vitamin K-Dependent Factors Protein S and GAS6 and TAM Receptors in SARS-CoV-2 Infection and COVID-19-Associated Immunothrombosis.* Cells, 2020. **9**(10).
49. Norris, R.A., et al., *The many facets of the matricelluar protein periostin during cardiac development, remodeling, and pathophysiology.* J Cell Commun Signal, 2009. **3**(3-4): p. 275-86.
50. Rached, M.T., et al., *FoxO1 expression in osteoblasts regulates glucose homeostasis through regulation of osteocalcin in mice.* Journal of Clinical Investigation, 2010. **120**(1): p. 357-368.
51. Oury, F., et al., *Osteocalcin regulates murine and human fertility through a pancreas-bone-testis axis.* The Journal of Clinical Investigation, 2013. **123**(6): p. 2421-2433.
52. Viegas, C.S.B., et al., *Nanoencapsulation of Gla-Rich Protein (GRP) as a Novel Approach to Target Inflammation.* International Journal of Molecular Sciences, 2022. **23**(9): p. 4813.
53. Hasanbasic, I., I. Rajotte, and M. Blostein, *The role of γ-carboxylation in the anti-apoptotic function of gas6.* Journal of Thrombosis and Haemostasis, 2005. **3**(12): p. 2790-2797.
54. Mao, S., et al., *Overexpression of GAS6 Promotes Cell Proliferation and Invasion in Bladder Cancer by Activation of the PI3K/AKT Pathway.* Onco Targets Ther, 2020. **13**: p. 4813-4824.
55. Heo, S.C., et al., *Periostin Accelerates Bone Healing Mediated by Human Mesenchymal Stem Cell-Embedded Hydroxyapatite/Tricalcium Phosphate Scaffold.* PLOS ONE, 2015. **10**(3): p. e0116698.
56. Rios, H., et al., *periostin Null Mice Exhibit Dwarfism, Incisor Enamel Defects, and an Early-Onset Periodontal Disease-Like Phenotype.* Molecular and Cellular Biology, 2005. **25**(24): p. 11131-11144.
57. Kühn, B., et al., *Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair.* Nature Medicine, 2007. **13**(8): p. 962-969.

58. Kulman, J.D., et al., *Primary structure and tissue distribution of two novel proline-rich gamma-carboxyglutamic acid proteins.* Proc Natl Acad Sci U S A, 1997. **94**(17): p. 9058-62.
59. Khazi, F.R., K.C. Chu, and K.A. High, *Transmembrane Gla Protein 4 as a Novel Modulator of ERK2.* Blood, 2006. **108**(11): p. 544-544.
60. Franceschi, C., et al., *The Continuum of Aging and Age-Related Diseases: Common Mechanisms but Different Rates.* Frontiers in Medicine, 2018. **5**.
61. Knapen, M.H.J., et al., *Three-year low-dose menaquinone-7 supplementation helps decrease bone loss in healthy postmenopausal women.* Osteoporosis International, 2013. **24**(9): p. 2499-2507.
62. Mandatori, D., et al., *Three-dimensional co-culture system of human osteoblasts and osteoclast precursors from osteoporotic patients as an innovative model to study the role of nutrients: Focus on vitamin k2.* Nutrients, 2021. **13**(8).
63. Stock, M., et al., *A Dual Role of Upper Zone of Growth Plate and Cartilage Matrix-Associated Protein in Human and Mouse Osteoarthritic Cartilage: Inhibition of Aggrecanases and Promotion of Bone Turnover.* Arthritis Rheumatol, 2017. **69**(6): p. 1233-1245.
64. Binkley, N.C., et al., *Vitamin K supplementation reduces serum concentrations of under-γ-carboxylated osteocalcin in healthy young and elderly adults.* The American Journal of Clinical Nutrition, 2000. **72**(6): p. 1523-1528.
65. Booth, S.L., et al., *Dietary Phylloquinone Depletion and Repletion in Older Women.* The Journal of Nutrition, 2003. **133**(8): p. 2565-2569.
66. Sokoll, L.J. and J.A. Sadowski, *Comparison of biochemical indexes for assessing vitamin K nutritional status in a healthy adult population.* The American Journal of Clinical Nutrition, 1996. **63**(4): p. 566-573.
67. Koitaya, N., et al., *Low-dose vitamin K2 (MK-4) supplementation for 12 months improves bone metabolism and prevents forearm bone loss in postmenopausal Japanese women.* Journal of Bone and Mineral Metabolism, 2014. **32**(2): p. 142-150.
68. Inaba, N., T. Sato, and T. Yamashita, *Low-Dose Daily Intake of Vitamin K₂ (Menaquinone-7) Improves Osteocalcin γ-Carboxylation: A Double-Blind, Randomized Controlled Trials.* Journal of Nutritional Science and Vitaminology, 2015. **61**(6): p. 471-480.
69. Nakamura, E., et al., *Low-dose menaquinone-4 improves γ-carboxylation of osteocalcin in young males: a non-placebo-controlled dose–response study.* Nutrition Journal, 2014. **13**(1): p. 85.
70. Lee, S.J., I.K. Lee, and J.H. Jeon, *Vascular calcification—new insights into its mechanism.* International Journal of Molecular Sciences, 2020. **21**(8).
71. Pazár, B., et al., *Basic Calcium Phosphate Crystals Induce Monocyte/Macrophage IL-1β Secretion through the NLRP3 Inflammasome In Vitro.* The Journal of Immunology, 2011. **186**(4): p. 2495-2502.
72. Poznyak, A.V., et al., *Macrophages and Foam Cells: Brief Overview of Their Role, Linkage, and Targeting Potential in Atherosclerosis.* Biomedicines, 2021. **9**(9).
73. Hjortnaes, J., et al., *Valvular interstitial cells suppress calcification of valvular endothelial cells.* Atherosclerosis, 2015. **242**(1): p. 251-260.
74. Sheen, C.R., et al., *Pathophysiological Role of Vascular Smooth Muscle Alkaline Phosphatase in Medial Artery Calcification.* Journal of Bone and Mineral Research, 2015. **30**(5): p. 824-836.
75. Schinke, T. and G. Karsenty, *Vascular calcification - A passive process in need of inhibitors.* Nephrology Dialysis Transplantation, 2000. **15**(9): p. 1272-1274.
76. Luo, G., et al., *Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein.* Nature, 1997. **386**(6620): p. 78-81.

77. Price, P.A., S.A. Faus, and M.K. Williamson, *Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves.* Arterioscler Thromb Vasc Biol, 1998. **18**(9): p. 1400-7.
78. Murshed, M., et al., *Extracellular matrix mineralization is regulated locally; different roles of two gla-containing proteins.* J Cell Biol, 2004. **165**(5): p. 625-30.
79. Riphagen, I.J., et al., *Prevalence and Effects of Functional Vitamin K Insufficiency: The PREVEND Study.* Nutrients, 2017. **9**(12).
80. Liu, Y.P., et al., *Inactive matrix Gla protein is causally related to adverse health outcomes: a Mendelian randomization study in a Flemish population.* Hypertension, 2015. **65**(2): p. 463-70.
81. Okubo, Y., et al., *Osteoinduction by recombinant human bone morphogenetic protein-2 at intramuscular, intermuscular, subcutaneous and intrafatty sites.* Int J Oral Maxillofac Surg, 2000. **29**(1): p. 62-6.
82. Rosen, V., *BMP2 signaling in bone development and repair.* Cytokine Growth Factor Rev, 2009. **20**(5-6): p. 475-80.
83. Rafael, M.S., et al., *Insights into the association of Gla-rich protein and osteoarthritis, novel splice variants and γ-carboxylation status.* Mol Nutr Food Res, 2014. **58**(8): p. 1636-46.
84. Viegas, C.S., et al., *Gla-rich protein is a potential new vitamin K target in cancer: evidences for a direct GRP-mineral interaction.* Biomed Res Int, 2014. **2014**: p. 340216.
85. Roy, M.E. and S.K. Nishimoto, *Matrix Gla protein binding to hydroxyapatite is dependent on the ionic environment: calcium enhances binding affinity but phosphate and magnesium decrease affinity.* Bone, 2002. **31**(2): p. 296-302.
86. Viegas, C.S.B., et al., *Gla-Rich Protein Acts as a Calcification Inhibitor in the Human Cardiovascular System.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2015. **35**(2): p. 399-408.
87. Palmer, C.R., et al., *Association between vitamin K(1) intake and mortality in the Danish Diet, Cancer, and Health cohort.* Eur J Epidemiol, 2021. **36**(10): p. 1005-1014.
88. Geleijnse, J.M., et al., *Dietary intake of menaquinone is associated with a reduced risk of coronary heart disease: the Rotterdam Study.* J Nutr, 2004. **134**(11): p. 3100-5.
89. Dalmeijer, G.W., et al., *Phylloquinone concentrations and the risk of vascular calcification in healthy women.* Arterioscler Thromb Vasc Biol, 2014. **34**(7): p. 1587-90.
90. Kelly, J.M., et al., *The Contribution of Lipids to the Interindividual Response of Vitamin K Biomarkers to Vitamin K Supplementation.* Mol Nutr Food Res, 2019. **63**(24): p. e1900399.
91. Li, Y., et al., *Vitamin K1 Inhibition of Renal Crystal Formation through Matrix Gla Protein in the Kidney.* Kidney and Blood Pressure Research, 2019. **44**(6): p. 1392-1403.
92. te Velde–Keyzer, C.A. and M.H. de Borst, *Vitamin K supplementation and vascular health after kidney transplantation.* American Journal of Transplantation, 2022. **22**(1): p. 318-319.
93. Rahimi Sakak, F., et al., *Glycemic control improvement in individuals with type 2 diabetes with vitamin K2 supplementation: a randomized controlled trial.* European Journal of Nutrition, 2021. **60**(5): p. 2495-2506.
94. Beulens, J.W.J., et al., *Dietary Phylloquinone and Menaquinones Intakes and Risk of Type 2 Diabetes.* Diabetes Care, 2010. **33**(8): p. 1699-1705.
95. Franceschi, C., et al., *Inflammaging: a new immune–metabolic viewpoint for age-related diseases.* Nature Reviews Endocrinology, 2018. **14**(10): p. 576-590.
96. Xu, K., et al., *Do inflammaging and coagul-aging play a role as conditions contributing to the co-occurrence of the severe hyper-inflammatory state and deadly coagulopathy during COVID-19 in older people?* Experimental Gerontology, 2021. **151**.
97. Rea, I.M. and H.D. Alexander, *Triple jeopardy in ageing: COVID-19, co-morbidities and inflamm-ageing.* Ageing Research Reviews, 2022. **73**.

98. Alberro, A., et al., *Inflammaging markers characteristic of advanced age show similar levels with frailty and dependency.* Scientific Reports, 2021. **11**(1).
99. Ohsaki, Y., et al., *Vitamin K suppresses the lipopolysaccharide-induced expression of inflammatory cytokines in cultured macrophage-like cells via the inhibition of the activation of nuclear factor κB through the repression of IKKα/β phosphorylation.* J Nutr Biochem, 2010. **21**(11): p. 1120-6.
100. Pan, M.H., et al., *Inhibition of TNF-α, IL-1α, and IL-1β by Pretreatment of Human Monocyte-Derived Macrophages with Menaquinone-7 and Cell Activation with TLR Agonists in Vitro.* Journal of Medicinal Food, 2016. **19**(7): p. 663-669.
101. Zhang, M., et al., *Vitamin K2 suppresses proliferation and inflammatory cytokine production in mitogen-activated lymphocytes of atopic dermatitis patients through the inhibition of mitogen-activated protein kinases.* Biological and Pharmaceutical Bulletin, 2021. **44**(1): p. 7-17.
102. Desai, A.P., et al., *Vitamin K & D Deficiencies are independently associated with COVID-19 disease severity.* Open Forum Infectious Diseases, 2021. **8**(10).
103. Linneberg, A., et al., *The association of low vitamin k status with mortality in a cohort of 138 hospitalized patients with covid-19.* Nutrients, 2021. **13**(6).
104. Pizzino, G., et al., *Oxidative Stress: Harms and Benefits for Human Health.* Oxidative Medicine and Cellular Longevity, 2017. **2017**: p. 1-13.
105. Vervoort, L.M., J.E. Ronden, and H.H. Thijssen, *The potent antioxidant activity of the vitamin K cycle in microsomal lipid peroxidation.* Biochem Pharmacol, 1997. **54**(8): p. 871-6.
106. Mishima, E., et al., *A non-canonical vitamin K cycle is a potent ferroptosis suppressor.* Nature, 2022. **608**(7924): p. 778-783.
107. Li, J., et al., *Novel role of vitamin k in preventing oxidative injury to developing oligodendrocytes and neurons.* J Neurosci, 2003. **23**(13): p. 5816-26.
108. Li, J., H. Wang, and P.A. Rosenberg, *Vitamin K prevents oxidative cell death by inhibiting activation of 12-lipoxygenase in developing oligodendrocytes.* J Neurosci Res, 2009. **87**(9): p. 1997-2005.
109. Berenjian, A., et al., *Vitamin K series: current status and future prospects.* Critical Reviews in Biotechnology, 2015. **35**(2): p. 199-208.
110. EFSA Panel on Dietetic Products, N., et al., *Dietary reference values for vitamin K.* EFSA Journal, 2017. **15**(5): p. e04780.
111. *Dietary reference values for food energy and nutrients for the United Kingdom. Report of the Panel on Dietary Reference Values of the Committee on Medical Aspects of Food Policy.* Rep Health Soc Subj (Lond), 1991. **41**: p. 1-210.
112. Przygoda, B.; Wierzejska, R.; Matczuk, E.; Kłys, W.; Jarosz, M.Witaminy. In Normy ˙zywienia dla populacji Polski i ich zastosowanie;

Jarosz, M., Rychlik, E., Sto´s, K., Charzewska, J., Eds.; Narodowy Instytut Zdrowia Publicznego—Pa´nstwowy Zakład Higieny:

Warsaw, Poland, 2020; pp. 185–189.

113. Regulska-Ilow, B., et al., Es*timation of Vitamin K Content and Its Sources in the Diet of the Polish Participants of the PURE Study. N*utrients, 2022. 14**(9**): p. 1917.
114. Schurgers, L.J., et al., Nu*tritional Intake of Vitamins K1 (Phylloquinone) and K2 (Menaquinone) in The Netherlands. J*ournal of Nutritional & Environmental Medicine, 1999. 9(**2**): p. 115-122.
115. Theuwissen, E., et al., Lo*w-dose menaquinone-7 supplementation improved extra-hepatic vitamin K status, but had no effect on thrombin generation in healthy subjects. B*ritish Journal of Nutrition, 2012. 10**8(9**): p. 1652-1657.

116. Garber, A.K., et al., Co*mparison of phylloquinone bioavailability from food sources or a supplement in human subjects. J* Nutr, 1999. 12**9(6)**: p. 1201-3.
117. Matschiner, J.T. and R.G. Bell, Me*tabolism and vitamin K activity of cis phylloquinone in rats. J* Nutr, 1972. 10**2(5)**: p. 625-9.
118. van Summeren, M.J.H., et al., The *effect of menaquinone-7 (vitamin K2) supplementation on osteocalcin carboxylation in healthy prepubertal children. B*ritish Journal of Nutrition, 2009. 10**2(8)**: p. 1171-1178.
119. Jensen, M.B., P. Ložnjak Švarc, and J. Jakobsen, Vit*amin K (phylloquinone and menaquinones) in foods – Optimisation of extraction, clean-up and LC–ESI-MS/MS method for quantification. F*ood Chemistry, 2021. 34**5:** p. 128835.
120. Xu, Y., et al., Ex*traction and Determination of Vitamin K(1) in Foods by Ultrasound-Assisted Extraction, SPE, and LC-MS/MS. M*olecules, 2020. 25**(4)**.
121. Roeck-Holtzhauer, Y.D., I. Quéré, and C. Claire, Vit*amin analysis of five planktonic microalgae and one macroalga. J*ournal of Applied Phycology, 2004. 3: p. 259-264.
122. Tarento, T.D.C., et al., Mi*croalgae as a source of vitamin K1. A*lgal Research, 2018. 36**:** p. 77-87.
123. Jensen, M.B., et al., Vit*amin K (phylloquinone and menaquinones) in foods – Cost-effective quantification by LC-ESI-MS/MS. F*ood Chemistry, 2022. 38**5.**
124. Lee, S., et al., An*alysis of vitamin K1 in commonly consumed foods in Korea. J*ournal of the Korean Society of Food Science and Nutrition, 2015. 44**(8)**: p. 1194-1199.
125. Palmer, C.R., et al., De*velopment of a Vitamin K Database for Commercially Available Food in Australia. F*rontiers in Nutrition, 2021. 8.
126. Kim, H., et al., Co*mparison of extraction methods for the determination of phylloquinone content in legumes and nuts. F*ood Science and Biotechnology, 2015. 24**(5)**: p. 1603-1607.
127. Bonrath, W. and T. Netscher, Ca*talytic processes in vitamins synthesis and production. A*pplied Catalysis A: General, 2005. 28**0:** p. 55-73.
128. Thor, H., et al., The *metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells. J* Biol Chem, 1982. 25**7(2**0): p. 12419-25.
129. Baj, A., et al., Co*nvergent Synthesis of Menaquinone-7 (MK-7). O*rganic Process Research & Development, 2016. 20**(6)**: p. 1026-1033.
130. Mahdinia, E., A. Demirci, and A. Berenjian, En*hanced Vitamin K (Menaquinone-7) Production by Bacillus subtilis natto in Biofilm Reactors by Optimization of Glucose-based Medium. C*urr Pharm Biotechnol, 2018. 19**(1**1): p. 917-924.
131. Sato, T., et al., Pr*oduction of Menaquinone (vitamin K2)-7 by Bacillus subtilis. J*ournal of Bioscience and Bioengineering, 2001. 91**(1**): p. 16-20.
132. Daines, M.A., et al., The *Synthesis of Naturally Occurring Vitamin K and Vitamin K Analogues. C*urrent Organic Chemistry, 2003. 7(**1**6): p. 1625-1634.
133. USA Drug Agency Bans Menadione. New York Times [Internet]. 1963 Mar 30;8.
134. European Commission. Opinion on Vitamin K1 (Phytonadione); SCCP/1105/07; Scientific Committee on Consumer Safety: Brussels, Belgium, 2007; pp. 1–31.
135. Center for Food Safety and Applied Nutrition. Food Additives and Ingredients – Food Additive Status List [Internet]. Silver Spring, MD: US Federal Drug Administration; [cited 2022 Sept. 16]. Available from: http://www.fda.gov/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/ucm091048.htm#ftnM.
136. Chainho, P., et al., No*n-indigenous species in Portuguese coastal areas, coastal lagoons, estuaries, and islands. E*stuarine Coastal and Shelf Science, 2015. 16**7.**
137. Engelen, A., et al., Ci*rcumglobal Invasion by the Brown Seaweed Sargassum muticum. O*ceanography and marine biology, 2015. 53**:** p. 81-126.
138. Ito, T., et al., The *effects of irradiance, temperature, and desiccation on the photosynthesis of a brown alga, Sargassum muticum (Fucales), from a native distributional range in Japan. J*ournal of Applied Phycology, 2021. 33**(3**): p. 1777-1791.

139. Zelený, D., Co-*occurrence based assessment of species habitat specialization is affected by the size of species pool: reply to Fridley et al. (2007).* Journal of Ecology, 2009. 97**(1**): p. 10-17.
140. Sánchez, Í. and C. Fernández, Re*source availability and invasibility in an intertidal macroalgal assemblage. M*arine Ecology Progress Series, 2006. 31**3:** p. 85-94.
141. Peter, A.S.Â.h., et al., In*vasion of Sargassum muticum in Limfjorden (Denmark) and its possible impact on the indigenous macroalgal community. M*arine Ecology Progress Series, 2000. 20**7:** p. 79-88.
142. Kevin, H.B.-S., Di*rect and indirect effects of the introduced alga Sargassum muticum on benthic, subtidal communities of Washington State, USA. M*arine Ecology Progress Series, 2004. 27**7:** p. 61-78.
143. Critchley, A.T., W.F. Farnham, and S.L. Morrell, An *account of the attempted control of an introduced marine alga, Sargassum muticum, in Southern England. B*iological Conservation, 1986. 35**(4**): p. 313-332.
144. Giakoumi, S., et al., Ma*nagement priorities for marine invasive species. S*cience of The Total Environment, 2019. 68**8:** p. 976-982.
145. Freitas, R., et al., Hi*ghlighting the Biological Potential of the Brown Seaweed Fucus spiralis for Skin Applications. A*ntioxidants, 2020. 9(**7**): p. 611.
146. Kurnialahi, I.D., et al., Ant*ioxidant activity of tropical seaweed sargassum muticum fucoidan. A*ACL Bioflux, 2020. 13**(1**): p. 230-240.
147. Flórez-Fernández, N., et al., Ult*rasound-assisted extraction of fucoidan from Sargassum muticum. J*ournal of Applied Phycology, 2017. 29**(3**): p. 1553-1561.
148. Darvin, M.E., et al., Ca*rotenoids in Human Skin In Vivo: Antioxidant and Photo-Protectant Role against External and Internal Stressors. A*ntioxidants, 2022. 11**(8**).
149. Krinsky, N.I., Th*e antioxidant and biological properties of the carotenoids. A*nn N Y Acad Sci, 1998. 85**4:** p. 443-7.
150. Vieira, F.A., et al., Si*ngle-step extraction of carotenoids from brown macroalgae using non-ionic surfactants. S*eparation and Purification Technology, 2017. 17**2:** p. 268-276.
151. Vieira, F.A. and S.P.M. Ventura, Ef*ficient Extraction of Carotenoids from Sargassum muticum Using Aqueous Solutions of Tween 20. M*arine Drugs, 2019. 17**(5**): p. 310.
152. Mazumder, A., et al., Ex*traction of alginate from Sargassum muticum: process optimization and study of its functional activities. J*ournal of Applied Phycology, 2016. 28**(6**): p. 3625-3634.
153. Flórez-Fernández, N., H. Domínguez, and M.D. Torres, A *green approach for alginate extraction from Sargassum muticum brown seaweed using ultrasound-assisted technique. I*nternational Journal of Biological Macromolecules, 2019. 12**4:** p. 451-459.
154. Susano, P., et al., Mi*tigating the negative impacts of marine invasive species – Sargassum muticum - a key seaweed for skincare products development. A*lgal Research, 2022. 62**.**
155. Santos, F., et al., Un*raveling the lipidome and antioxidant activity of native bifurcaria bifurcata and invasive sargassum muticum seaweeds: A lipid perspective on how systemic intrusion may present an opportunity. A*ntioxidants, 2020. 9(**7**): p. 1-20.
156. Terme, N., et al., Ra*dical scavenging activity of lipids from seaweeds isolated by solid-liquid extraction and supercritical fluids. O*CL, 2018. 25**(5**): p. D505.
157. Pérez-Larrán, P., et al., Ant*ioxidant and Antitumoral Properties of Aqueous Fractions from Frozen Sargassum muticum. W*aste and Biomass Valorization, 2020. 11**(4**): p. 1261-1269.
158. Silva, J., et al., Se*aweeds' neuroprotective potential set in vitro on a human cellular stress model. M*olecular and Cellular Biochemistry, 2020. 47**3(1**-2): p. 229-238.
159. Manrique, R., et al., Ef*fects of salinity on the CO2 permeation across lipid bilayer for microalgae biofixation: a molecular dynamics study. J*ournal of Applied Phycology, 2018. 30**(1**): p. 55-61.
160. American Type Culture Collection – THP-1 TIB 202; Handling Information [Internet]. [cited 2022 Sept. 20]. Available from: https://www.atcc.org/products/tib-202

161. Reynolds, J.L., Hu*man Vascular Smooth Muscle Cells Undergo Vesicle-Mediated Calcification in Response to Changes in Extracellular Calcium and Phosphate Concentrations: A Potential Mechanism for Accelerated Vascular Calcification in ESRD.* Journal of the American Society of Nephrology, 2004. 15**(1**1): p. 2857-2867.
162. Smith, P.K., et al., Me*asurement of protein using bicinchoninic acid. A*nal Biochem, 1985. 15**0(1**): p. 76-85.
163. Wiechelman, K.J., R.D. Braun, and J.D. Fitzpatrick, In*vestigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. A*nal Biochem, 1988. 17**5(1**): p. 231-7.
164. Nakano, T., et al., Va*scular smooth muscle cell-derived, Gla-containing growth-potentiating factor for Ca(2+)-mobilizing growth factors. J* Biol Chem, 1995. 27**0(1**1): p. 5702-5.
165. Jäpelt, R.B. and J. Jakobsen, An*alysis of vitamin K1 in fruits and vegetables using accelerated solvent extraction and liquid chromatography tandem mass spectrometry with atmospheric pressure chemical ionization. F*ood Chemistry, 2016. 19**2:** p. 402-408.
166. Bruni, R., et al., Ra*pid techniques for the extraction of vitamin E isomers from Amaranthus caudatus seeds: ultrasonic and supercritical fluid extraction. P*hytochem Anal, 2002. 13**(5**): p. 257-61.
167. Munteanu, I.G. and C. Apetrei, An*alytical Methods Used in Determining Antioxidant Activity: A Review. I*nt J Mol Sci, 2021. 22**(7**).
168. Fiedor, J. and K. Burda, Po*tential Role of Carotenoids as Antioxidants in Human Health and Disease. N*utrients, 2014. 6(**2**): p. 466-488.
169. Wolfe, K.L. and R.H. Liu, Ce*llular Antioxidant Activity (CAA) Assay for Assessing Antioxidants, Foods, and Dietary Supplements.* Journal of Agricultural and Food Chemistry, 2007. 55**(2**2): p. 8896-8907.
170. Kellett, M.E., P. Greenspan, and R.B. Pegg, Mo*dification of the cellular antioxidant activity (CAA) assay to study phenolic antioxidants in a Caco-2 cell line. F*ood Chemistry, 2018. 24**4:** p. 359-363.
171. Félix, R., et al., Ev*aluating the In Vitro Potential of Natural Extracts to Protect Lipids from Oxidative Damage. A*ntioxidants, 2020. 9(**3**): p. 231.
172. Park, E.K., et al., Op*timized THP-1 differentiation is required for the detection of responses to weak stimuli. I*nflammation Research, 2007. 56**(1**): p. 45-50.
173. Yoon, W.-J., et al., Br*own alga Sargassum muticum inhibits proinflammatory cytokines, iNOS, and COX-2 expression in macrophage RAW 264.7 cells [Kahverengi alg Sargassum muticum RAW 264,7 makrofaj hücrelerindeki iltihaba sebep olan sitokinler, iNOS ve COX-2 ekspresyonunu engeller]. T*urkish Journal of Biology, 2010. 34**:** p. 25-34.