FACULTY OF PHARMACEUTICAL SCIENCES

LIPOSOMAL ENCAPSULATION OF ERUCIN, EXTRACTED FROM ARUGULA LEAVES, GIVEN ITS POTENTIAL FOR THE PREVENTION AND TREATMENT OF MULTIPLE DISEASES

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Summary:

Erucin is an isothiocyanate obtained as one of the various end products arising from an enzymatic hydrolysis, catalyzed by myrosinase, of the glucosinolate glucoerucin. Glucosinolates represent a class of sulfur-containing secondary metabolites that are highly concentrated in plants from the family Brassicaceae, including the rucola plant.

Promising pharmacological properties have been attributed to this secondary metabolite, which can contribute to the prevention and treatment of multiple diseases. Erucin possess anti-oxidant, anti-inflammatory, biocidal and goitrogenic properties and has potential preventive effects against certain forms of cancers, diabetes, neurodegenerative and cardiovascular disorders. However, formulating erucin as a pharmaceutical faces numerous challenges due to its unfavorable pharmacokinetic characteristics. Specifically, erucin exhibits low solubility in water, limited stability, reactivity and temperature lability.

The objective of this master dissertation is to enhance the knowledge regarding the optimization of the extraction of erucin from fresh rucola leaves. The extraction procedure compromises two steps, consisting of a hydrolysis step conducted in a neutral medium and an extraction step in an organic medium, which can be performed simultaneously or sequentially. During the extraction step, the organic solvents ethanol and ethyl acetate were employed. The highest erucin yields were obtained with ethyl acetate.

Subsequently, pure erucin was encapsulated in the lipidic bilayer of the liposomes by exploiting the simil-microfluidic method. The dimensional properties of the liposomes were assessed using Open-Source Dynamic Light Scattering. The measurement of the encapsulation efficiency and load capacity were carried out using a tangential flow filtration technique. The encapsulation procedure aims to enhance the stability of erucin in a pharmaceutical formulation, protect the isothiocyanate against degradation, and enhance its bioavailability within the human body.

Samenvatting:

Erucin is een isothiocyanaat dat ontstaat als één van de vele eindproducten uit een enzymatische hydrolysereactie, gekatalyseerd door myrosinase, uitgaande van het glucosinolaat glucoerucin. Glucosinolaten zijn secundaire metabolieten die in hoge concentraties worden aangetroffen in kruisachtige groenten, waaronder de rucola plant.

Er worden veelbelovende farmacologische eigenschappen toegeschreven aan dit secundaire metaboliet, die kunnen bijdragen aan de behandeling en preventie van verschillende aandoeningen. Zo zou erucin anti-oxidatieve, ontstekingsremmende, biocide en goitrogene eigenschappen hebben en vertoont het potentieel preventieve effecten tegen bepaalde vormen van kanker, diabetes, neurodegeneratieve en cardiovasculaire aandoeningen. Anderzijds zijn er echter tal van uitdagingen bij het formuleren van erucin als geneesmiddel vanwege zijn ongunstige farmacokinetische eigenschappen. Erucin wordt namelijk gekenmerkt door een lage oplosbaarheid in water, geringe stabiliteit, reactiviteit en thermolabiliteit.

In deze masterthesis werd de optimalisatie van de extractie van erucin uit verse rucola bladeren beoogt. Deze extractieprocedure is tweedelig en bestaat uit een hydrolysestap in neutraal midden en een extractiestap in een organisch medium, die simultaan of achtereenvolgens kunnen worden uitgevoerd. Tijdens de extractiestap wordt gebruik gemaakt van de organische solvents ethanol en ethyl acetaat. De hoogste erucin opbrengsten werden verkregen met ethyl acetaat.

Vervolgens zal pure erucin geëncapsuleerd worden in de lipide dubbellaag van de liposomen waarbij gebruik wordt gemaakt van de simil-microfluidic methode. De dimensionele eigenschappen van de liposomen worden verkregen aan de hand van Open-Source Dynamic Light Scattering. Het berekenen van de encapsulatie efficiëntie en de ladingscapaciteit werden uitgevoerd via een tangential flow filtration techniek. De encapsulatie procedure heeft als doel de stabiliteit van erucin te verhogen in een farmaceutische formulatie, de isothiocyanaat te beschermen tegen degradatie en zijn biologische beschikbaarheid te doen toenemen in het menselijk lichaam.

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TABLE OF CONTENTS

Abbreviations:

AFM	Atomic Force Microscope
AUC	Area Under the Curve
$CH_3COOC_2H_5$	Ethyl acetate
C_2H_5OH	Ethanol
osDLS	Open-Source Dynamic Light Scattering
EE%	Encapsulation Efficacy
ERN	Erucin
FID	Flame Ionization Detector
GC	Gas Chromatography
GSL(s)	Glucosinolate(s)
GST	Glutathione S-transferase
HPLC	High Performance Liquid chromatography
HFE	Hydrolysis followed by extraction
H_2S	Hydrogen sulfide
ITC(s)	Isothiocyanate(s)
LET	Liposomal encapsulation technology
LC%	Loading capacity
MS	Mass spectrometry
NaCl	Sodium chloride
$Na_3PO_4 \cdot 12H_2O$	sodium phosphate tribasic dodecahydrate
Na_2SO_4	Sodium sulfate anhydrous
NTU	Nephelometric Turbidity Units
NQO1	NAD(P)H-quinone oxidoreductase 1
PC	Phosphatidylcholine
PDI	Polydispersity index
R^2	Coefficient of determination
RID	Refractive Index Detector
rpm	Revolutions per minute
SFR	Sulforaphane
SHE	Simultaneous hydrolysis and extraction

SMF	Simil-microfluidic
SPM	Scanning Probe Microscope
STM	Scanning Tunneling Microscope
TFF	Tangential Flow Filtration
TLC	Thin layer chromatography
UDP(G)	Uridine diphosphate (glucose)
UV	Ultraviolet
VWD	Variable Wavelength Detector

1. INTRODUCTION

1.1. HISTORICAL BACKGROUND OF NANOTECHNOLOGY

Nanotechnology is a dynamic and rapidly evolving field that holds great promise for advancing a broad range of scientific disciplines, involving the manipulation and engineering of materials at the nanoscale, typically ranging from 1 to 100nm.(1,2) At this scale, materials exhibit unique properties that differ from those observed at larger scales, such as electric conductance, chemical reactivity, magnetism, optical effects and mechanical properties.(1,3,4) The term “nano” is derived from a Greek prefix, meaning “dwarf” or “very small”, and serves in the context of modern science as a reference to the minuscule dimensions at which nanotechnology operates.(1,2,4)

Nanoparticles share a rich history that can be traced back to the ancient Greek philosopher Democritus, who first proposed the idea of atoms.(2) However, the term "nanoparticle" was officially coined in 1959 by Richard Feynman, an American physicist often considered as the father of modern nanotechnology. Feynman discussed in his famous lecture "There's Plenty of Room at the Bottom", at the annual meeting of the American Physical Society, his visionary ideas on manipulating atoms and molecules to fabricate products on the macroscale.(1,2,4,5) Consequently, the nanometer scale, in honor of Feynman’s contributions, is frequently referred to as the Feynman scale.(4) In 1974 defined the Japanese scientist, Norio Taniguchi, the term “nanotechnology” for the first time.(1,2) During the 1980s and 1990s, significant advancements occurred with the development of the Scanning Tunneling Microscope (STM) by physicists Gerd Binning and Heinrich Rohrer. This breakthrough introduced the general category of the Scanning Probe Microscope (SPM) techniques.(1–5) The STM enabled the imaging of solid surfaces based on the quantum mechanical tunneling effect, which led afterwards to the creation of the Atomic Force Microscope (AFM). AFM, a high-resolution surface imaging instrument, was developed by Binning, Quate and Gerber and operates by utilizing interatomic or intermolecular forces, facilitating the visualization and manipulation of individual atoms and molecules.(1–5) This discovery laid the foundation for all SPMs and continues to be extensively utilized nowadays in research.(1–5)

On a particular note, recent years have shown promising results in the application of nanotechnology to human health, especially in the field of cancer treatment.(2,6) Nanoparticles can be engineered to selectively target cancer cells and enable the direct delivery of therapeutic agents to tumors.(6,7) This approach holds great promise in minimizing the systemic toxicity and improving treatment outcomes compared to conventional chemotherapy and radiotherapy approaches.(1,2,6,8) Furthermore, nanotechnology has found diverse bio-applications in the fields of cosmetics, medical diagnosis, cosmetics, tissue engineering, drug delivery, drug targeting and molecular imaging.(1–6)

Despite the considerable potential of nanoparticles in diverse applications, there remain concerns regarding their impact on human health and the environment. Their small size allows them to be inhaled, raising concerns about potential respiratory risks.(1,3,5) Additionally, during the production process, the release of nanoparticles into the air or water can contribute to what is known as "Nano Pollution".(1,3,5) It is crucial to establish regulations and guidelines encompassing the health, safety, long-term impact and environmental implications of nanoparticle usage.(5) Employing biological systems, such as plants, can present a viable alternative for achieving a more sustainable nanoparticle synthesis due to their beneficial effects on the environment.(3)

1.2. ERUCIN

1.2.1. Arugula plant

Arugula, also known as rocket, is an edible plant belonging to the Brassicaceae family, which is characterized by a high concentration of glucosinolates (GSLs), amounting to about 1 percentage of the plant dry weights.(9–12) Other frequently consumed cruciferous vegetables who also contain a high amount of GSLs are cabbages, Brussels sprouts, cauliflower, kale and broccoli.(13,14) Every vegetable can be characterized by its own unique GSL profile. For instance, the predominant GSL in Brussels sprouts, cabbage, cauliflower and kale is sinigrin.(15) While arugula and broccoli have a high concentration of two GSLs, namely glucoerucin and glucoraphanin.(16–19) This master dissertation is focused on the arugula plant, which has the highest amounts of GSLs in its seedpods and

seeds, with relatively lower levels in the leaves.(16,20–22) Moreover, the seedpods of arugula are mainly composed of glucoraphanin, whereas the seeds and leaves of arugula contain primarily glucoerucin.(23) Furthermore, notable variations are found in GSL concentrations in arugula leaves, attributable to their geographical origin.(24,25) Specifically, arugula accessions originating from Mediterranean countries exhibite 2-fold higher levels of GSLs compared to those derived from Asia and Middle East Asia.(24)

1.2.2. Glucosinolates

Glucosinolates, also named as β- thioglucoside N-hydroxysulfates, are a class of sulfur- and nitrogen-containing secondary metabolites that are responsible for the pungent taste and aroma of these vegetables.(9,12,20,26) They are distinguished from each other because of their remarkable structural diversity arising from over 120 variable side chains.(9,13,20,27) According to the structure differences, GSLs are classified into ten different categories including sulfur-containing, aliphatic straight-chain, aliphatic branched chain, olefins, aliphatic straight and branched chain alcohols, aliphatic straight chain ketones, aromatic, ω-hydroxyalkyl, indoles and multiply glycosylated types, from which the sulfur-containing side chains constitute approximately one-third.(9,18,20) The most frequently employed classification of GSLs is based on the precursor amino acid from which they are derived, differentiating 3 major groups, namely aliphatic, aromatic and indolic GSLs.(18,20,26,28) Aliphatic GSLs originate from the amino acids alanine, leucine, methionine, valine, or isoleucine. Conversely, aromatic GSLs are derived from phenylalanine or tyrosine, while indole GSLs are biosynthesized from tryptophan.(19,29)

GSLs themselves do not possess significant bioactivity, but their metabolites do, which makes the ITC formation crucial to generate a biological effect.(8) However, they have a higher stability, within cytoplasm until brought into contact with myrosinase, and well water-soluble compared to their conversion products.(10,19,21,33) In particular, ITCs provided with a β-hydroxyl group are prone to spontaneously cyclize to oxazolidine-2-thione, which can reduce their biological activity and are reported to have negative biological effects by interfering with the thyroxine synthesis.(18,19,24,27)

1.2.3. Hydrolysis of glucosinolates

Myrosinase, also referred to as β-thioglucoside glucohydrolase, is an enzyme that mediates the conversion of GSLs into erucin (4-methylthiobutyl isothiocyanate; ERN) by breaking down the anomeric carbon-sulfur bond.(9,12,30,31) In intact plant tissue, the GSL substrate is stored in the vacuoles of S-cells, separated from myrosinase, which is localized in aqueous vacuoles known as “myrosin” cells.(9,20,27,32,33) Upon damage to the plant tissue, such as by chewing or chopping the plant, the endogenous enzyme myrosinase is released, allowing it to react with the GSLs.(20,22,30) Subsequently, the enzymatic conversion depends upon the availability of myrosinase.(14,15,17,31)

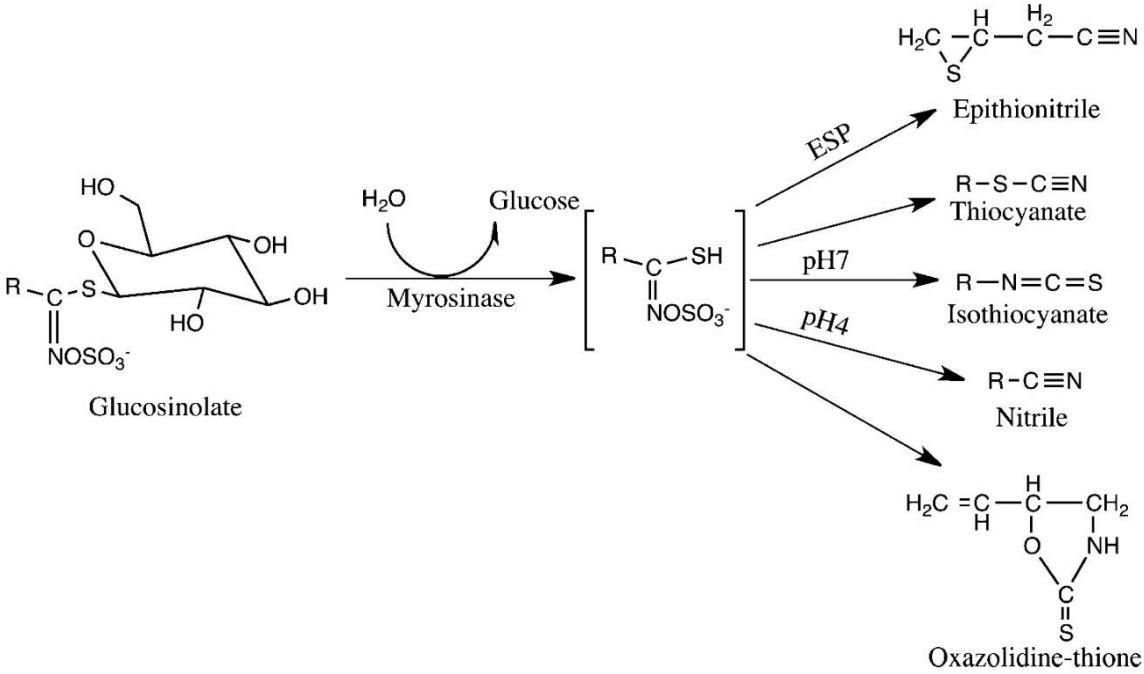

Figure 1.1: Schematic representation of the diverse array of products generated through the glucosinolate-myrosinase reaction under various conditions(25)

The β-thioglucoside glucohydrolase catalyzes the cleavage of the glucose moiety, leading to the formation of an unstable intermediate product, aglucone thiohydroxymate-O-sulfonate.(9,19,27) The aglucone intermediate will spontaneously rearrange into several products (Figure 1.1), depending on the specific side chain of the GSL.(9,34) Additionally, the hydrolysis conditions and the presence of cofactors will also affect the final product.(27,34) In addition to isothiocyanates (ITCs), which are generated by a Lossen rearrangement, other products, including thiocyanates, nitriles, epithionitriles, indoles or

less commonly, alcohols, and cyanide, can also be generated from the GSL substrate.(20,21,24,25,27,35)

There are two main routes of isothiocyanate generation mediated by myrosinase. First, the endogenous myrosinase present in specialized cells. The second route occurs when ingested GSLs, that have not yet undergone hydrolysis, are converted to ITCs, to a lesser extent, by the myrosinase activity of human gut microflora.(35) However, the efficiency of the β-thioglucoside glucohydrolase, present in the colon, is reported to be comparatively lower.(9,17,20,36) After absorption, mercapturic acids are formed by the conjugation of ITCs to glutathione through a mercapturic acid pathway.(17,20,25,31) Subsequently, the conjugated ITC undergoes a series of consecutive conversion processes, resulting in the formation of ITC-cysteinylglycine, ITC-cysteine, and ITC-N-acetylcysteine conjugates.(17) The catalysis of this phase II metabolization reaction is carried out by glutathione S-transferase (GST).(36)

The conditions to maximally optimize the enzymatic conversion to ERN are by working in a neutral or alkaline environment, at low temperatures and in the presence of water.(17,26,30) The best results are achieved by using a phosphate buffer of 0.1M (pH 6-7) at room temperature.(22,26,37) This occurs because the nitrile formation is favored at a lower pH range and the acidic environment has a negative impact on the hydrolysis reaction.(19,27,30) According to literature, nitriles are considered to be potential toxins, causing dose-dependent lesions in the liver, pancreas and notably in the kidney.(19,27) Also the incubation time effects the hydrolysis where 2 hours of incubation, under continuous shaking, would obtain the best results.(17,26) The enzyme myrosinase is susceptible to heat and will denature, resulting in the inactivation of the enzyme, if cooking temperatures will be applied during the hydrolysis process.(15,27,29,37) Although, the process of mild heating exerts a potential stimulating effect on ITCs formation due to the optimal enzyme activity of myrosinase at a temperature of 55°C.(17,34,35)

A dynamic equilibration exists between ERN and SFR (Figure 1.2) within certain tissues, where the sulfoxide group of SFR is reduced to a thioether group, creating ERN in vivo.(8)

This conversion may be facilitated by human colonic bacteria after absorption. However, the ratio of SFR metabolite to ERN metabolite varies depending on the tissue location, even when only a single GSL source is administered.(14,17,21,38)

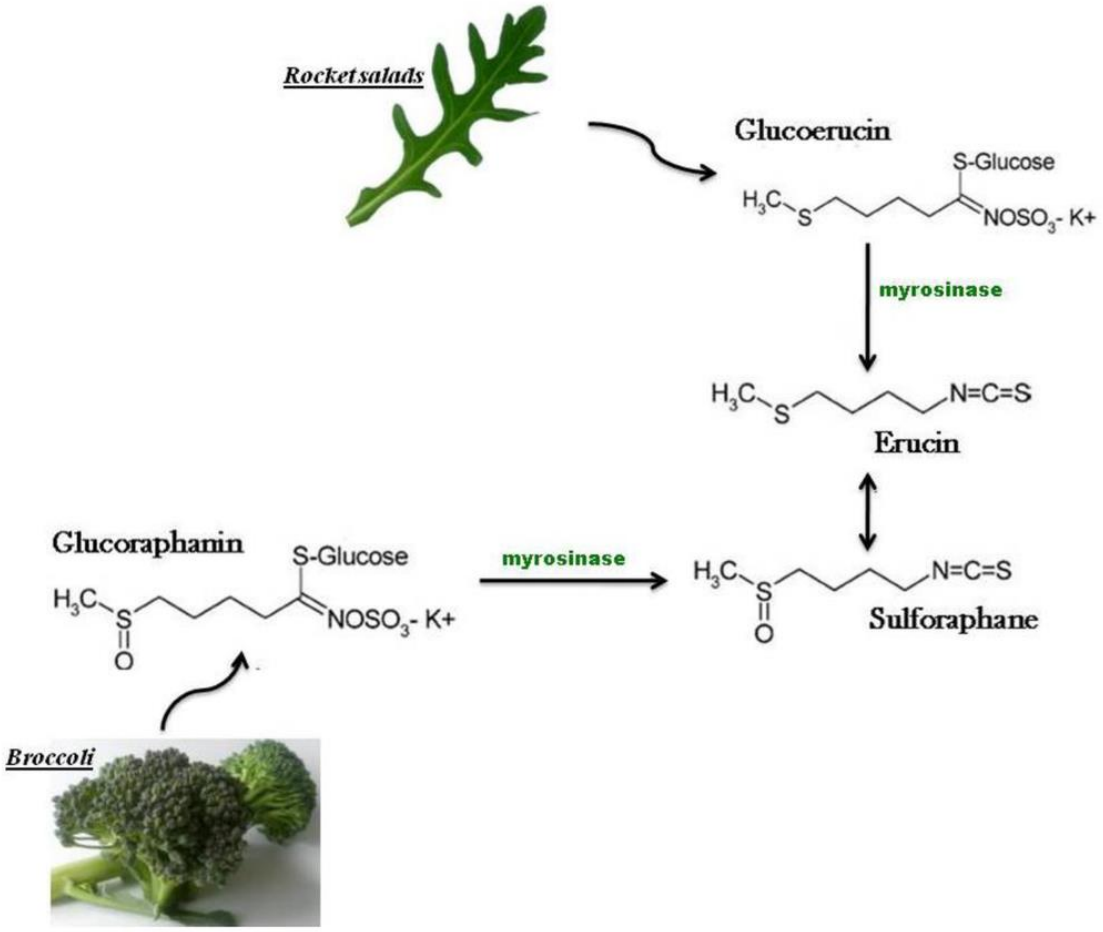

Figure 1.2: Equilibration between erucin and sulforaphane(39)

1.3. ERUCIN ANALYSIS, STATE OF ART

The identification and quantification of ERN can be challenging due to several factors, including its volatile nature, low stability, temperature lability, reactivity and low solubility in water.(8,22,26,29,31) The potential for glucoerucin to generate a variety of unique breakdown products further adds to the difficulty of accurately analyzing ERN in complex matrices.(20)

To analyze ERN, it is mostly necessary to convert glucoerucin to the desired product.(26) The conversion can be achieved by crushing the rucola leaves, in presence of water, to allow the release of the endogenous enzyme myrosinase and the hydrolyzation to obtain ITCs from GSLs. After the hydrolysis, the ITCs are typically extracted using a volatile, slightly polar solvent such as dichloromethane, chloroform or ethyl acetate.(19,21)

Chemical synthesis of GSLs (Figure 1.3) is another frequently used method to obtain ERN, starting from glucose and amino acids. The bio-precursor of glucoerucin is methionine, making glucoerucin classified as an 'aliphatic' glucosinolate.(28) The biosynthesis of GSLs can be broken down into three major steps. The first step involves chain elongation of the amino acid by adding methylene groups, followed by N-hydroxylation of the precursor amino acid to form dihomo-methionine.(20,29) The dihomo-methionine is then decarboxylated to an aldoxime, catalyzed by the cytochrome P450 CYP79F1.(9,29) The second step consists of the GSL core biosynthesis, which includes the conversion to thiohydroximic acid and the S-glycosyl transfer from uridine diphosphate glucose (UDPG). The transfer is catalyzed by UDPG:thiohydroximate glucosyltransferase and followed by a sulfation in assistance of 3′-Phosphoadenosine-5′-phosphosulfate:desulfoglucosinolate sulfotransferase that serves as a donor.(9,20,29) Finally, side chain modifications can occur to complete the biosynthesis process.(9) The chemically synthesized glucoerucin can be further converted to erucin in presence of the exogeneous enzyme myrosinase.

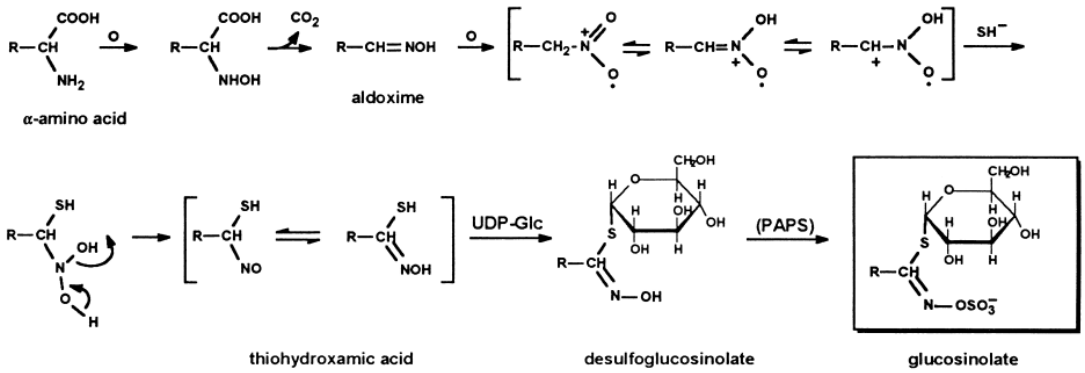

Figure 1.3: The distinct biosynthetic stages encompassing the chemical synthesis of a glucosinolate core(9)

Historically, paper or thin-layer chromatography (TLC) and reversed phase extraction were used to identify ITCs.(9,19) However, nowadays reversed phase high performance liquid chromatography (HPLC) coupled with a ultraviolet (UV) detector is the most commonly used technique for analyzing ITCs.(9,19,20) Gas chromatography (GC) analysis, assisted by flame ionization detector (FID), is also a viable option, although the high temperatures involved can compromise the stability of the thiocyanates, necessitating derivatization.(19,26) For this reason, GC is now typically used to access the purity of the ITCs.(30,31) The most common derivatization techniques involve conjugation procedures, employing N-(tert-butoxycarbonyl)-L-cysteine methyl ester (Figure 1.4), relying on the formation of a stable dithiocarbamate ester derivative.(17) Additionally, mass spectrometry (MS) combined with electrospray ionization is a useful tool for the detection and quantification of ERN, although its greatest application is the confirmation of the identities of the compounds.(20,26,30)

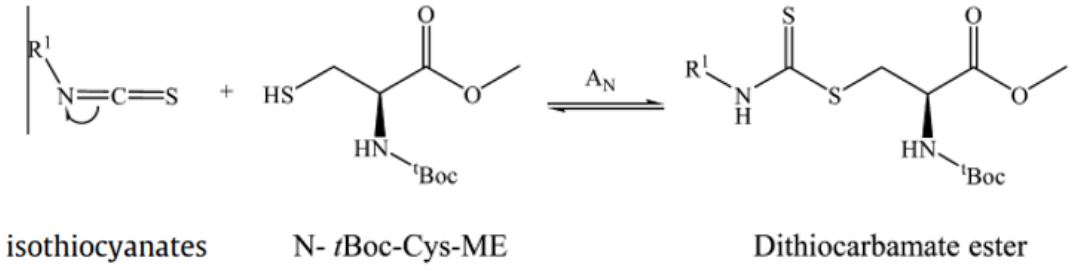

Figure 1.4: Derivatization procedure to optimize ITC analysis(26)

1.4. LIPOSOMES

1.4.1. General introduction liposomes

Liposomes have emerged as versatile and highly investigated nanostructures in the field of pharmaceutical and biomedical sciences. Their composition, characterized by biodegradability, high biocompatibility and low immunogenicity , has attracted significant attention.(7,40–43) These small artificial vesicles are characterized by their spherical shape and composed of lipid bilayer membranes that encapsulate an aqueous volume, mimicking the structure of cell membranes.(7,40–45) The choice of bilayer components, such as cholesterol and natural phospholipids, will determine the “fluidity” and the charge

of the bilayer.(7,41,44) Notably, unsaturated phosphatidylcholine (PC) form more permeable and less stable bilayers, compared to saturated phospholipids with long acyl chains.(7,40,41)

With particle sizes typically ranging from 30nm to several micrometers, liposomes consist of one or more lipid bilayers surrounding an aqueous core, with the polar head groups oriented towards the interior and exterior aqueous phase.(7,40,41) This unique amphiphilic structural arrangement (Figure 1.5) offers liposomes the ability to encapsulate and deliver a wide range of substances, including hydrophilic, hydrophobic, and amphiphilic compounds, within their aqueous core or lipid bilayers.(7,8,40,44–46) The incorporation of the pharmaceutical depends on the solubility in aqueous solution.(40,46) Highly lipophilic compounds are predominantly entrapped within the lipid bilayer, while intensely hydrophilic drugs are confined entirely within the aqueous compartment.(7,40,44) Drugs with an intermediate partition coefficient can partition between the lipid and aqueous phases in both the bilayer and the aqueous core.(7) Consequently, liposomes hold promise as effective carriers for targeted drug delivery and are suitable for diverse routes of administration, such as parenteral, oral, pulmonary, nasal, ocular and transdermal.(7,41,44,47)

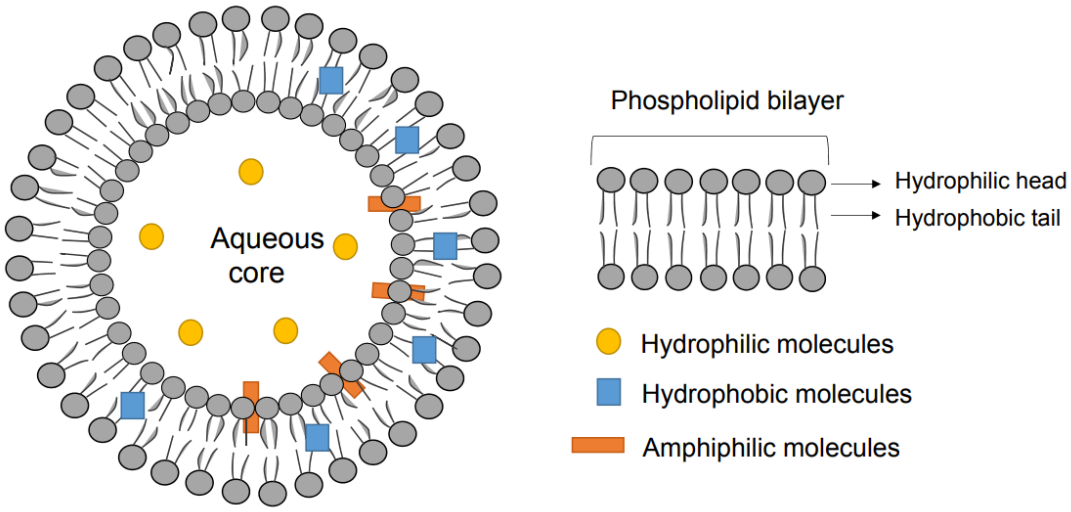

Figure 1.5: Representation of the general structure of liposomes(40)

Liposomal encapsulation technology (LET) is a novel drug delivery technique that utilizes liposomes to protect and deliver drugs to specific target organs.(7) Liposomes form a protective barrier around their pharmaceutical, shielding them from degradation by enzymes in mouth and stomach, digestive juices, and other substances in the body that could potentially decrease the bioavailability of the active ingredient.(7) This results in the protection from oxidation and degradation processes.(7,46) This protective phospholipid barrier remains intact until the liposomes reach their intended target.(7) Additionally, liposomes can enhance water solubility, increase the bioavailability, protect against degradation thereby enhancing the chemical stability, increase the half-life, reduce toxicity and improve therapeutic efficacy.(7,40–42,45,46)

1.5. THERAPEUTICAL APPLICATIONS OF ERUCIN

Cruciferous flowers have a rich historical background of both medicinal and culinary utilization.(10) Notable historical figures, such as the Greek physician Hippocrates and philosopher Pythagoras, have both documented the therapeutic effects of mustard juice, which contains natural ITCs.(27) Moreover, rocket has been included in traditional pharmacopeia for a diverse array purposes, encompassing its use as an antiphlogistic agent, treatment for eye infections, deodorant, digestive aid, laxative, astringent, diuretic, digestive, emollient, and rubefacient.(10,18)

These compounds have emerged as H_2S (hydrogen sulfide)-donors with favorable pharmacokinetic characteristics after oral administration and are therefore considered as potential antihypertensive agents.(29,48) ERN slowly stimulates the sustained release of H_2S within vascular cells, inhibits noradrenaline-induced vasoconstriction, and lowers blood pressure in hypertensive rats.(48,49) Additionally, naturally occurring ITCs possess a broad range of biological activities, including antifungal, antibacterial, and antimicrobial activities.(25,35,50) The antibacterial effects of ITCs on gram-positive bacteria may be attributed to their ability to bind to sulfhydryl sites.(27)

Epidemiological research has demonstrated that consuming high amounts of arugula on a daily basis has been related to a substantial decrease in the relative risk of certain cancers,

including but not limited to pancreatic, thyroid, lung, colorectal, bladder and prostate cancers, by up to 50%.(8,9,17,20,30,31) The cancer-preventive potential of these metabolites is mainly attributed to their ability to induce enzymes responsible for the phase II detoxification process.(17,30) Phase II enzymes, such as GST, UDP-glucuronosyltransferase and NAD(P)H-quinone oxidoreductase 1 (NQO1), primarily generate conjugation products that are more hydrophilic, thereby providing protection against harmful substances by reducing their bioavailability and facilitating their removal from the body.(16,18,20,51) Additionally, they have potential antiproliferative, apoptosis-promoting in human cancer cells, anti-inflammatory, redox-regulating, and phase-1 enzyme inhibitory roles.(9,17,30,31,36) Among the phase I enzymes, cytochrome P450s are the most significant ones that catalyze the oxidation, reduction, and hydrolysis of various toxic and carcinogenic substances.(20) ERN has also be reported to suppress microtubule dynamics, resulting in the inhibition of the proliferation of MCF-7 breast cancer cells.(14,24) Besides anti-cancerogenic properties, ITCs have also shown protective effects against cardiovascular disease, neurodegeneration and diabetes.(13,19,25,29)

ITCs are known to possess potent insecticidal properties and act as a defensive compound against invertebrates and generalist herbivores due to their pungency, which leads to repelling insects.(9,10,20,26,27) Experimental evidence indicates that elevated levels of GSLs can induce metabolic stress in herbivorous invertebrates, resulting in compromised growth.(20) They are also likely involved in host plant recognition by predators and may therefore be used as an insect feeding attractant.(9,13,27)

The anti-thyroid activity of ITCs with a β-hydroxyl group has been attributed to the spontaneous formation of oxazolidine-2-thione, a cyclization product that interferes with the synthesis of thyroid hormones.(20,27) Although this compound has structural similarities to several known teratogens, it has not been found to be active in this regard in preclinical trials with rodents.(27) On the other hand, ERN has been identified as a goitrogen, meaning it interferes with the function of the thyroid gland, regulating the body's metabolism, and is associated with the inhibition of iodine uptake, resulting in reduction of

the thyroid hormone synthesis and thyroid enlargement.(13) This effect is due to the conversion of ERN in vivo to the thiocyanate ion.(13,20,27)

GSL glucoerucin exhibits both direct and indirect antioxidant activity, in contrast to ERN which has primarily been identified as an indirect antioxidant.(17,18) ERN hinders the initiation of peroxidation reactions through the decomposition of hydroperoxides and hydrogen peroxide, which are major contributors to the production of reactive radical species.(17,18) Furthermore, ERN is rich in various nutrients, such as vitamins A, C, E and K, along with flavonoids, carotenoids, folates, and minerals.(10,17,22,52)

2. OBJECTIVES

Erucin is an isothiocyanate with promising potential in the prevention and treatment of several diseases. However, its inherent characteristics such as volatility, low stability, temperature lability, reactivity and low solubility in water limits the achievement of high concentrations after oral administration and thereby its therapeutical potential. The usage of controlled drug delivery systems, such as liposomes, can encapsulate the extracted hydrophobic erucin within their lipid bilayers, enhancing the systemic bioavailability and therapeutic efficacy by protecting it from degradation and increasing its solubility in aqueous environments.

The objective is to enhance the knowledge regarding the optimalization of the extraction of erucin from fresh rucola leaves by comparing the yield of erucin using different solvents, including ethyl acetate and ethanol, and different methods, differentiating in the performance of the hydrolysis and extraction simultaneously or sequentially. The hypothesis is that the use of ethyl acetate as well as the simultaneous execution of both reactions will result in the highest erucin concentrations.

The simil-microfluidic technique is an effective method to produce liposomes, aiming a load of 1%. With this technique the encapsulation of extracted erucin from arugula, dissolved in ethanol, is possible. Making use of a triple injection, a system patented by our research group, may be more suitable for this purpose compared to a coaxial injection. Open-Source Dynamic Light Scattering, tangential flow filtration technique and the turbidity meter will be capable of characterizing the obtained liposomal suspension. Our hypothesis is that this technique will be able to encapsulate erucin within liposomes with a loading capacity near 1% and a high encapsulation efficiency.

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Summary

The fresh arugula leaves were provided by the local grocery store in Fisciano, Italy and subsequently stored under refrigeration until needed. The phosphate buffer was prepared using sodium phosphate tribasic dodecahydrate ($Na_3PO_4 \cdot 12H_2O$) (CAS: 10101-89-0; Sigma-Aldrich; St. Louis; Missouri; United States). Both Sodium chloride (NaCl) (CAS: 7647-14-5; Sigma-Aldrich; St. Louis; Missouri; United States) and Sodium sulfate anhydrous (Na_2SO_4) (CAS: 7757-82-6; Carlo Erba; Val de Reuil; France) were employed after the hydrolysis. The organic solvents ethyl acetate ($CH_3COOC_2H_5$) (CAS: 141-78-6) and ethanol 96°/pure ethanol (C_2H_5OH) (CAS: 64-17-5) were both obtained from the supplier Carlo Erba based in Val de Reuil; France. The ERN standard (SML-343-25MG) was purchased from Sigma-Aldrich headquartered in St. Louis; Missouri; United States. PC was purified from Soy Lecithin obtained from ACEF (Piacenza; Italy) by Serena.

3.1.2. Phosphate buffer

The phosphate buffer was prepared using $Na_3PO_4 \cdot 12H_2O$ with a molecular weight of $380.12 \frac{mg}{mmol}$ at a concentration of 0.1M within a pH range of 6-7, in accordance with the observed optimal hydrolysis conditions for ERN formation.(22,26,37) During the calculations for the buffer preparation, both the desired buffer volume and the water content of the utilized arugula leaves were taken into consideration. The humidity was determined gravimetrically by subjecting the leaves to an oven until a stable mass was achieved, and by employing a moisture analyzer. This resulted in a total water content of 90% relative to the total mass of the arugula leaves. The required mass of $Na_3PO_4 \cdot 12H_2O$ to obtain the desired concentration of the phosphate buffer was determined using equation 3.1.

$$m(g) = 0.1 \frac{mmol}{mL} * \left(V_b + \frac{Mleaves * 0{,}9}{0.997 \frac{mg}{mL}}\right) * 380.12 \frac{mg}{mmol} * 1000 \frac{g}{mg} \quad \text{(Eq 3.1)}$$

Where: V_b = volume of buffer (mL)

Mleaves = mass of the arugula leaves (mg)

3.1.3. Sodium Chloride (NaCl)

NaCl, commonly referred to as salt, is employed subsequent to the simultaneous performance of hydrolysis and extraction (SHE) in order to accelerate the liquid-liquid extraction and facilitate the transfer of ERN from the water phase to the $CH_3COOC_2H_5$ layer.(53) The addition of salt decreases the solubility of ERN within the aqueous phase, resulting in an augmented partitioning of ERN into the $CH_3COOC_2H_5$ layer.(53) This phenomenon is known as “salting out” and is attributed to the high solubility of NaCl in water, compared to $CH_3COOC_2H_5$.(53,54) The effectiveness of the salting-out capability is influenced by various factors, including the size, ionic charge, and the concentration of the cation present.(53)

In addition, NaCl could also be employed during the hydrolysis step to assist in the disruption of the plant tissue, achieved through osmotic effects. The presence of NaCl in the external environment leads to a lower ionic concentration outside the cells, resulting in the influx of water into the cells. As a consequence, the cells swell and eventually rupture under increased internal pressure. The disruption of the plant tissue facilitates the release of ERN and myrosinase, sequestered within specialized cells, thereby promoting the hydrolysis reaction.(9,20,27,32,33) In this master dissertation, the distortion of the plant tissue was achieved through the application of mechanical force using a hand blender.

3.1.4. Phosphatidylcholine (PC)

PC, the predominant phospholipid in eukaryotic cellular membranes and in lecithin, is frequently used in formulating liposomes due to its presence of phospholipid bilayers, which enhances the encapsulation capacity of liposomes.(55,56) Lecithin, a complex mixture of triglycerides, phospholipids and glycolipids, naturally occurs in various sources such as soybeans, egg yolks, sunflower, rice beans, and rapeseed.(40,41,55) However, in the field of biochemistry, lecithin specifically refers to the pure extracted PC.(55) Soy lecithin, rich in PC, is favored for large-scale liposome production due to its greater stability compared to other lecithin sources.(55)

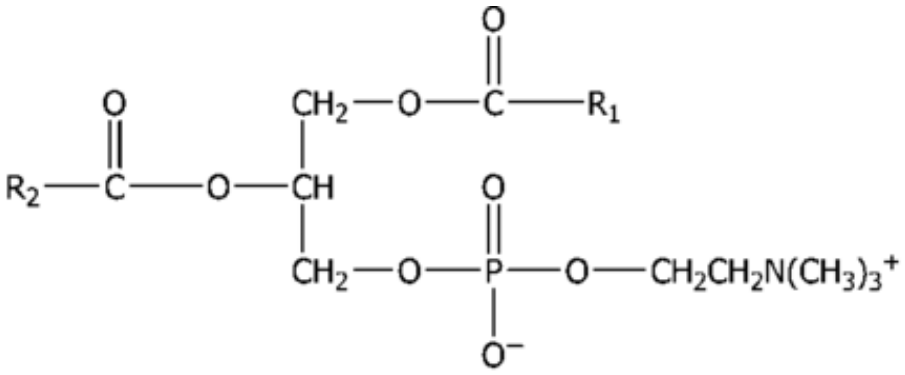

Figure 3.1: Chemical structure of phosphatidylcholine(57)

PC is composed of two fatty acid chains, a glycerol backbone, a phosphate group, and a choline molecule. This chemical arrangement provides PC with amphiphilic properties, characterized by both hydrophilic and hydrophobic regions, enabling its formation of bilayer structures.(57) The hydrophilic part consists of the phosphate and choline group, which face the surrounding aqueous environment, while the hydrophobic tails, comprised of the two fatty acid chains, create a protective lipid layer. ERN is slightly soluble in water, resulting in a greater compatibility with the hydrophobic part of the amphiphilic molecule, thereby favoring its presence within the lipid layer. In this master dissertation, the purification of PC from lecithin, extracted from soy beans, in pure C_2H_5OH was performed by Serena from the Eng4Life lab.

3.2. INSTRUMENTS

3.2.1. Summary

A moisture analyzer from OHAUS (Parsippany; New Jersey; United States) was used to determine the humidity of the fresh rocket leaves. An electronic analytical balance from Precisa (Dietikon; Switzerland) was used for accurately measuring the mass of the leaves. A hand blender was utilized during the sample preparation for the hydrolysis. The pH was measured with an Edge benchtop pH meter (Hanna Instruments; Woonsocket; Rhode Island; United States). The extraction process involved different energy-adding tools, including the Mechanical stirrer AT-M (FALC; Civitanova Marche; Italy), the Hot Plate Magnetic Stirrer Model Tk23 (technoKartell; Noviglio; Italy) or the Ultrasonic Processor (Sonics; Newtown; Connecticut; United States). The Neya bench top centrifuge (REMI;

Mumbai; India), Separation funnel 250mL (Schott Duran; Mainz; Germany), paper filter, diaphragm vacuum pump (Edwards vacuum; Burgess Hill; United Kingdom) were separation tools employed during the laboratory experiments. The Rotavapor (Heidolph; Schwabach; Germany) and Orbital shaker (Stuart; Barcelona; Spain) were used to evaporate the organic solvent used during the extraction. The calibration curve of ERN was obtained utilizing a micropipette and vortex (biosan; Riga; Latvia).

A simil-microfluidic (SMF) bench scale apparatus (designed by Eng4Life; Salerno; Italy) with chronometer to control the flow rates, were used to encapsulate ERN in liposomes. The determination of the characteristics of the produced liposomes were obtained by Open-Source Dynamic Light Scattering (osDLS) (designed by Eng4Life; Salerno; Italy) and a Turbidity meter (PCE Instruments; Meschede; Germany), along with a Tangential Flow Filtration (TFF) (Pall corporation; New York; United States) in combination with a syringe pump of 50mL (Terumo; Shibuya; Tokyo; Japan) and a plastic clamp.

All the samples were analyzed with HPLC (Agilent technologies; Santa Clara; California; United States) with the method described below in section 3.2.2. For the sample preparation a syringe filter of 25mm diameter with an PTFE membrane and pore size of 0.45µm was employed (ISOLAB; Wertheim; Germany) in combination with a syringe of 1mL or 3mL (Terumo; Shibuya; Tokyo; Japan).

3.2.2. High Performance Liquid Chromatography (HPLC)

HPLC is an analytical device used to separate and identify ERN present in the sample and to quantify it, making use of two external calibration curve created with an ERN standard of 25mg, differentiating in the used injection volume. The standard solutions were prepared with pure C_2H_5OH for a set of ERN solutions at concentrations of 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, 0.195, 0.097mg/mL for the calibration curve using an injection volume of 30µL. The other calibration curve added an additional 1:2 dilution, amounting to 0.049mg/mL. The ratios of the Area Under the Curve (AUC) of ERN for each solution were plotted against the concentrations to form the standard curves.

The HPLC equipment employed in the laboratory consists of 5 individual compartments, each serving its own functionality. The employed HPLC method, described in section 3.2.2., is reported by the Zhejiang University of Science and Technology, located in Hangzhou, China.(21)

The first compartment, known as the sampler (G1329B), is responsible for accurately injecting 10 or 30µL, depending on the employed HPLC method, from the vial. It incorporates a needle wash and column purge to prevent contamination from previous analyses during the subsequent sample analysis. The second compartment consists of the quaternary pump (G1311C), which regulates the flow rate at 0.75mL/min. It has awareness of the bottle fillings and controls the composition of the mobile phase, allowing for the establishment of a gradient if required. The initial conditions of the mobile phase employed in our experiment consists of 10% methanol in water, changing linearly over 35min to 90% methanol, which is maintained for additional 2min to ensure column purging. The total analysis duration is 37min. The column compartment (G1316A) is equipped with an apolar Kinetic 2.6µm XB-C18 (4.6×150 mm) column purchased from Phenomenex (Torrance, California, United States), which is maintained at a temperature of 30°C. The characteristics of the column will determine the separation and retention time of the ERN during the HPLC analysis. The employed method follows an isochromatic approach, where a Variable Wavelength Detector (VWD; G1314B) is set at a fixed wavelength of 241nm to detect ERN around the 25th minute. The fifth compartment consists of a Refractive Index Detector (RID), which is not relevant to our experiment and thus not utilized.

The sample preparation involves filtering the sample through a syringe filter using either a 1mL or 3mL syringe. The primary objective of the filtration step is to eliminate solid parts larger than 0.45µm, thereby protecting the integrity of the column. Afterwards, the samples are transferred into 1.5mL HPLC vials for subsequent HPLC analysis.

3.2.3. Simil-microfluidic (SMF) apparatus

The SMF apparatus, utilized in this master dissertation, makes use of the ethanol injection method coupled with the microfluidic fluid dynamic principles and consists of 2 peristaltic pumps: a water pump and an anhydrous C_2H_5OH pump.(42,43) These pumps are connected to corresponding inlet and outlet tubes, which transport the continuous flow of fluids, provided by the pumps, within the SMF apparatus. The lipids/C_2H_5OH feed solution, in which ERN and PC are dissolved, has a flow rate of 4.5mL/min and will be directly injected into the polar phase with a flow rate of 45mL/min through a needle, creating a laminar flow of the C_2H_5OH phases in the aqueous phase.(42,43) At the interface between both solutions, the spontaneously self-assembled internal formation of liposomes, to obtain minimal internal energy, takes place due to the reduced solubility of the solvent in water, the ratio of 1:10 between the low volumetric flow rates and the ratio of 1:5 between the internal diameters of the tubes.(7,42,43) The end product is an ERN dilution of 1:11 in the resulting liposomal suspension on a nanoscale.

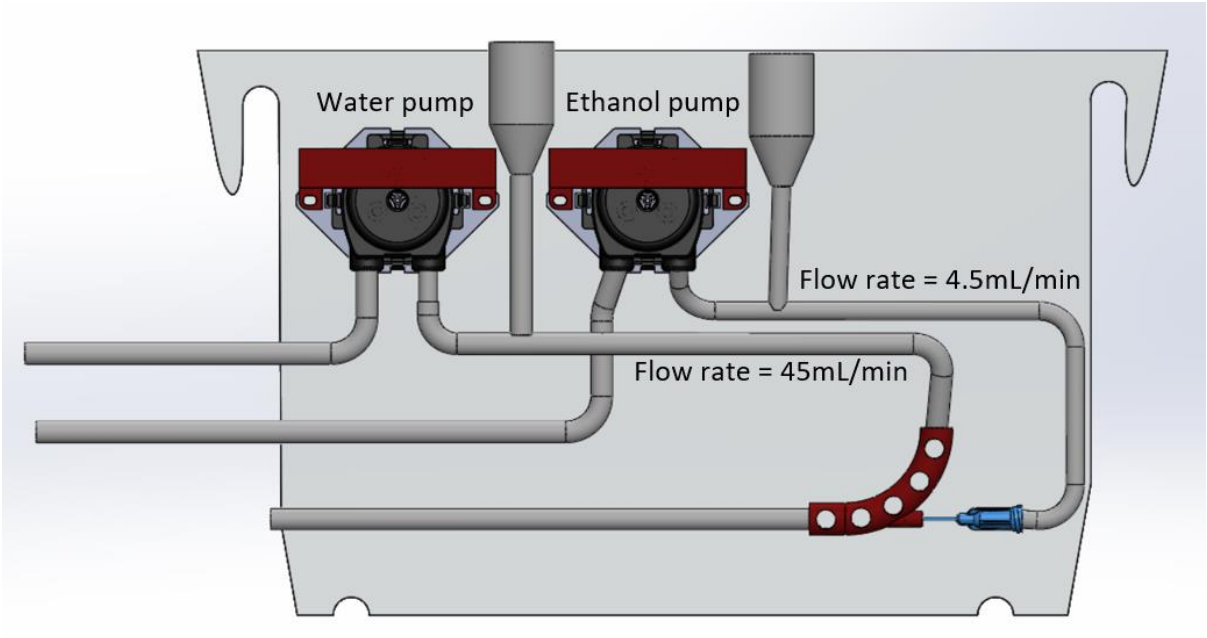

Figure 3.2: Simil-microfluidic apparatus incorporating 2 peristaltic pumps with corresponding tubes and flow rates

3.2.4. Tangential Flow Filtration (TFF)

Figure 3.3: Tangential Flow Filtration device(58)

TFF is used for the quantitative determination of non- encapsulated ERN in liposomes by separating the liposomes from the external medium. It is an indirect method to determine the encapsulated concentration of ERN within the hydrophilic bilayer of the liposomes. A syringe of 50mL is connected to the TFF feed inlet to facilitate the transport of the liposome suspension throughout the TFF system. A tubing is attached to the retentate outlet of TFF and closed off using a plastic clamp, inducing a pressure drop that forces the movement of the liquid through the TFF membrane. The membrane employed possesses a defined pore size of 300kDa, selectively permitting the passage of particles equal to or smaller than 300kDa. Consequently, the liposomes will be retained by the membrane, separating them from the external phase. The resulting permeate, containing the non-encapsulated ERN, will be collected for subsequent analysis to determine the concentration of ERN with HPLC.

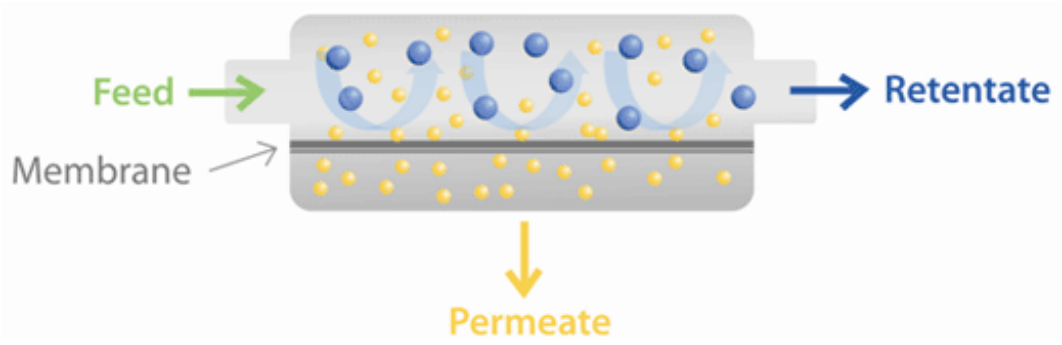

Figure 3.4: mechanism of action of a tangential flow filtration device(59)

3.2.5. Open-Source Dynamic Light Scattering (osDLS)

OsDLS, also known as photon correlation spectroscopy, is a technique that utilizes the phenomenon of light scattering to determine the size of particles in a solution.(40) During the osDLS measurement, a laser beam is directed through the cuvette containing the liposomal solution. As the laser light interacts with the liposomes. The scattered light carries information about the size and motion of the particles. The osDLS instrument detects the scattered light and analyzes its fluctuations over time. These fluctuations are caused by the Brownian motion, which are defined as the random movement of nanoparticles suspended in a liquid, of the liposomes, which is influenced by their size.(5) By mathematically analyzing the intensity fluctuations of the scattered light, osDLS can determine the hydrodynamic diameter of the liposomes.(43) The hydrodynamic diameter obtained through osDLS represents the effective size of the liposomes in solution, it provides valuable information about the size distribution and polydispersity index (PDI) of the liposomal sample.(43)

3.3. METHODS

3.3.1. Extraction of erucin from rucola leaves

3.3.1.1. SHE with ethyl acetate

The first procedure used for extracting ERN from arugula leaves was the SHE method (Figure 3.5), based on the procedure reported by the Zhejiang university and was slightly modified according to the findings and the equipment available in the lab.(60) The sample preparation consisted of a grinding step, which crushed 100g fresh leaves of arugula with a hand blender, after adding a buffer solution from 0.1M and a pH of 7.0 in a ratio of 1 to 2 (buffer/leaves) to allow GSL hydrolysis. The organic solvent $CH_3COOC_2H_5$ was added to the mixture in a ratio of 1 to 1.17 (leaves/$CH_3COOC_2H_5$) to perform the extraction step using the mechanical stirrer AT-M, followed by an incubation step under continuous shaking at 100 revolutions per minute (rpm) at room temperature. After 4 hours of incubation, NaCl was added in a ratio of 1 to 15 (NaCl/leaves), followed by another incubation step of 15 minutes under the same conditions as described before. Afterwards, a filtration step was performed by using a paper filter to remove the solid particles. Mostly, an additional centrifugation step occurred to remove the remaining solid particles.

On the liquid residue of the filtration or centrifugation, a washing step was executed with 100mL of $CH_3COOC_2H_5$. Afterwards, the separation funnel was mechanically shaken to start the liquid-liquid extraction. The extraction process was repeated by adding 50mL of $CH_3COOC_2H_5$. Due to the density differences between $CH_3COOC_2H_5$ and water, it was possible to decant the bottom, aqueous, phase and the remaining $CH_3COOC_2H_5$ layer was consequently collected. The water phase was again added to the separation funnel, followed by 50mL of $CH_3COOC_2H_5$ to perform a second extraction. The final $CH_3COOC_2H_5$ extracts were combined, creating approximately 250mL of extract. Sometimes it was indicated to add anhydrous Na_2SO_4 to the collected $CH_3COOC_2H_5$ phase to remove the remaining water before analyzing it with HPLC.

The combined $CH_3COOC_2H_5$ layers were dried under vacuum at room temperature using a rotary evaporator to remove the solvent. The dried residue was dissolved in 15mL of $CH_3COOC_2H_5$ and agitated using an orbital shaker until the majority of the residue was dissolved. Subsequently, the sample was subjected to analysis by HPLC.

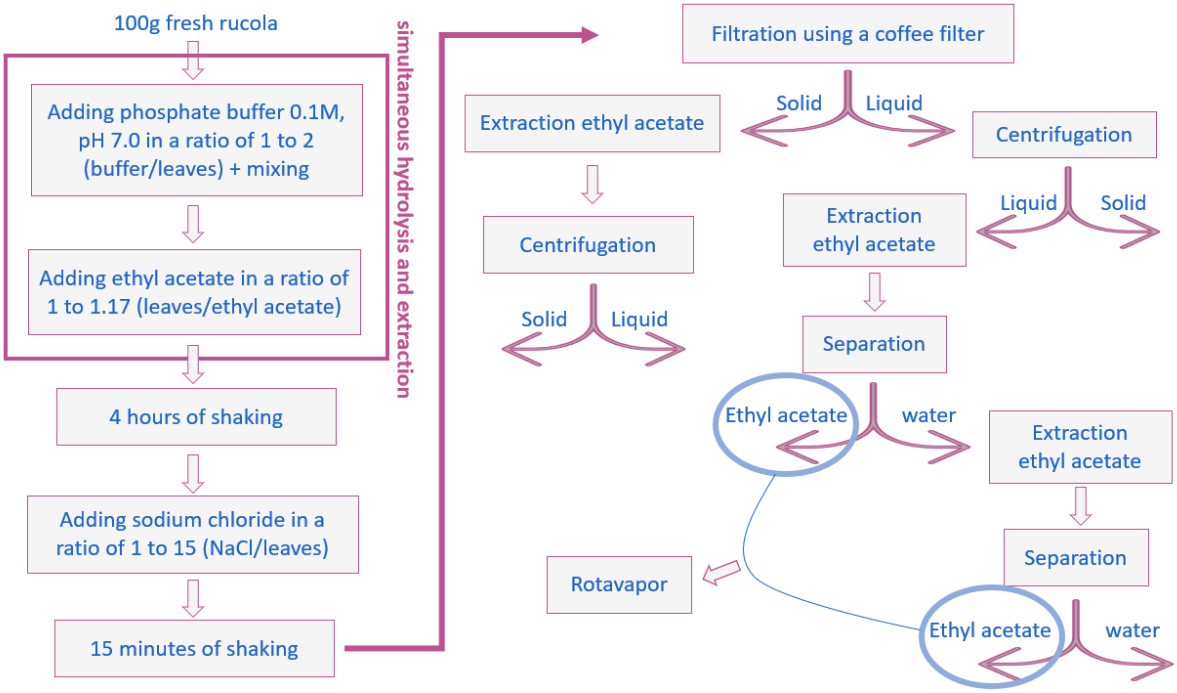

Figure 3.5: Schematic representation of the SHE method with ethyl acetate

3.3.1.2. HFE on solid part with 96° ethanol

The second method consisted of a hydrolysis followed by extraction (HFE) (Figure 3.6), where the organic solvent $CH_3COOC_2H_5$ was substituted by 96° C_2H_5OH. The sample preparation consisted again of a grinding step, which crushed 100g fresh leaves of arugula with a hand blender, after adding a buffer solution from 0.1M and a pH of 7.0 in a ratio of 1 to 1 (buffer/leaves) to allow sufficient GSL hydrolysis. After an incubation step of 2 hours using a Hot Plate Magnetic Stirrer Model Tk23 at room temperature under continuous shaking at 100rpm, in a closed environment provided with parafilm, a filtration step was performed by using a paper filter.

On the solid residue of the filtration, the organic solvent 96° C_2H_5OH was added to the mixture in a ratio of 1 to 1.17 (leaves/ C_2H_5OH) to perform the extraction step. After another

incubation step of 2 hours using a Hot Plate Magnetic Stirrer Model Tk23, performed under the same conditions as described above, the mixture was divided over 2 plastic tubes of 50mL and were put in the Neya bench top centrifuge (25°C, 9500rpm, 10min) to separate the liquid part from the solid part. After bringing over the liquid phase to other tubes, 15mL of 96° C_2H_5OH was added to the remaining pellet of each tube to perform a second extraction step, followed by mixing the tubes with a vortex and mechanically shaking until homogenous. The tubes were again put in the Neya bench top centrifuge, under the same conditions, to separate the liquid once again from the solid. After both extraction steps, the supernatant was analyzed by HPLC to quantify the amount of ERN extracted from the arugula leaves.

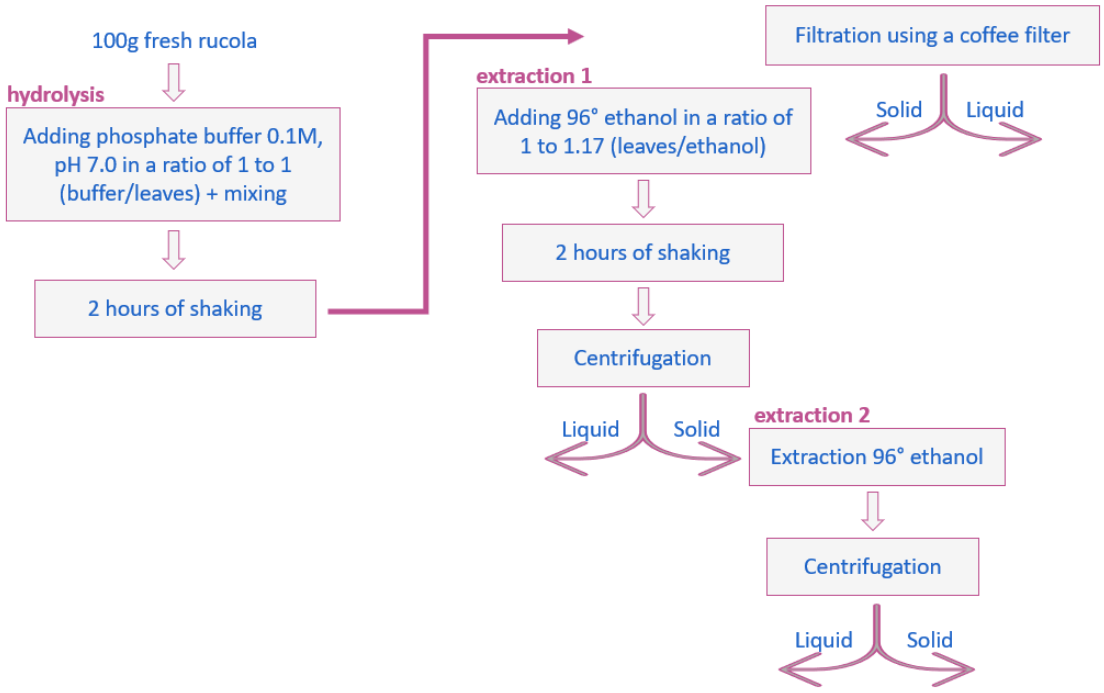

Figure 3.6: Schematic representation of the HFE on solid part with 96° ethanol

3.3.1.3. HFE on hydrolysis product with 96° ethanol

The third method made again use of a HFE method (Figure 3.7), where the organic solvent used was also 96° C_2H_5OH. The sample preparation consisted again of a grinding step, which crushed 100g fresh leaves of arugula with a hand blender, after adding a sulfate buffer from 0.1M until a pH of 7.0, measured with a pH meter, was reached to achieve ideal conditions for the GSL hydrolysis. After an incubation step of 2 hours using a Hot Plate

Magnetic Stirrer Model Tk23, at room temperature under continuous shaking at 50rpm, in a closed environment provided with parafilm, 50mL of 96° EtOH was added to the mixture. The solid was not separated anymore from the liquid. After 1 hour of incubation, an aliquot was taken from the mixture and submitted to the Neya bench top centrifuge (25°C, 9500rpm, 10min). After separation, the supernatant was used for subsequent analysis with HPLC. This procedure was repeated until a total incubation time of 3 hours was reached.

After the hydrolysis, a sample was taken from the mixture to employ an alternately extraction method utilizing a sonicator to provide energy through mechanical steering. Precautions were taken to prevent excessive heating of the mixture. The incubation step was carried out in an ice buffer by altering 1 minute of sonication, followed by minute of cooling down the mixture, amounting 15 minutes of agitation in total. At every 5-minute interval of sonication, an aliquot was taken to assess the impact of sonication on the extraction process. The sample was transferred to an Eppendorf tube and subjected the Neya bench top centrifuge (25°C, 9500rpm, 10min). After centrifugation, the supernatant was analyzed using the HPLC method described in section 3.2.2.

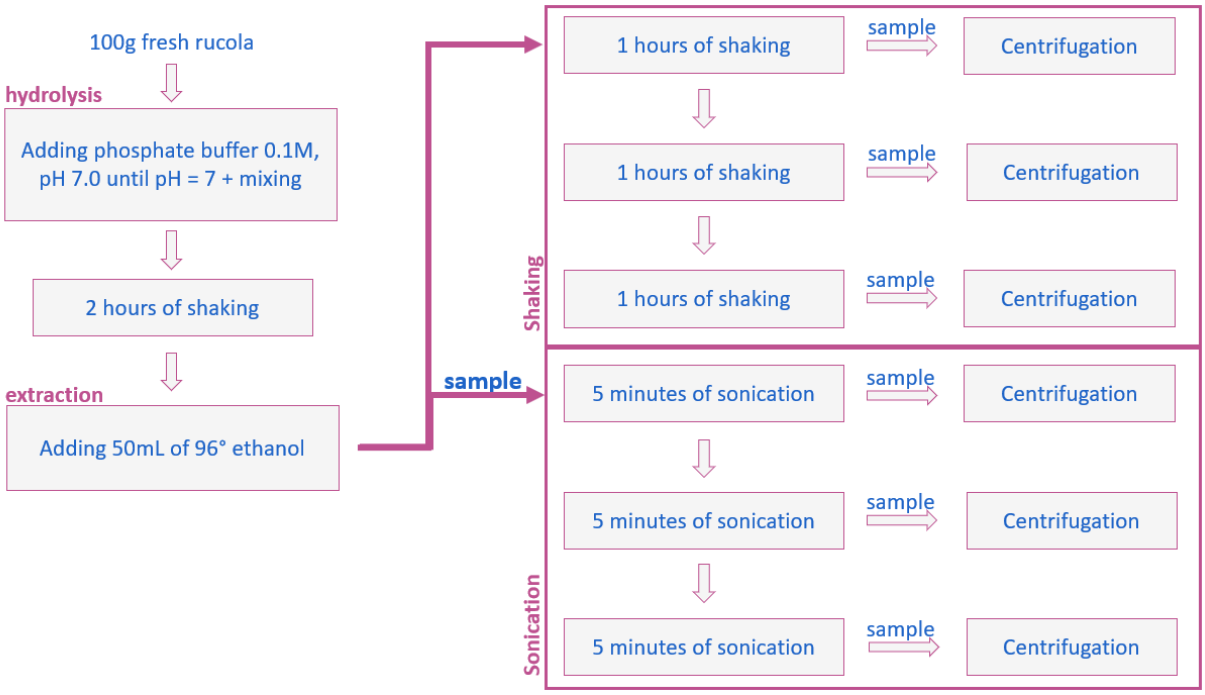

Figure 3.7: Schematic representation of the HFE on hydrolysis product with 96° ethanol

3.3.2. Encapsulation of erucin in liposomes using SMF

First, a washing step of the pumps and tubes is needed to avoid contamination of previous analysis and the waste will be collected at the end of the outlet tubes. The water pump was washed with distilled water, while the C_2H_5OH pump was purged with pure C_2H_5OH. During the washing, it is important to control all two of the flow rates by using a chronometer and collecting the amount of liquid from the outlet tubes in an graduated cylinder of 50mL for minimum 30 seconds. To accurately control the flow rate, it is important that only the inlet tube of that pump is transporting liquid because it is not possible to switch off individual pumps. After this washing step, the inlet tube of the C_2H_5OH pump was placed in a lipid solution of 22.5mL anhydrous C_2H_5OH, containing 0.56mg/mL ERN and 67.3mg/mL PC, while the inlet tube of the water phase remained in the deionized water. The outlet tube of the water pump was transferred to another flask to collect the formed liposome suspension. The lipid C_2H_5OH solution with a measured flow rate of 4.7mL/min, was rapidly injected into the aqueous phase with a flow rate of 48mL/min through a needle, creating a laminar flow of the C_2H_5OH phases in the water phase. After collecting all the formed liposomes, another washing step will be performed with the solvents corresponding to the specific pumps. The SMF apparatus is thoroughly described in section 3.2.3.

3.3.3 Determination of the characteristics of liposomes

3.3.3.1. Open-Source Dynamic Light Scattering (osDLS)

The sample preparation consisted of the cleaning of the glass cuvette by using distilled water and a soft paper to ensure the removal of all the remaining nanoparticles. Afterwards, shaking of the nanosuspension was performed to ensure a representative taken aliquot. 1000µL aliquot of the liposome suspension was transferred into the appropriate reading cuvette for analysis utilizing a glass pipette with a rubber. Before osDLS analysis, performed at room temperature, it is important to ensure the absence of air bubbles. Sometimes an additional dilution step is necessary if the intensity of scattered light is higher than 30MHz. The mode of action of the osDLS is elaborately outlined in section 3.2.5.

3.3.3.2. Tangential Flow Filtration (TFF)

A syringe of 50mL, containing distilled water, was connected to the feed inlet of TFF system in order to remove the 0.5M natrium hydroxide, that was initially present, throughout the device and obtain a neutral pH. To ensure selective collection of the permeate, a tubing was attached to the retentate outlet of TFF and closed off with a plastic clamp, preventing collecting particles that didn't pass through the filtration membrane. Once a neutral pH was reached, through precise measurement utilizing a pH meter, the syringe was loaded with the liposome suspension. As the liposome suspension got transported through the TFF system, the resulting permeate, containing the non-encapsulated ERN, was collected for subsequent HPLC analysis to determine the concentration of ERN with HPLC. TFF is meticulously described in section 3.2.4.

3.3.3.3. Turbidity meter

The usage of the turbidity meter is straightforward. First, the 2mL cuvette was cleaned using distilled water to ensure there is no residue or contamination. Subsequently, the cuvette was filled with the sample of interest. The turbidity meter is employed to quantify the turbidity of the aliquot, providing a numerical value expressed in Nephelometric Turbidity Units (NTU).(43)

4. RESULTS AND DISCUSSION

4.1. OPTIMIZATION OF THE EXTRACTION PROCESS

4.1.1. SHE with ethyl acetate

The majority of methods reported in literature perform the hydrolysis and extraction separately. However, the ERN yield seems to be higher by performing them simultaneously.(60) Conversely, the standard procedures utilize arugula seeds rather than arugula leaves for ERN extraction. This preference is due to the fact that the seeds of the rocket contain approximately 4-fold higher levels of GSLs, leading to a higher concentration of ERN, than arugula leaves. (16,20,21) The decreased ERN content can be attributed to the composition of the arugula leaves, consisting of 90 percent water and 10 percent solid mass, of which ERN represents a maximum of 1.77 percent. (9–12)

The objective of the buffer solution, calculated using equation 3.1, is to obtain a maximally enzymatic conversion from GSLs into ERN by creating a neutral environment. As previously described, the optimal conditions for the hydrolysis are working in a neutral environment, at low temperatures and in the presence of water. (17,26,30) The nitrile formation is favored at a lower pH range and the acidic environment has a negative impact on the hydrolysis reaction.(27,30) The continuous shaking facilitates the contact between the substrate and its enzyme, leading to an improved conversion. To ensure the preservation of the activity of myrosinase, it is crucial to avoid denaturation by heat. Therefore, temperature monitoring during the incubation process is desirable.(15,27) The temperature was not controlled in our lab as each incubation step was carried out at room temperature and the blending did not sensibly increase the temperature of the mixture, rendering temperature control unnecessary. However, it is worth noting that risk of deactivating myrosinase due to denaturation occurs only at temperatures exceeding 70 degrees Celsius. Nevertheless, ERN, the desired product, is more susceptible to thermal degradation compared to its catalyst. Hence, caution must be exercised to prevent excessive heat exposure that could lead to the degradation of ERN.

Figure 4.1: Presence of solid particles complicating the separation of the Ethyl acetate layer (upper layer) from the water layer (bottom layer)

The filtration step is necessary due to the high presence of solid particles, which complicates the separation of two layers (Figure 4.1). Therefore, the solid part is preferably removed from the mixture to facilitate the separation of the two layers. Sometimes the filtration step is inadequate to remove the entire number of solids, requiring an additional centrifugation step. It is also possible to perform the filtration using a vacuum pump instead of a paper filter, which is even more efficient in separating the liquid from the solid phase. In our experiment, it was not possible to employ this filtration technique because the qualities of $CH_3COOC_2H_5$ caused the interruption of the paper filter, compromising the procedure.

Table 4.1: Dilutions of erucin standard for the construction of the calibration curve with an injection volume of 10µL

Sample	Concentration (mg/mL)	Absorbance (mAU*s)	Retention time (min)
S1	12.5	32011.2	25.355
S2	6.25	16333.0	25.259
S3	3.13	8432.5	25.227

S4	1.56	4273.1	25.183
S5	0.781	2110.4	25.152
S6	0.390	1065.0	25.249
S7	0.195	545.8	25.283
S8	0.0975	281.5	25.148
S9	0.0488	141.1	25.151

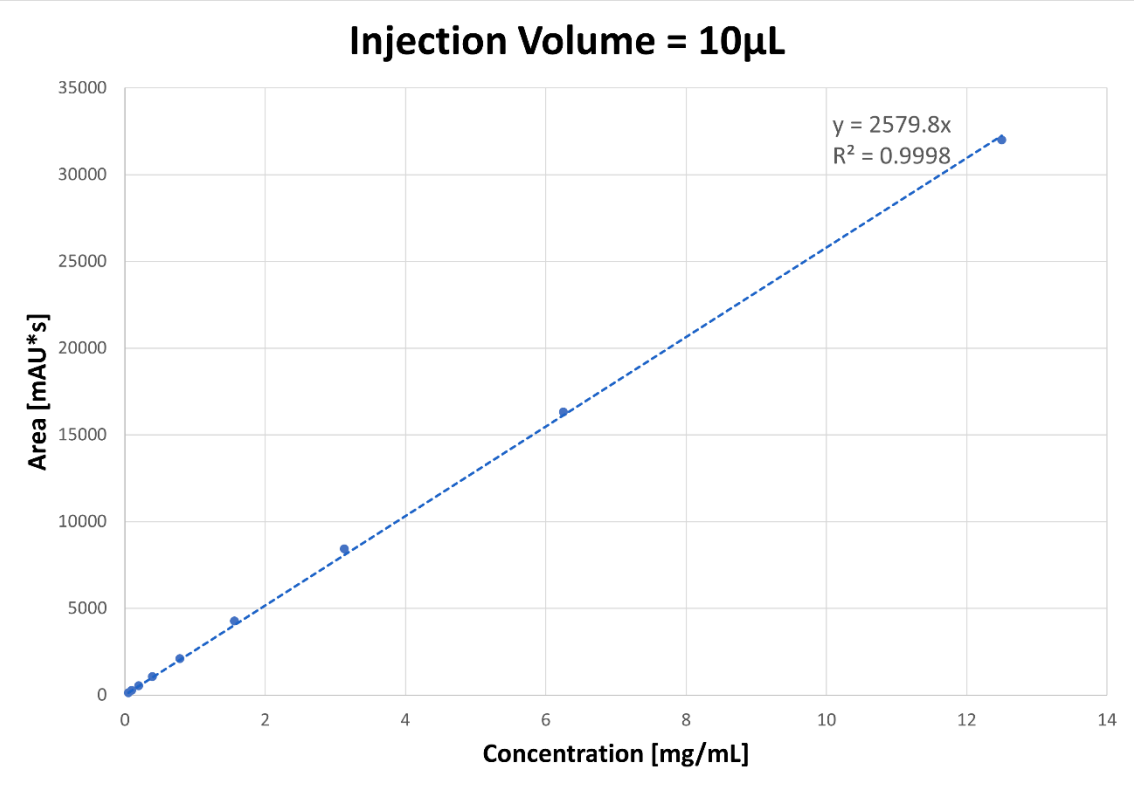

Figure 4.2: Calibration curve to quantify the erucin concentration

To enhance linearity, the undiluted S0 was excluded from the calibration curve. Additionally, two additional points (S8 and S9) were added in order to improve the accuracy of extrapolation from the AUC to the corresponding concentration in mg/mL. These points were also necessary as the concentrations obtained from the extracted samples fell within a lower range than the current calibration curve. The calibration curve demonstrates excellent linearity over the concentration range of 0.05–12.5 mg/mL, with a coefficient of determination (R^2) value of 0.9998, which indicates a strong correlation between the measured AUC values and the actual concentrations of ERN. Consequently, the calibration curve can be confidently used for quantification and accurate determination of ERN

concentrations within the specified range. The calibration curve was obtained using the HPLC method, reported in section 3.2.2.

Table 4.2: Calculations regarding SHE combined ethyl acetate phases

	Experiment 1	Experiment 2
AUC (mAU*s)	34.161	80.730
Rt (min)	25.418	25.245
Total Volume of $CH_3COOC_2H_5$ (mL)	258	217
Conc (mg/mL)	0.0132	0.0311
Mass (µg)	3416.3	5226.0
Amount (µg/g dry)	341.6	522.6

By utilizing the retention times of the ERN standard as a point of reference, our focus lies on the peaks falling within the time range of 25.1 and 25.4 minutes. Both peaks obtained from the two experiments described in Table 4.2, specifically 25.418 and 25.245 minutes, are comparable to this range, suggesting that they could potentially correspond to the presence of ERN. To facilitate comparison of values and ensure consistency in the quantification of ERN, considering the arbitrary unit used in literature, the concentrations of ERN are expressed in µg/g of dry rocket.(51) Expressing the ERN concentrations in terms of dry weight of arugula leaves, rather than the employed volume of extracts, allows a meaningful comparison of ERN content between different samples, regardless of the specific volume employed. The amounts (µg/ g dry) in Table 4.2 were determined using equation 4.1.

$$\text{Amount} = \frac{\text{AUC}}{\text{A}} * V_{total}(mL) * 1000\frac{\mu g}{mg} * 0.1 \qquad \text{(Eq 4.1)}$$

Where: AUC = Area Under the Curve obtained from HPLC (mAU*s)

A = slope from calibration curve = 2579.8

0.1 = considering the 90% water content of rocket leaves

It is important to address the dissimilarities between the two executed experiments in order to gain a comprehensive understanding of the reported results in Table 4.2. In the second experiment, a decrease in the used volume of $CH_3COOC_2H_5$ was implemented, specifically employing 50mL instead of 100mL to perform the first extraction step. The usage of a lower

volume aimed to achieve a higher concentration of ERN. However, this reduction may potentially compromise the extraction power of the organic solvent due to a decreased concentration gradient that drives the equilibration between water and $CH_3COOC_2H_5$. Additionally, in the first experiment, the ethyl layers obtained from both washing steps were combined and subjected to HPLC analysis. On the contrary, in the second experiment, separated analysis were conducted on the $CH_3COOC_2H_5$ layers after each washing step. The value referred to in table 4.2 represents the cumulative amounts of ERN obtained from both HPLC analysis. Notably, in the $CH_3COOC_2H_5$ layer, collected after the second was, the presence or ERN was no longer detectable, indicating that the first washing step inhibited high efficiency in extracting all the ERN from the aqueous layer.

During the repetition of the first experiment, a mixture compromising solid parts, $CH_3COOC_2H_5$ and water was subjected to an overnight incubation period at room temperature, thereby extending the contact time for the extraction process. This prolonged contact duration likely resulted in a greater opportunity for the solute molecules, including ERN, to distribute themselves more effectively between the two phases, resulting in a significant increase of the quantified amount of ERN given that ERN is only slightly soluble in water. The first experiment obtained an ERN content of 341.6µg/g dry rocket, while the second experiment demonstrated an even higher quantity of 522.6µg ERN/g dry rocket.

In literature, a study conducted on broccoli seeds using SHE, reported a mean ERN content of 933,34µg/g, including 3 different types of broccoli seeds, namely LS-1, LS-2 and Xinfeng seeds.(60) The method was also performed on crude broccoli, reporting a mean ERN content of 1910µg/g.(60) Both of these reference values are higher than the values obtained in our experiments. Another study conducted on Broccoli Green Dragon, utilizing methylene chloride as an extraction solvent, reported an ERN content of 2528.1µg/g.(21) These finding are not unusual and can be attributed to several factors. One significant reason may be the use of different cruciferous plants, with broccoli being used in the mentioned studies, whereas our experiments were performed on arugula. The department of Food Science and Technology of the Ohio State University revealed in their investigation that the highest levels of ERN formation were observed in arugula seeds, followed by

arugula leaves, while broccoli sprouts exhibited the lowest amounts.(22) It is also well-known that arugula seeds are more concentrated in the GSL glucoerucin compared to their leaves.(22) Remarkable is that these findings cannot be extrapolated to broccoli since the reported value of crude broccoli is approximately twice as high compared to the broccoli seeds.(16)

Table 4.3: Calculations regarding extraction on the solid part after filtration

	Experiment 1
AUC (mAU*s)	76.9119
Rt (min)	25.279
Total Volume of $CH_3COOC_2H_5$ (mL)	14
Conc (mg/mL)	0.0298
Mass (µg)	417.4
Amount (µg/g dry)	41.7

An additional experiment was performed on the solid parts, derived from the filtration step using a paper filter. This solid mass was transferred to a 50 plastic tube, into which 14mL of $CH_3COOC_2H_5$ was added. Subsequently, intense shaking and vortexing were employed to ensure homogenization of the mixture. The plastic tube was then subjected to the Neya bench top centrifuge (25°C, 9500rpm, 5min), enabling the separation of the supernatant from the solid pellet for subsequent HPLC analysis. (Figure 3.5) The obtained mass was 41.7µg ERN/g dry, reported in Table 4.3, indicating that the extraction efficiency achieved with SHE is less than 100 percent, signifying the presence of a residual quantity of ERN within the solid part.

Table 4.4: Calculations regarding the rotary evaporator

	Remaining extract	$CH_3COOC_2H_5$
AUC (mAU*s)	31.1905	93.1889
Rt (min)	25.357	25.358
Total Volume of $CH_3COOC_2H_5$ (mL)	16	16
Conc (mg/mL)	0.0121	0.0361
Mass (µg)	193.4	578.0
Amount (µg/g dry)	19.3	57.8

The rotary evaporator was employed on the combined $CH_3COOC_2H_5$ phases, aiming to enhance the ERN concentration by reconstituting the vacuum dried residue in a reduced volume of $CH_3COOC_2H_5$ compared to the initial quantity of the organic solvent. It was observed that the highest levels of ERN were detected in the evaporated $CH_3COOC_2H_5$ with a content of 57.8µg ERN/g dry, as indicated in Table 4.4. In contrast, the reconstituted sample exhibit an ERN content of 19.3µg/g, suggesting that ERN possesses a volatile nature. Nevertheless, in order to obtain a more concentrated end product, a feasible approach is to terminate the process before complete evaporation of the sample, thereby achieving a lower volume of the evaporated $CH_3COOC_2H_5$ resulting in a higher concentration of the evaporated ERN.

4.1.2. HFE on solid part with 96° ethanol

The extraction procedure has undergone significant modifications in order to optimize the extraction of ERN from rocket leaves and improve the yield. One of the major changes implemented was the adoption of the HFE method, which involves the separation of the hydrolysis and extraction step. This differs from the previously used SHE method, allowing a better control and optimization of each individual step. To enhance the conversion of glucoerucin into ERN, adjustments were made to the experimental parameter. Specifically, the volume of the phosphate buffer, used in the hydrolysis, was doubled, resulting in a total volume of 100mL water for 100g of arugula leaves. In accordance with findings reported in literature, the duration of the incubation step was reduced to 2 hours.(17,26) Our previously performed experiment, using the rotary evaporator, highlighted the volatile nature of ERN, necessitating a change in the incubation procedure. To prevent loss of ERN, the incubation was performed in a closed environment, obtained by sealing the measuring cup with parafilm, and a Hot Plate Magnetic Stirrer Model Tk23 equipped with a rotating magnet. This provided gentle agitation to the mixture during the incubation, facilitating the ERN formation.

In addition, the extraction step was modified as well by substituting the $CH_3COOC_2H_5$ by the organic solvent C_2H_5OH as the extraction solvent. In this case, the usage of pure C_2H_5OH is not necessary since there are already trace amounts of water present from the

arugula leaves and buffer used for the hydrolysis. Complete removal of the total amount of water is not feasible by using a paper filter during the filtration step. It is important to acknowledge that C_2H_5OH is a significantly less powerful extraction solvent compared to $CH_3COOC_2H_5$. Additionally, water and C_2H_5OH form an azeotropic mixture, meaning that they have limited separation ability due to their mutual solubility. This property makes it difficult to remove water from C_2H_5OH after the extraction process, complicating the subsequent encapsulation of ERN in liposomes. The advantage of using C_2H_5OH lies in its compatibility with the human body, particularly for oral formulations of the medicines. Moreover, if the objective is to encapsulate ERN in liposomes, C_2H_5OH may be preferred due to its compatibility with the liposome encapsulation process. Furthermore, as with the hydrolysis step, the incubation step was performed in a closed environment using parafilm, preserving not only the volatile nature of ERN but also preventing the evaporation of the organic solvent, C_2H_5OH, during the incubation process.

Table 4.5: Calculations regarding HFE water after filtration

	Experiment 1	**Experiment 2**
AUC (mAU*s)	5.657	3.533
Rt (min)	25.284	25.263
Total Volume of buffer (mL)	92	127
Conc (mg/mL)	0.0022	0.0017
Mass (µg)	201.7	173.9
Amount (µg/g dry)	20.2	17.4

The water, recovered from the filtration step, is collected to quantify the ERN content lost in the buffer. The HPLC analysis reveals that 20.2µg ERN/g dry was found in the water of the first experiment, while the second experiment yielded 17.4µg ERN/g dry (Table 4.5). ERN exhibits limited solubility in water, indicating that only a small percentage will be present in the aqueous phase. Since the overall ERN content, extracted from the leaves, is very low in this experiment, reported in Table 4.6, a significant amount of ERN got lost in the water phase during the performance of the hydrolysis. This highlights the importance of preventing this phenomenon in future experiments to obtain the maximum possible concentration of ERN in the extracted end product. To address this issue, it becomes essential to create a method that eliminates the need for a filtration step.

Table 4.6: Calculations regarding HFE ethanol after centrifugation

	Experiment 1		Experiment 2	
	1st extraction	2nd extraction	1st extraction	2nd extraction
AUC (mAU*s)	8.019	1.382	4.758	3.102
Rt (min)	25.284	25.302	25.274	25.297
Total Volume of C_2H_5OH (mL)	136	54	30	30
Conc (mg/mL)	0.0031	0.0005	0.0018	0.0012
Mass (µg)	422.7	28.92	55.3	36.1
Amount (µg/g dry)	42.3	28.9	5.5	3.6

Subsequent experiments focused on extracting ERN from the solid part, obtained after the filtration. Preliminary experiments conducted on the solid residue of the filtration demonstrated a significant amount of ERN remaining, even after the performance of an extraction with $CH_3COOC_2H_5$. In both experiments, a consistent trend was observed. The resulting supernatant after conducting a centrifugation step, obtained from the first extraction, contained larger amounts of ERN compared to the second extraction. The solid residue from the first extraction was reused for performing the second extraction step, indicating that C_2H_5OH is capable of extracting ERN from the solid material, albeit with a low extraction power compared to $CH_3COOC_2H_5$. In literature, methylene chloride is acknowledged for having the highest extraction rate of ERN. However, due to the well-documented toxic properties and potential risks to human health, our laboratory opted not to utilize it in our laboratory.(21)

It is important to consider the utilization of different quantities of C_2H_5OH during the extraction process. The objective of employing varied volumes of the extraction solvent was to gain a better understanding into the impact on the concentration gradient as well as to evaluate their effect on the ERN concentration. When a large volume of C_2H_5OH is utilized, the concentration of ERN will be significantly reduced, despite the presence of a higher concentration gradient. The higher concentration gradient occurs due to the larger differences in ERN concentrations between the rocket leaves and C_2H_5OH. As a

consequence, this phenomenon has the potential to enhance the efficacy of the extraction process, resulting in higher ERN concentrations.

It is notable that the ERN content extracted using this method was found to be very low, reported in Table 4.6. These findings indicate that this particular extraction method is not ideal for obtaining significant amounts of ERN out of rucola leaves. Furthermore, the resulting ERN solution may not be suitable for subsequent encapsulation in liposomes. The low extraction efficiency suggests that an optimized extraction method is required to improve the yield of ERN from arugula leaves and generate a more concentrated ERN solution that is usable for further applications.

In absence of studies specifically using C_2H_5OH as an extraction solvent, a study conducted on broccoli seeds using the HFE method with $CH_3COOC_2H_5$ will be used as a reference. The study, executed by the Zhejiang university, reported a mean ERN content of 360µg/g, including the same 3 types of broccoli seeds as described previously.(60) The repetition of the method on crude broccoli obtained a mean ERN content of 170µg/g.(60) These values, although significantly lower than those obtained with performance of the SHE method, are still higher than the ERN content obtained in our experiments (Table 4.6). The disparity in ERN content between the reference study and our experiments can be attributed to various factors and modifications employed in our study, as previously discussed.

4.1.3. HFE on hydrolysis product with 96° ethanol

In the optimized HFE method, the specific volume of phosphate buffer added was not explicitly defined. Instead, the 0,1M phosphate buffer solution was added to the rucola leaves, crushed using a hand blender, until a pH of 7 was reached, amounting approximately 1mL buffer in total. Enzymatic reactions, such as the hydrolysis of glucoerucin to ERN, are known to be influenced by the pH of the reaction medium. Maintaining a neutral pH range is reported in literature to be favorable for maximizing myrosinase activity and subsequently enhancing the conversion of glucoerucin to ERN,

potentially leading to improved ERN yield. However, in the previously conducted experiments, the pH of the hydrolysis step was never measured or controlled. Consequently, the influence of pH on the enzymatic reaction and its impact on the overall ERN extraction efficiency remains unknown in our previously performed experiments.

The incubation steps in the optimized HFE method were carried out in a closed environment using parafilm and a Hot Plate Magnetic Stirrer Model Tk23 equipped with a rotating magnet. The stirring speed during the incubation was set at 50rpm specifically to avoid excessive temperature increase during the incubation processes. Higher stirring speeds combined with the higher viscosity of the mixture, due to the use of a lower volume of phosphate buffer, resulted in elevated temperatures. Furthermore, the exclusion of an additional filtration step prevents the loss of ERN during the hydrolysis, resulting in potentially higher yield of ERN. Consequently, the elimination of the filtration step necessitates a subsequent centrifugation step to separate the C_2H_5OH from the pellet. This is needed due to the substantial presence of solid particles remaining after the extraction, thereby complicating the further HPLC analysis. In order to minimize the sample size, the centrifugation process (25°C, 9500rpm, 5min) was performed by making use of Eppendorf tubes.

Table 4.7: Calculations regarding experiment 1 HFE ethanol after centrifugation

Experiment 1	EtOH 1 hour	EtOH 2 hour	EtOH 3 hour
AUC (mAU*s)	6.396	36.289	42.654
Rt (min)	25.073	25.034	25.040
Total Volume of C_2H_5OH (mL)	50	50	50
Conc (mg/mL)	0.0025	0.0141	0.0165
Mass (µg)	124.0	703.3	826.7
Amount (µg/g dry)	12.4	70.3	82.7

Through the systematic sample collection at hourly intervals during the extraction process, the optimal duration of incubation could be determined, rather than relying on literature-reported values. HPLC analysis of the taken samples, reported in Table 4.7, revealed that the highest ERN content was achieved after 3 hours of incubation, resulting in a concentration of 82.7µg ERN/g dry.

Due to the insufficient amounts of ERN obtained using the improved HFE method, the current HPLC method, described in 3.2.2., was adjusted by elevating the injection volume to 30µL to enhance the sensitivity and obtain higher AUC values. However, this modification rendered the previous calibration curve, established with a 10µL injection volume, no longer suitable for accurate extrapolation of AUC values to their corresponding concentrations (mg/mL). As a result, a new calibration curve was necessary, obtained by the optimized HPLC method.

The injection volume should be carefully selected, ensuring that it remains within the range of 1 to 5 percent of the total volume of the stationary phase.(61) The total volume of the stationary phase was calculated by considering the cross sectional area, obtained using equation 4.2. Additionally, the maximum feasible injection volume that could be employed in our new method, was calculated using equation 4.3 with the assumption that 70% of the stationary phase is occupied and only 30% can be filled with the eluent. The selected injection volume of 30µL is below the calculated value of 37µL obtained with Eq 4.3.

$$\text{Cross sectional area (mm}^2\text{)} = \frac{\pi * d^2}{4} = 16.62\text{mm}^2 \qquad \text{(Eq 4.2)}$$

Where: d = diameter of LC-column (mm) = 4.6mm

$$\text{Maximum } V_{inj}\text{(mL)} = 16.62\text{mm}^2 * l * 0.001\frac{\text{mL}}{\text{mm3}}\ 0.3 * 0.05 = 0.037\text{mL} \qquad \text{(Eq 4.3)}$$

Where: l = length of LC-column (mm) = 150mm

Table 4.8: Dilutions of erucin standard for the construction of the calibration curve with an injection volume of 30µL

Sample	Concentration (mg/mL)	Absorbance (mAU*s)	Retention time (min)
S2	6.25	35772.9	25.226
S3	3.13	17282.6	25.155
S4	1.56	9819.3	25.185
S5	0.781	2945.3	25.148
S6	0.390	2624.6	25.119

S7	0.195	1432.4	25.096
S8	0.0975	629.5	25.086

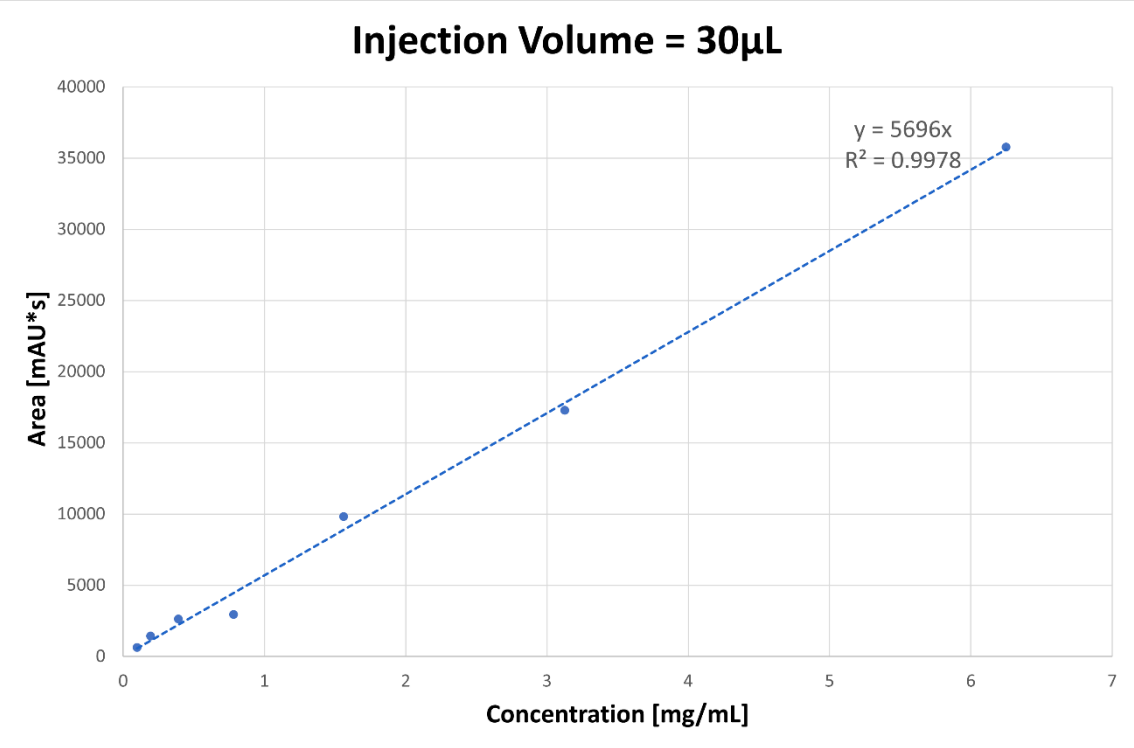

Figure 4.3: Calibration curve to determine the erucin concentration

To improve linearity, S0 and S1 was excluded from the calibration curve. To further enhance accuracy, it was contemplated to exclude S5 as well from the calibration curve. However, this seemed unnecessary given the purposes of our study. The calibration curve exhibited excellent linearity over the concentration range of 0.0975–6.25 mg/mL, as indicated by a high R^2 value of 0.9979. Consequently, the calibration curve can be used for quantification and accurate determination of ERN concentrations within the specified range.

Table 4.9: Calculations regarding experiment 2 HFE ethanol after centrifugation

Experiment 2	EtOH 1 hour	EtOH 2 hour	EtOH 3 hour
AUC (mAU*s)	76.982	84.157	84.179
Rt (min)	25.183	25.136	25.132
Total Volume of C_2H_5OH (mL)	50	50	50

Conc (mg/mL)	0.0135	0.0148	0.0148
Mass (µg)	675.8	738.7	738.9
Amount (µg/g dry)	67.6	73.9	73.9

Through the repetition of the experiment, consistent results were observed, confirming the optimal extraction duration of 3 hours. The obtained quantities were comparable to those obtained in the previously performed experiment, reported in Table 4.7, amounting 82.7µg ERN/g dry. The repeated experiment yielded an amount of 73.9µg ERN/g dry (Table 4.9). This similarity can be attributed to the use of the exact same amount of C_2H_5OH for the extraction process and the application of identical experimental conditions.

Table 4.10: Calculations regarding experiment 2 HFE ethanol after sonication

Experiment 2	**EtOH 1 sonication**	**EtOH 2 sonication**	**EtOH 3 sonication**
AUC (mAU*s)	38.456	13.364	10.446
Rt (min)	25.136	25.121	25.126
Total Volume of C_2H_5OH (mL)	50	50	50
Conc (mg/mL)	0.0068	0.0023	0.0018
Mass (µg)	337.6	117.3	91.7
Amount (µg/g dry)	33.8	11.7	9.2

In the repeated experiment, the incubation was primarily performed using the Hot Plate Magnetic Stirrer Model Tk23 equipped with a rotating magnet. However, a sample was subjected to the extraction process utilizing a sonicator. The addition of the mechanical agitation could potentially influence the extraction yield positively. Precautions were taken to prevent excessive heating of the mixture caused by the known temperature increase associated with the sonicator. Although, the results reported in Table 4.10 showed a degradation of ERN, compared to the previously obtained amounts of ERN in Table 4.9. Since both experiments were conducted concurrently and underwent the same hydrolysis, only the extraction method differs, making the results interesting to compare.

4.2. ENCAPSULATION OF ERUCIN STANDARD IN LIPOSOMES

Because our extraction experiments were inadequate to obtain a highly enough concentrated solution of ERN to achieve the desired objective of a 0.1% ERN load in liposomes, the encapsulation was only conducted once using the undiluted standard S0 with a concentration of 25mg/mL. This approach allowed the employment of SMF apparatus with a coaxial pump system, where ERN and PC were both present in C_2H_5OH phase in absence of water. ERN possesses slight solubility in water, making it more suitable to dissolve the isothiocyanate within an C_2H_5OH solution. If ERN exhibited strong hydrophilic properties, an aqueous solution would have been more appropriate, leading to ERN encapsulation in the core of the liposomes instead of in the hydrophobic bilayer. The outcome of the method was a clear nanosuspension of liposomes in a solution consisting of 10% C_2H_5OH and 90% water, as determined by the chosen flow rate ratio of 1:10. The limpidity of the suspension increases with larger nanoparticle size and higher concentration of liposomes.

By controlling the flow rate of the water pump, 24mL of water was collected within a 30-second time interval, resulting in a flow rate of 48mL/min. The microfluidics-like equipment employed in our experiment has a maximum achievable flow rate of 50mL/min. For the C_2H_5OH pump, a total volume of 4.7mL C_2H_5OH was collected over 1 minute, corresponding to a flow rate of 4.7mL/min. Additionally, it is crucial for both tubes to have comparable lengths to ensure an equivalent injection time in the aqueous tube. The diameter of the C_2H_5OH pump was measured to be 3mm, while the diameter of the water pump was 0.6mm. Both the flow rate ratio of 1:10 and diameter ratio of 1:5 between the two phases contributed to the formation of the internal lipid structure of the liposomes.

The calculations were conducted with the aim of achieving a 1% ERN load, which was the desired target for this experiment. From the standard solution S0 of 25mg/mL a sample of 500µL was withdrawn, resulting in a mass of 12.5mg ERN. The purified PC solution exhibited a concentration of 134.6mg/mL in 11mL anhydrous C_2H_5OH. To attain a dilution of 1:2, an additional 11mL of anhydrous C_2H_5OH was added, resulting in a final concentration of 67.3mg/mL. Thus, the total volume of the C_2H_5OH solution, containing

both ERN and PC, amounts 22.5mL. This calculation accounts for the 11mL of PC, 11mL C_2H_5OH, and 0.5mL of the initial ERN standard.

In summary:

Concentration of standard = $25\frac{mg}{mL}$ * 0.5mL = x * 22.5mL

$x = 0.56\frac{mg}{mL}$ ERN

Total concentration of lipids = $67.3\frac{mg}{mL}$ PC

% ERN maximally encapsulated = $\frac{0.56\frac{mg}{mL}\ \text{ERN}}{67.3\frac{mg}{mL}\ \text{PC}}$ *100% = 0.83% (<1%)

Final concentration after encapsulation = $0.56\frac{mg}{mL}$ * $4.7\frac{mL}{min}$ = x * (4.7 + 48) $\frac{mL}{min}$

$x = 0.05\frac{mg}{mL}$ ERN

It is also possible, although not obligated, to incorporate cholesterol into the C_2H_5OH phase in a ratio of 2.5:1 (PC/cholesterol) to mimic the typical composition of a cell membrane.(42,43) Cholesterol is known to enhance the structural stability and decrease the permeability of the formed liposomes.(40,41,62) In their absence, liposomes can interact with proteins present in the human body resulting in a destabilization of the formed liposomal structure.(40) If the addition of this hydrophobic compound would be considered, the added amount should be considered in the total lipid concentration for calculating the maximally possible percentage of ERN that can be encapsulated into liposomes.

If our extraction experiments yield a highly concentrated solution of ERN, the introduction of a third pump with anhydrous C_2H_5OH becomes necessary. The SMF apparatus comprises 3 peristaltic pumps: a water pump, an C_2H_5OH /water pump containing ERN and an anhydrous water pump for PC. The coaxial pump method would not be suitable for the ERN encapsulation in liposomes due to the insoluble properties of lecithin in water, which would result in the precipitation of PC in the C_2H_5OH/water mixture containing ERN. Both C_2H_5OH phases with a collective flow rate of 5mL/min, would be injected into the aqueous phase with a flow rate of 50mL/min through a needle, creating a laminar flow of the C_2H_5OH phases in the water phase. The ratio between the flow rates and the internal

diameters of the tubes would be maintained throughout this procedure. Consequently, the resulting liposomal suspension would exhibit an ERN dilution ratio of 1:11.

The employment of the C_2H_5OH injection method in conjunction with microfluidic fluid dynamic principles is accompanied by several drawbacks. One notable disadvantage is the heterogeneity, represented by the PDI, observed in the dimensional features of the formed liposomes, what occurs at a too rapid injection velocity.(62) Moreover, the liposomes generated through this method are very dilute and the complete removal of C_2H_5OH is difficult due to the its high solubility in water.(7,42,47,62) Furthermore, the method is not suitable for lipids characterized by poor solubility in C_2H_5OH, leading to suboptimal encapsulation efficacy (EE%) when hydrophilic compounds are incapsulated in the aqueous core of the liposomal structure.(40,47) Nevertheless, the method is relatively straightforward to execute, as it does not require the application of high pressures or temperature. Additionally, the method employs only a limiting amount of solvents and is characterized with a high level of reproducibility.(40,42,62) On the other hand, C_2H_5OH has the advantageous property to be an acceptable solvent for an in vivo drug delivery applications, at lower concentrations, according to the European pharmacopoeia because of it is relatively safe.(40,62)

4.2.1. Characteristics liposomes

4.2.1.1. Dimensional characterization

Nanoparticles are too small to investigate using visible light, meaning that the dimensional characterization of the liposomal vesicles needs to be characterized by the intensity of scattered light measured by the osDLS device, which should fall within the range of 15 to 30MHz.(1) If the measured value falls below 15MHz, it indicates that the concentration of liposomal suspension is insufficient, and a more concentrated suspension is required to obtain accurate osDLS analysis results. Conversely, if the value exceeds 30MHz, it suggests that the liposomal suspension is too concentrated, necessitating an additional dilution step, which is typically the case.

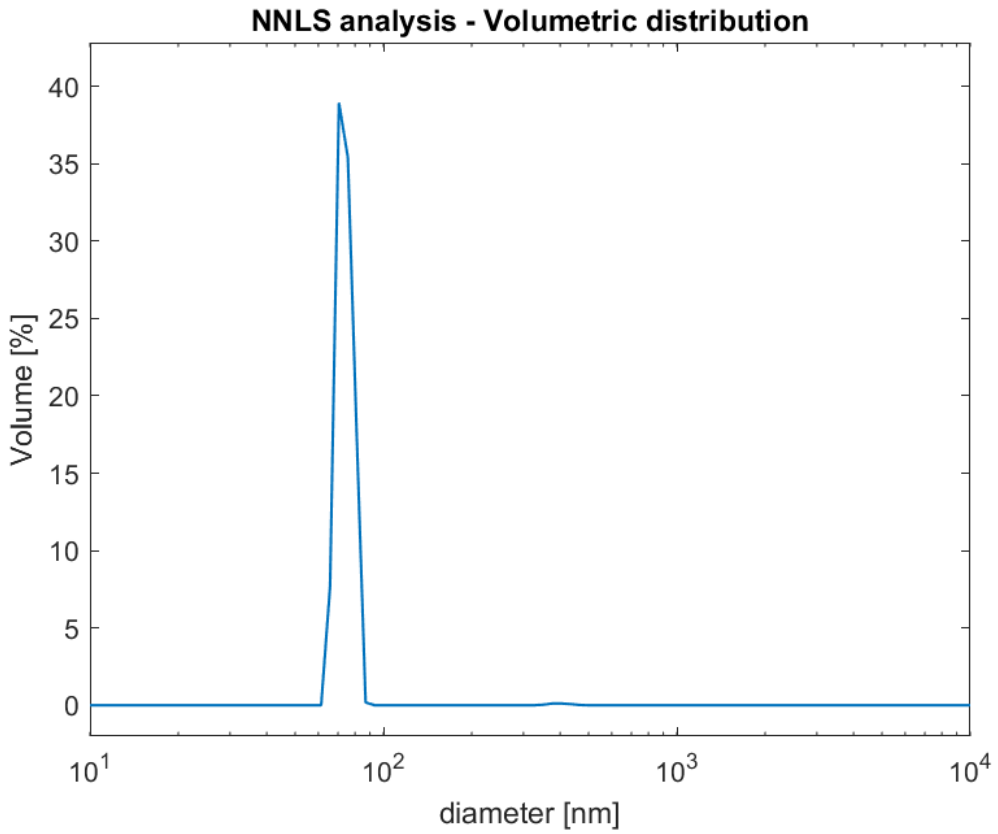

Figure 4.4: Volume[%] in function of the diameter[nm]

The x-axis is plotted on a logarithmic scale, where the amplitude of the gaussian bell curve corresponds to the abundance of liposomes within the sample, as determined by the average diameter of the nanoparticles present in the liposome suspension, measured in nanometer.(43) On the other hand, the width of the peak represents the variability in diameter among the formed liposomal structures, also expressed in nanometer. A wider peak indicates a higher level of variability in liposome sizes. The mean diameter of our nanosuspension amounts to 60-70nm and PDI was 0.17, as presented in Figure 4.4.

The size of the vesicles is an crucial parameter in determining the circulation half-life of liposomes, particularly in the context of inhalation and parental administration.(7,40,41) Smaller liposomes tend to exhibit longer circulation times within the body compared to large liposomes, which are more rapidly eliminated from the blood circulation.(40) Furthermore, both size and number of bilayers impact the EE%, where increased liposome size and decreased number of bilayers results in a higher EE% for encapsulated hydrophilic

compounds.(7,41) In the field of drug delivery, the desired vesicles size ranges usually falls between 50 and 200nm.(40,41,45,62) An elevated lipid concentration leads to the formation of the phosphatidyl-coated liposomal vesicles, characterized by an increased particle size as well as positively influencing the EE% owing to the augmented thickness of the membranes.(62) However, excessively high lipid concentrations should be avoided to prevent the occurrence of aggregation.(62)

OsDLS offers several advantages, including its simplicity and reliability, enabling measurements across an extensive range from a few nanometers to several micrometers.(33) However, it is important to acknowledge the limitations of this technique, such as the challenge in distinguishing single particles from aggregates and its high sensitivity in detecting low levels of impurities.(33)

4.2.1.2. Encapsulation efficiency and loading capacity (LC%)

The encapsulated concentration of ERN was determined by subtracting the ERN concentration present in the external solution from the total ERN present in the liposome suspension. To assess the total dispersed quantity of ERN in the external solution, TFF was employed by passing the nanosuspension through a TFF membrane and quantifying the permeate using HPLC with photodiode array detector. The total ERN content of the liposomal dispersion was determined through sonication in order to achieve the destruction of the liposomal structure and the liberation of ERN. Also this solution will be subjected to HPLC analysis because it is the only method available for a quantified analysis of ERN.

The total volume present in the TFF is 40mL meaning that theoretically the application of 1 syringe of 50mL with distilled water is enough to remove the 0.5M natrium hydroxide present in the filter. Although multiple washing steps will be applied to ensure a neutral pH before injecting the liposome suspension in the TFF membrane. The choice of natrium hydroxide is due to its inert properties because it doesn’t interrupt with the compounds of the membrane. The afterwards application of air through the TFF system is to ensure that there will take no dilution place of the liposome suspension during the filtration process.

This is essential since the permeate will be subjected to subsequent HPLC analysis to quantify the ERN concentration.

Table 4.11: Calculations regarding the liposomal encapsulation

Liposomes	Total	TFF	Total – TFF
AUC (mAU*s)	108.383	8.029	-
Rt (min)	25.140	25.107	-
Total Volume (mL)	242.5	22.5	-
Conc (mg/mL)	0.0571	0.0014	0.0557
Mass (µg)	13842.8	31.7	13811.1

There is only a small concentration of ERN present in the external medium due to the lowly concentrated ERN solution employed. Consequently, there is an exceptionally high EE%, approaching nearly 100%, as determined by Equation 4.4. This signifies that almost all of the ERN is successfully encapsulated within liposomes. Furthermore, the theoretical load measures 0.91% and the effective load 0.87%, calculated using equation 4.5 and 4.6, which closely aligns with the targeted LC% of 1%.

$$EE\% = \frac{\text{Total conc} - \text{TFF permeate conc}}{\text{Total conc}} * 100\% \quad \text{(Eq 4.4)}$$

$$\rightarrow EE\% = \frac{0.0557 \frac{mg}{mL}}{0.0571 \frac{mg}{mL}} * 100\% = 97.53\%$$

$$\text{Theoretical LC\%} = \frac{\text{mass encapsulated ERN}(\mu g)}{\text{mass the total lipids}(\mu g)} * 100\% \quad \text{(Eq 4.5)}$$

$$\text{mass of the total lipids} = 67.3 \frac{mg}{mL} * 22.5mL = 1514.25mg$$

$$\rightarrow \text{Theoretical LC\%} = \frac{13811.1 \mu g}{1514250 \mu g} * 100\% = 0.91\%$$

$$\text{Effective LC\%} = EE\% * \text{Theoretical LC\%} \quad \text{(Eq 4.6)}$$

$$\rightarrow \text{Effective LC\%} = 95.53\% * 0.91\% = 0.87\%$$

4.2.1.3. Turbidity characterization

A turbidity meter is an instrument that operates on the principle of measuring the scattering and absorption of light caused by suspended particles, including nanoparticles, in a liquid sample. It is used to quantify the turbidity of the liposomal suspension.(63) Greater

transparency in a liposomal suspension results in lower turbidity values due to reduced scattering of light from its original direction.(63)

The turbidity of the nanosuspension was quantified at 53NTU, indicating a relatively limpid suspension. The optical properties of nanofluids can vary, ranging from transparent to opaque depending on the characteristics and concentration of the dispersed particles.(5)

5. CONCLUSION

This master dissertation aimed to increase the knowledge about the optimalization of the extraction of ERN from fresh rucola leaves by comparing the yield of ERN using different extraction solvents and methods. The utilization of the SHE method with $CH_3COOC_2H_5$ as extraction solvent resulted in the highest ERN yield among all tested methods. Furthermore, the HFE method on the hydrolysis product using 96° C_2H_5OH as extraction solvent exhibited the second highest ERN yield. Conversely, the HFE method on the solid part with 96° C_2H_5OH as the extraction solvent resulted in the lowest ERN concentrations due to the loss of ERN during the hydrolysis step. Overall, these findings support the hypothesis that $CH_3COOC_2H_5$ is a more powerful extraction solvent compared to C_2H_5OH for ERN extraction from rucola leaves.

The research conducted in this study successfully demonstrated the extraction of ERN from rucola leaves. However, the obtained ERN concentrations were insufficient to achieve the desired LC% of 1% in liposomes. For this reason, the ERN encapsulation was only conducted once using the undiluted standard S0 with a concentration of 25mg/mL. This indicates the need for further optimization to achieve the desired outcome.

The SMF technique using the SMF apparatus is an effective method to produce liposomes. This method enables the encapsulation of extracted ERN from arugula leaves, dissolved in C_2H_5OH. In this dissertation, the coaxial injection method was more suitable compared to the triple injection system, primarily due to the dissolution of the ERN standard in anhydrous C_2H_5OH.

The encapsulation resulted in an EE% of nearly 97.53%, a theoretical LC% of 0.91% and an effective LC% of 0.87%, which closely aligns with the targeted load of 1%. The liposomal suspension consisted of 10% C_2H_5OH and 90% water, due to the chosen flow rate and diameter ratio. Since the obtained values were sufficiently high, the liposomal production was considered successful and optimization of the process was considered to be unnecessary.

6. REFERENCES

1. Tabassum N. AN EMPIRICAL EXPLORATION OF THE NANOTECHNOLOGY. Int J Adv Res (Indore) [Internet]. 2020 Jul 31;8(7):885–915. Available from: https://www.journalijar.com/article/33775/an-empirical-exploration-of-the-nanotechnology/
2. Bayda S, Adeel M, Tuccinardi T, Cordani M, Rizzolio F. The history of nanoscience and nanotechnology: From chemical-physical applications to nanomedicine. Vol. 25, Molecules. MDPI AG; 2020.
3. Jaiswal S. APPLICATIONS OF NANOPARTICLES: A STEP TOWARDS A GREEN ENVIRONMENT. International Journal of Biological Innovations. 2022;04(01):64–70.
4. Mansoori GA, Soelaiman TAF. Nanotechnology-An Introduction for the Standards Community [Internet]. Vol. 2, Journal of ASTM International. 2005. Available from: www.astm.org
5. Salaheldeen Elnashaie S, Danafar F, Hashemipour Rafsanjani H. From Nanotechnology to Nanoengineering. In: Nanotechnology for Chemical Engineers. Springer Singapore; 2015. p. 79–178.
6. Escoffre JM, Mannaris C, Geers B, Novell A, Lentacker I, Averkiou M, et al. Doxorubicin liposome-loaded microbubbles for contrast imaging and ultrasound-triggered drug delivery. IEEE Trans Ultrason Ferroelectr Freq Control. 2013;60(1):78–87.
7. Akbarzadeh A, Rezaei-Sadabady R, Davaran S, Woo Joo S, Zarghami N, Hanifehpour Y, et al. Liposome: classification, preparation, and applications. 2013 [cited 2023 May 31]; Available from: http://www.nanoscalereslett.com/content/8/1/102
8. Singh S, Singh G, Attri S, Kaur P, Rashid F, Bedi N, et al. Development and optimization of nanoparticles loaded with erucin, a dietary isothiocyanate isolated from Eruca sativa: Antioxidant and antiproliferative activities in ehrlich-ascites carcinoma cell line. Front Pharmacol. 2023 Jan 25;13.

9. Fahey JW, Zalcmann AT, Talalay P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants $ [Internet]. Available from: www.elsevier.com/locate/phytochem
10. Alruwaih NA, Yaylayan VA. Comparative evaluation of bioactive compounds in lyophilized and tray-dried rocket (Eruca sativa). J Food Process Preserv. 2017 Oct 1;41(5).
11. Forlenzo P. Estrazione e nano-incapsilazione di principi attivi di origine naturale. [PowerPoint presentation] Laurea in Ingegneria Chimica. Univesità degli Studi di Salerno. [updated anno accademico 2020-2021; cited 2023 Apr 23]. Available from: Presentazione_Pierpaolo_Forlenza finale.pptx - Google Presentaties.
12. Rollin P, Tatibouët A. Glucosinolates: The synthetic approach. Vol. 14, Comptes Rendus Chimie. Elsevier Masson SAS; 2011. p. 194–210.
13. Possenti M, Baima S, Raffo A, Durazzo A, Giusti AM, Natella F. Glucosinolates in Food. In: Reference Series in Phytochemistry. Springer Science and Business Media B.V.; 2017. p. 87–132.
14. Azarenko O, Jordan MA, Wilson L. Erucin, the major isothiocyanate in Arugula (Eruca sativa), inhibits proliferation of MCF7 tumor cells by suppressing microtubule dynamics. PLoS One. 2014 Jun 20;9(6).
15. Clarke JD, Hsu A, Riedl K, Bella D, Schwartz SJ, Stevens JF, et al. Bioavailability and inter-conversion of sulforaphane and erucin in human subjects consuming broccoli sprouts or broccoli supplement in a cross-over study design. Pharmacol Res. 2011;64(5):456–63.
16. Zhu N, Soendergaard M, Jeffery EH, Lai RH. The impact of loss of myrosinase on the bioactivity of broccoli products in F344 rats. J Agric Food Chem. 2010 Feb 10;58(3):1558–63.
17. Bricker G V., Riedl KM, Ralston RA, Tober KL, Oberyszyn TM, Schwartz SJ. Isothiocyanate metabolism, distribution, and interconversion in mice following consumption of thermally processed broccoli sprouts or purified sulforaphane. Mol Nutr Food Res. 2014 Oct 1;58(10):1991–2000.
18. Barillari J, Canistro D, Paolini M, Ferroni F, Pedulli GF, Iori R, et al. Direct antioxidant activity of purified glucoerucin, the dietary secondary metabolite

contained in rocket (Eruca sativa Mill.) seeds and sprouts. J Agric Food Chem. 2005 Apr 6;53(7):2475–82.

19. Karanikolopoulou S, Revelou PK, Xagoraris M, Kokotou MG, Constantinou-Kokotou V. Current Methods for the Extraction and Analysis of Isothiocyanates and Indoles in Cruciferous Vegetables. Analytica. 2021 Sep 24;2(4):93–120.
20. Mithen RF, Dekker M, Verkerk R, Rabot S, Johnson IT. The nutritional significance, biosynthesis and bioavailability of glucosinolates in human foods. Vol. 80, Journal of the Science of Food and Agriculture. John Wiley and Sons Ltd; 2000. p. 967–84.
21. You Y, Wu Y, Mao J, Zou L, Liu S. Screening of Chinese brassica species for anti-cancer sulforaphane and erucin. Afr J Biotechnol [Internet]. 2008;7(2):147–52. Available from: http://www.academicjournals.org/AJB
22. Lee M, Ralston RA, Schwartz SJ. The Development of a Tomato-Soy-Arugula Seed Beverage for Prostate Cancer Clinical Trial.
23. Lee MC, Schwartz SJ, Luis RS, Simons C. The Development, Sensory Evaluation and Interconversion of Bioactive Isothiocyanates in a Tomato-Soy-Arugula Seed.
24. Ku KM, Kim MJ, Jeffery EH, Kang YH, Juvik JA. Profiles of Glucosinolates, Their Hydrolysis Products, and Quinone Reductase Inducing Activity from 39 Arugula (Eruca sativa Mill.) Accessions. J Agric Food Chem. 2016 Aug 31;64(34):6524–32.
25. Bell L, Wagstaff C. Glucosinolates, myrosinase hydrolysis products, and flavonols found in rocket (eruca sativa and diplotaxis tenuifolia). Vol. 62, Journal of Agricultural and Food Chemistry. American Chemical Society; 2014. p. 4481–92.
26. Budnowski J, Hanschen FS, Lehmann C, Haack M, Brigelius-Flohé R, Kroh LW, et al. A derivatization method for the simultaneous detection of glucosinolates and isothiocyanates in biological samples. Anal Biochem. 2013;441(2):199–207.
27. Fenwick GR, Heaney RK. Glucosinolates and their Breakdown Products in Cruciferous Crops, Foods and Feedingstuffs. Vol. 11, Food Chemistry. 1983.
28. Blažević I, Montaut S, Burčul F, Olsen CE, Burow M, Rollin P, et al. Glucosinolate structural diversity, identification, chemical synthesis and metabolism in plants. Vol. 169, Phytochemistry. Elsevier Ltd; 2020.
29. Kamal RM, Razis AFA, Sukri NSM, Perimal EK, Ahmad H, Patrick R, et al. Beneficial Health Effects of Glucosinolates-Derived Isothiocyanates on

Cardiovascular and Neurodegenerative Diseases. Molecules [Internet]. 2022 Feb 1 [cited 2023 Jun 4];27(3). Available from: /pmc/articles/PMC8838317/

30. Matusheski N V., Wallig MA, Juvik JA, Klein BP, Kushad MM, Jeffery EH. Preparative HPLC method for the purification of sulforaphane and sulforaphane nitrile from Brassica oleracea. J Agric Food Chem. 2001;49(4):1867–72.
31. Melchini A, Costa C, Traka M, Miceli N, Mithen R, De Pasquale R, et al. Erucin, a new promising cancer chemopreventive agent from rocket salads, shows anti-proliferative activity on human lung carcinoma A549 cells. Food and Chemical Toxicology. 2009 Jul;47(7):1430–6.
32. Redovnikovic IR. Glucosinolates and their potential role in plant [Internet]. Available from: https://www.researchgate.net/publication/279766239
33. Bonnema G, Lee JG, Shuhang W, Lagarrigue D, Bucher J, Wehrens R, et al. Glucosinolate variability between turnip organs during development. PLoS One. 2019 Jun 1;14(6).
34. Hanschen FS, Klopsch R, Oliviero T, Schreiner M, Verkerk R, Dekker M. Optimizing isothiocyanate formation during enzymatic glucosinolate breakdown by adjusting pH value, temperature & dilution in Brassica vegetables & Arabidopsis thaliana. Sci Rep. 2017 Jan 17;7.
35. Pan Z, Meng Z, Tan M, Duan H, Ramaswamy HS, Qiu X, et al. Optimization the conversion of glucosinolate to isothiocyanate in yellow mustard seeds (Sinapis alba) by response surface methodology. Applied Food Research. 2022 Dec 1;2(2).
36. Vermeulen M, Van Den Berg R, Freidig AP, Van Bladeren PJ, Vaes WHJ. Association between consumption of cruciferous vegetables and condiments and excretion in urine of isothiocyanate mercapturic acids. J Agric Food Chem. 2006 Jul 26;54(15):5350–8.
37. 저작자표시-비영리-변경금지 2.0 대한민국 이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게.
38. Vermeulen M, Klöpping-Ketelaars IWAA, Van Den Berg R, Vaes WHJ. Bioavailability and kinetics of sulforaphane in humans after consumption of cooked versus raw broccoli. J Agric Food Chem. 2008 Nov 26;56(22):10505–9.

39. Melchini A, Traka MH. Biological profile of erucin: A new promising anticancer agent from cruciferous vegetables. Vol. 2, Toxins. 2010. p. 593–612.
40. Guimarães D, Cavaco-Paulo A, Nogueira E. Design of liposomes as drug delivery system for therapeutic applications. Vol. 601, International Journal of Pharmaceutics. Elsevier B.V.; 2021.
41. Nsairat H, Khater D, Sayed U, Odeh F, Al Bawab A, Alshaer W. Liposomes: structure, composition, types, and clinical applications. Vol. 8, Heliyon. Elsevier Ltd; 2022.
42. Bochicchio S, Dalmoro A, Lamberti G, Barba AA. Advances in nanoliposomes production for ferrous sulfate delivery. Pharmaceutics. 2020 May 1;12(5).
43. Bochicchio S, Dalmoro A, Bertoncin P, Lamberti G, Moustafine RI, Barba AA. Design and production of hybrid nanoparticles with polymeric-lipid shell-core structures: conventional and next-generation approaches. RSC Adv. 2018;8(60):34614–24.
44. He H, Lu Y, Qi J, Zhu Q, Chen Z, Wu W. Adapting liposomes for oral drug delivery. Vol. 9, Acta Pharmaceutica Sinica B. Chinese Academy of Medical Sciences; 2019. p. 36–48.
45. Yu B, Lee RJ, Lee LJ. Microfluidic Methods for Production of Liposomes. Vol. 465, Methods in Enzymology. 2009. p. 129–41.
46. Coimbra M, Isacchi B, Van Bloois L, Torano JS, Ket A, Wu X, et al. Improving solubility and chemical stability of natural compounds for medicinal use by incorporation into liposomes. Int J Pharm. 2011 Sep 20;416(2):433–42.
47. Çağdaş M, Sezer AD, Bucak S. Liposomes as Potential Drug Carrier Systems for Drug Delivery. In: Application of Nanotechnology in Drug Delivery. InTech; 2014.
48. Ciccone V, Piragine E, Gorica E, Citi V, Testai L, Pagnotta E, et al. Anti-Inflammatory Effect of the Natural H2S-Donor Erucin in Vascular Endothelium. Int J Mol Sci. 2022 Dec 1;23(24).
49. Martelli A, Piragine E, Citi V, Testai L, Pagnotta E, Ugolini L, et al. Erucin exhibits vasorelaxing effects and antihypertensive activity by H2S-releasing properties. Br J Pharmacol. 2020 Feb 1;177(4):824–35.

50. Pan Z, Meng Z, Tan M, Duan H, Ramaswamy HS, Qiu X, et al. Optimization the conversion of glucosinolate to isothiocyanate in yellow mustard seeds (Sinapis alba) by response surface methodology. Applied Food Research. 2022 Dec 1;2(2).
51. Prochaska HJ, Santamaria AB, Talalayt P. Rapid detection of inducers of enzymes that protect against carcinogens (cancer/chemoprotection/enzyme induction/quinone reductase/vegetables). Vol. 89, Medical Sciences. 1992.
52. Kaur P, Singh D, Singh G, Attri S, Singh D, Sharma M, et al. Pharmacokinetics and toxicity profiling of 4-(methylthio)butyl isothiocyanate with special reference to pre-clinical safety assessment studies. Toxicon. 2022 Jun 1;212:19–33.
53. He J, Li X, Shen Y, Zhang X. Salt Effect on the Liquid-Liquid Equilibrium for the Water-Ethylene Glycol-Ethylene Glycol Diacetate System. J Chem Eng Data. 2019 Jun 13;64(6):2445–54.
54. Gezahegn T, Tegegne B, Zewge F, Chandravanshi BS. Salting-out assisted liquid-liquid extraction for the determination of ciprofloxacin residues in water samples by high performance liquid chromatography-diode array detector. BMC Chem. 2019;13(3).
55. Le NTT, Cao V Du, Nguyen TNQ, Le TTH, Tran TT, Thi TTH. Soy lecithin-derived liposomal delivery systems: Surface modification and current applications. Vol. 20, International Journal of Molecular Sciences. MDPI AG; 2019.
56. Thomas AH, Catalá Á, Vignoni M. Soybean phosphatidylcholine liposomes as model membranes to study lipid peroxidation photoinduced by pterin. Biochim Biophys Acta Biomembr. 2016 Jan 1;1858(1):139–45.
57. Safety and efficacy of lecithins (Lipidol) for all animal species. EFSA Journal. 2016;14(8).
58. Specifications Materials of Construction Filter Media: Omega membrane (m-polyethersulfone) Housing: Polypropylene, glass reinforced [Internet]. 2020. Available from: www.pall.com/lab
59. What is Tangential Flow Filtration (TFF) and Where is it Used? » TBL Plastics [Internet]. [cited 2023 May 22]. Available from: https://tblplastics.com/tangential-flow-filtration-tff/

60. Lv C, Zhang Y, Zou L, Sun J, Song X, Mao J, et al. Simultaneous Hydrolysis and Extraction Increased Erucin Yield from Broccoli Seeds. ACS Omega. 2021 Mar 9;6(9):6385–92.
61. Guillarme D, Veuthey JL. Guidelines for the Use of UHPLC Instruments.
62. Gouda A, Sakr OS, Nasr M, Sammour O. Ethanol injection technique for liposomes formulation: An insight into development, influencing factors, challenges and applications. Vol. 61, Journal of Drug Delivery Science and Technology. Editions de Sante; 2021.
63. Omar AF Bin, MatJafri MZ Bin. Turbidimeter design and analysis: A review on optical fiber sensors for the measurement of water turbidity. Vol. 9, Sensors. 2009. p. 8311–35.

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