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# Development and characterization of chia (*Salvia hispanica* L.) plant-derived products as natural bioactive compounds

Daria Kuznetcova

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**ITMO UNIVERSITY**  
**Faculty of Biotechnologies**

**UNIVERSITY OF LORRAINE**  
**Graduate school SIReNa (Sciences et Ingénierie des Ressources Naturelles)**

**Thesis**

**Presented and publicly defended to obtain the title of**

**DOCTOR OF THE UNIVERSITY OF LORRAINE**

**Research area: «Génie biotechnologique et alimentaire» / «Biotechnology of foodstuff and biologically active substances»**

by **Daria KUZNETCOVA**

**Development and characterization of chia (*Salvia hispanica* L.)  
plant-derived products as natural bioactive compounds**

**December 15, 2020**

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**LIST OF ABBREVIATIONS**

**ALA** –  $\alpha$ -linolenic acid;

**APP** - amyloid precursor protein;

**CAT** – catalase;

**ChSL** - chia seed lipids;

**CLA** – conjugated linoleic acid;

**DCF** - 2',7'-dichlorofluorescein;

**DCFH-DA** - 2',7'-dichlorofluorescein-diacetate;

**DHA** – docosahexaenoic acid;

**DMEM** - Dulbecco's Modified Eagle Medium;

**ECACC** - European Collection of Authenticated Cell Cultures;

**EPA** – eicosapentaenoic acid;

**EU** – European Union;

**FA** – fatty acids;

**FAME** - fatty acid methyl esters;

**FAO** – Food and Agriculture Organization of the United Nations;

**FBS** - fetal bovine serum;

**GLUT4** - glucose transporter type 4;

**GPx** - glutathione peroxidase;

**GRd** - glutathione reductase;

**HBSS** - Hank's buffered salt solution;

**HDL** - high-density lipoprotein;

**HFF** - high-fat diet with a high fructose content;

**HPLC-MS** –high performance liquid chromatography with mass spectrometry;

**HSP** - heat shock proteins;

**LA** – linoleic acid;

**LDL** – low-density lipoprotein;

**Lyso-PC** - lysophosphatidyl-choline;

**MTT** - tetrazolium dye 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl-tetrazolium bromide;

**MUFA** – monounsaturated fatty acids;

**NE** – nanoemulsions;

**NL** – nanoliposomes;

**PA** - phosphatidic acid;

**PC** – phosphatidylcholine;

**PDI** - polydispersity index;

**PE** – phosphatidylethanolamine;

**PG** – phosphatidylglycerol;

**PGC-1a** - peroxisome proliferator-activated gamma receptor co-activator 1a;

**PI** – phosphatidylinositol;

**PL** – phospholipids;

**PPAR- $\alpha$**  - peroxisome proliferator-activated receptor-alpha;

**PUFA** – polyunsaturated fatty acids;

**ROS** - Reactive oxygen species;

**SFA** – saturated fatty acids;

**SOD** - superoxide dismutase;

**SREBP-1c** - sterol regulatory element-binding protein 1;

**TBARS** - reactive substances with thiobarbituric acid;

**TEM** - transmission electron microscopy;

**TG** – triacylglycerides;

**TNF-  $\alpha$**  - tumor necrosis factor alpha;

**USDA** - United States Department of Agriculture;

**VLDL** - very low-density lipoproteins;

**WHO** – World Health Organization.

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## INTRODUCTION

A healthy human diet is composed of macro-groups of nutrients such as proteins, carbohydrates, and lipids. It is important to maintain a balance of consumed substances within these groups for proper body function. A growing sedentary lifestyle in recent years and poor eating habits, including consumption of foods rich in saturated fats, has contributed to an increase worldwide in metabolic diseases, such as obesity, dyslipidemia, diabetes and cardiovascular disease.

For lipids in the diet, a balance is needed between the amount of ingested saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). Individual fatty acids (FA) play important roles in biological processes. Some FA are “essential” because they are necessary for normal body development, but cannot be synthesized *de novo* in the body, including linoleic acid (LA, omega-6) and  $\alpha$ -linolenic acid (ALA, omega-3). Since the mammals lack the enzyme involved in the bioconversion of LA to ALA, the only source of this FA is through the diet.

Omega-3 FA were first discovered in 1929 by G. Burr. (Scorletti *et al.*, 2013). Recently, a number of studies have also shown the relationship between an imbalance of omega-3 (n-3) and omega-6 (n-6) and risk of metabolic syndrome and cardiovascular diseases. In addition, recent studies point towards a link between lipid-related disorders and neurodegenerative diseases in the brain.

Fish and seafood are a rich source of long-chain omega-3 FA (such as eicosapentaenoic / EPA, docosahexaenoic / DHA), but it may not be a sustainable source for the public. Fish oil has a strong fishy taste, is not popular with consumers and cannot be included in vegetarian and vegan diets. In addition, some marine sources contain high levels of heavy metal, organic and other contaminants, and their allergenicity can be problematic for consumers (Timilsena *et al.*, 2017).

Today, the scientific community is actively exploring the effect of PUFA (in particular omega-3) on the prevention of the development and treatment of diseases mentioned earlier. As already noted, the main sources of omega-3 PUFA are fish and seafood, however, an active search is currently underway for alternative sources that are more accessible and safer, such as vegetable oils.

The main source of  $\alpha$ -linolenic FA are plants. The richest plant ALA source currently known is chia seeds (*Salvia hispanica* L.). These seeds also have a number of other nutritional benefits, including a significant amount of dietary fiber and protein, antioxidants and vitamins. Chia seed oil has a high degree of unsaturation (about 80% of PUFA), which also reduces its oxidative stability.

Development of methods for effective use of functional ingredients has been a topic of great interest. Biologically active substances and their compositions are often influenced by external factors, such as light, oxygen, humidity and temperature during processing and storage, which can lead to a decrease in stability resulting in a less active product. The effectiveness of the functional ingredient depends on its ability to pass the intestinal barrier, including resistance of stomach acidity and digestive enzymes, passage across the intestinal wall to reach the circulation. All of these factors will influence the efficacy of the bioactive product.

To address these issues that affect stability and activity, most functional substances are used or prepared in protective transporters, such as particles or capsules, to protect them from the external environment and minimize loss of stability and activity. Carriers are designed to provide a protective outer layer to help maintain the activity of the biofunctional element until it enters the target tissue.

Today, the development of nano- and microparticles as vectors for biologically active compounds is a priority for scientists working in various scientific areas, including that of food biotechnology. Such solutions have used plant-derived lipid extracts, for preparations of micro- or

nanoemulsions, and nanoliposomes, which can subsequently serve as a vector system with a high degree of biocompatibility for additional biologically active ingredients. The size of these nanoparticles is of great importance for the activity of the encapsulated substance. Indeed, studies report that a decrease in particle size has an effect on increasing the efficiency and bioavailability of biologically active compounds.

For this thesis research, we have developed and characterized nanoemulsions and nanoliposomes based on the lipid fraction of chia seeds as promising delivery systems for PUFA of plant origin and other biologically active substances. Nanoliposomes are particularly advantageous as universal vectors, since they have the properties of encapsulating hydrophilic and lipophilic biologically active substances, in contrast to nanoemulsions. The development of the technology for producing nanoliposomes based on the lipid fraction of chia seeds will make it possible to obtain a new form of biologically active substances with a wide range of applications not only in food but also in cosmetic and pharmaceutical fields as a delivery system.

Studies of chia seeds as a source of biologically active substances over the past 10 years have been of great interest to the scientific community because of its potential health benefits. The use of chia seed oil in different formulations has great potential. However, systematic studies on the chia-derived lipids, including phospholipid fraction and their use in the development of such vectors such as nanoemulsions (NE) and nanoliposomes (NL) are lacking. The development of such particles would be useful in studying the effects of chia seed derived oil and by-products that are rich in ALA on different metabolic processes.

This “*co-tutelle*” project was organized as follows. Theoretical studies included a review of the current level of the scientific knowledge through literature analysis of studies on chia plant, seeds and the lipid fraction, as well as methods for chia seed oil extraction and other lipid obtaining research. Also, modern methods of processing vegetable oils for use as biologically active ingredients, including nanotechnological processes, as well as bioactivities of chia research were

studied. Based on this literature analysis, and identified research niches, the main goal of the scientific work was defined, and the research tasks were set.

Experimental studies of the thesis work were performed in part at the basis of the Faculty of Biotechnologies and the International Scientific Centre “Biotechnology of the Third Millennium”, ITMO University, St. Petersburg, Russia. The work presented here was carried out in two laboratories of the National School of Agronomy and Food Industry (ENSAIA), University of Lorraine, Nancy, France. The work related to the extraction of the chia seed lipid fraction and their physicochemical analysis, as well as the preparation of NE and NL, their characterization and stability studies were performed at the “Laboratory of Biomolecule Engineering” (LIBio). Some preliminary tests for the extraction of lipids and the properties of chia seed mucilage were carried out at the ITMO University facilities. Cytotoxicity tests of the obtained soft nanoparticles and some antioxidant tests were conducted with the Quality of Diet and Aging team (QUALIVIE) of the Research Unit “Animals and Functionality of Animal Products” (UR AFPA). The combined experience of scientific teams of the two universities allowed this thesis project to move forward not only from the point of food product production, but also in taking the first step to address a global health issue. Indeed, research at UR AFPA on diet quality and aging, focuses on lipids effect on processes in the brain, which is directly related to investigation of chia seeds as a rich plant source of omega-3 fatty acids. In LIBio, researchers conduct studies in biochemistry and physical chemistry in order to develop natural nanoparticles for food and other purposes.

The thesis is presented in six chapters, starting with a bibliography on lipids and health, with a focus on chia seeds and oils, and nanotechnology as vectors, followed by a section describing materials and methods, and then the results and discussion presenting the preparation and physicochemical characterization of chia-based NL and NE, and cell studies, and some additional research of obtained particles. The final chapter presents the conclusions and perspectives, ending with the bibliography, list of publications and communications and appendix.

## CHAPTER I. LITERATURE REVIEW

### 1.1 ROLE OF LIPIDS IN BIOLOGICAL PROCESSES

Lipids are one of the most important structures in living organisms, cells and tissues. Also, lipids are important in human health, where brain lipid homeostasis plays a key role in cognitive functions including motor function, learning ability and memory.

The founder of lipid chemistry is the French chemist Michel Eugene Chevreul (Leray, 2017). Some researchers define lipids as a wide range of natural products, including fatty acids (FA) and their derivatives, steroids, terpenes, carotenoids and bile acids, which are highly soluble in organic solvents; there are other attempts to describe a group of lipids, however, the definition is imprecise due to the heterogeneity and characteristics of some of the compounds included in this group. Lipids can be classified based on different parameters, including physical properties at room temperature (oils and fats), polarity (polar and neutral), role in human health (essential and non-essential fatty acids), or structural features (simple and complex). The neutral ones include FA, fatty alcohols, glycerides, and sterols, can be separated from polar lipids - glycerophospholipids and glyceroglycolipids. However, this is conditional due to the polarity of some short-chain FA. Thus, structural classification is considered optimal, with derivatives considered as building elements (free FA, fatty alcohols), as well as simple or complex lipids (phospholipids and glycolipids) (Akoh, 2017). According to LIPID MAPS<sup>®</sup> Structure database, one of the most complete classification of lipids found, lipids could be derived into 8 major classes: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, phenol lipids, saccharolipids and polyketides. In the frame of our study, we are primarily interested in the 3 first groups. The representatives of first group are fatty acids (FA), for the second are triacylglycerols (TAG, especially oils) and for the third one there are phospholipids which are the focus of interest

in this particular research on chia seed lipids, and particularly on plant-derived polyunsaturated fatty acids (PUFA).

There is a standard nomenclature for the classification of FA developed by the International Union of Pure and Applied Chemists (IUPAC) and International Union of Biochemistry (IUB) (Akoh, 2017).

**Table 1.** Classification of FA (Akoh, 2017)

<b>Systematic name of FA</b>	<b>Commonly used name</b>	<b>Short name</b>
<b>Saturated FA</b>		
Methanoic	Formic	C1:0
Ethanoic	Acetic	C2:0
Propanoic	Propionic	C3:0
Butanoic	Butyric	C4:0
Pentanoic	Valeric	C5:0
Hexanoic	Caproic	C6:0
Heptanoic	Enanthic	C7:0
Octanoic	Caprylic	C8:0
Nonanoic	Pelargonic	C9:0
Decanoic	Capric	C10:0
Undecanoic	-	C11:0
Dodecanoic	Lauric	C12:0
Tridecanoic	-	C13:0
Tetradecanoic	Myristic	C14:0
Pentadecanoic	-	C15:0
Hexadecanoic	Palmitic	C16:0
Heptadecanoic	Margaric	C17:0
Octadecanoic	Stearic	C18:0
Nonadecanoic	-	C19:0
Eicosanoic	Arachidic	C20:0

<b>Systematic name of FA</b>	<b>Commonly used name</b>	<b>Short name</b>
Docosanoic	Behenic	C22:0
Tetracosanoic	Lignoceric	C24:0
Hexacosanoic	Cerotic	C26:0
Octacosanoic	Montanic	C28:0
Tricontanoic	Melissic	C30:0
Dotriacontanoic	Lacceroic	C32:0
<b>Unsaturated FA</b>		
<i>c</i> -9-Dodecenoic	Lauroleic	C12:1 n-3
<i>c</i> -5-Tetradecenoic	Physeteric	C14:1 n-9
<i>c</i> -9-Tetradecenoic	Myristoleic	C14:1 n-5
<i>c</i> -9-Hexadecenoic	Palmitoleic	C16:1 n-7
<i>c</i> -7, <i>c</i> -10, <i>c</i> -13-Hexadecatrienoic	-	C16:3 n-3
<i>c</i> -4, <i>c</i> -7, <i>c</i> -10, <i>c</i> -13-Hexadecatetraenoic	-	C16:4 n-3
<i>c</i> -9-Octadecenoic	Oleic	C18:1 n-9
<i>c</i> -11-Octadecenoic	<i>cis</i> -Vaccenic (Asclepic)	C18:1 n-7
<i>t</i> -11-Octadecenoic	Vaccenic	N/A
<i>t</i> -9-Octadecenoic	Elaidic	N/A
<i>c</i> -9, <i>c</i> -12-Octadecadienoic	Linoleic (LA)	C18:2 n-6
<i>c</i> -9- <i>t</i> -11-Octadecadienoic	Rumenic (CLA isomer)	N/A
<i>c</i> -9, <i>c</i> -12, <i>c</i> -15-Octadecatrienoic	Linolenic (ALA)	C18:3 n-3
<i>c</i> -6, <i>c</i> -9, <i>c</i> -12-Octadecatrienoic	$\gamma$ -Linolenic	C18:3 n-6
<i>c</i> -6, <i>c</i> -9, <i>c</i> -12, <i>c</i> -15-Octadecatetraenoic	Stearidonic	C18:4 n-3
<i>c</i> -11-Eicosenoic	Gondoic	C20:1 n-9
<i>c</i> -9-Eicosenoic	Gadoleic	C20:1 n-11
<i>c</i> -8, <i>c</i> -11, <i>c</i> -14-Eicosatrienoic	Dihomo- $\gamma$ -linolenic	C20:3 n-6
<i>c</i> -5, <i>c</i> -8, <i>c</i> -11-Eicosatrienoic	Mead's	C20:3 n-9
<i>c</i> -5, <i>c</i> -8, <i>c</i> -11, <i>c</i> -14-Eicosatetraenoic	Arachidonic (AA)	C20:4 n-6
<i>c</i> -5, <i>c</i> -8, <i>c</i> -11, <i>c</i> -14, <i>c</i> -17-Eicosapentaenoic	Eicosapentaenoic (EPA)	C20:5 n-3
<i>c</i> -13-Docosenoic	Erucic	C22:1 n-9
<i>c</i> -11-Docosenoic	Cetoleic	C22:1 n-11
<i>c</i> -7, <i>c</i> -10, <i>c</i> -13, <i>c</i> -16, <i>c</i> -19-Docosapentaenoic	DPA, Clupanodonic	C22:5 n-3
<i>c</i> -4, <i>c</i> -7, <i>c</i> -10, <i>c</i> -13, <i>c</i> -16, <i>c</i> -19-Docosahexaenoic	DHA, Cervonic	C22:6 n-3
<i>c</i> -15-Tetracosenoic	Nervonic (Selacholeic)	C24:1 n-9

Most fatty acids are linear aliphatic molecules with a carboxylic acid end. The most common FA with 16-18 carbon atoms and a maximum of two unsaturated bonds, however, vary from the shortest propionic to 80 carbon molecules of mycolic acids of mycobacteria (Geiger, 2019).

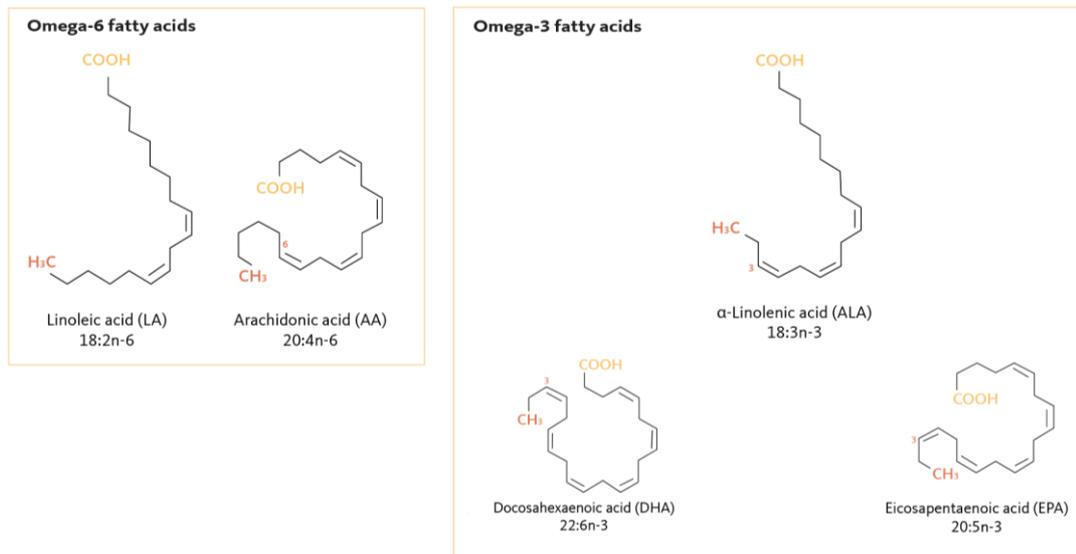
Along with the nomenclature names of FA, common names are widespread, which are associated with the source of the initial findings of the FA. In some cases, the names may be erroneous, but have become common names due to general usage (Akoh, 2017).

There are several main classes of FA - saturated and unsaturated FA, acetylenic FA, trans FA, branched, cyclic, hydroxyl and epoxy, furanoid FA (Akoh, 2017). We will focus on the first two groups of FA – saturated (SFA) and unsaturated, the latter of which can be subdivided into mono- (MUFA) and polyunsaturated FA (PUFA). FA can be esterified with glycerol in the form of TAG or to phospholipids with phosphoric acid.

FA called “saturated” because they are “filled” with hydrogen, most of them are straight hydrocarbon chains with an even number of carbon atoms. The most common SFA contain 12–22 carbon atoms. (Rustan & Drevon, 2001).

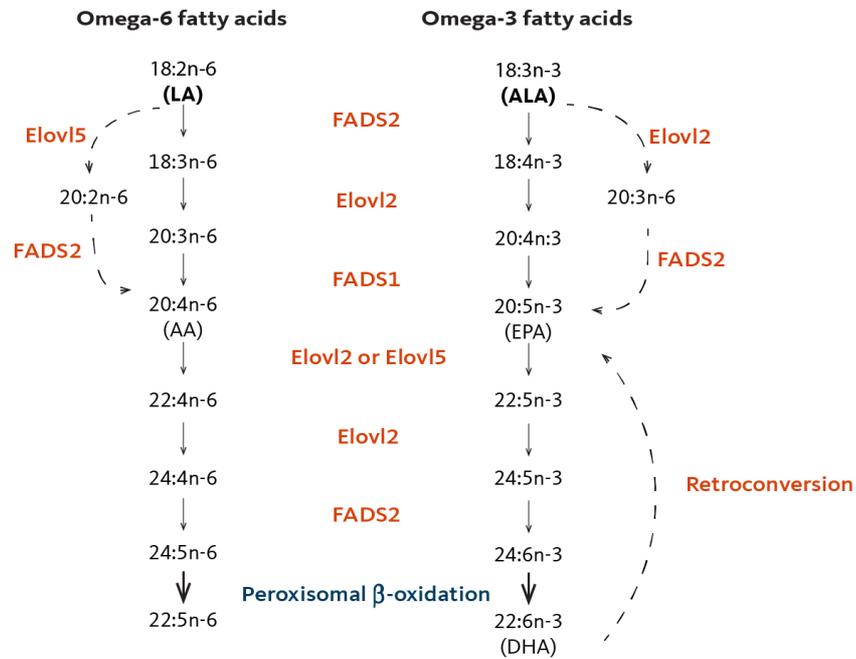
Unsaturated FA have two isomers, *cis*- and *trans*-, while most FA in biology are *cis*-isomers, with hydrogen atoms on either side of the double bond are oriented in the same direction. *Trans*- isomers can be provided by industrial processes, as well as in gastrointestinal tract of ruminants. MUFA have one carbon-carbon double bond, which can occur in different positions, the most common of them have a 16-22 carbon chain length. One of the most common MUFA in the *cis*- isoform is the omega-9 FA group, including oleic FA. In general, more than 100 monounsaturated FA species are known to science (Rustan & Drevon, 2001; Akoh, 2017; Geiger, 2019).

PUFA are grouped based on their metabolic characteristics (Akoh, 2017). PUFA omega-3 differ from omega-6 FA in the position of their first double bond, located between 3 and 4 carbon atoms rather than between 6 and 7 carbon atoms. According to the nomenclature, the numbering starts at the end of methyl group - the opposite of the acid one - hence the name, fatty acid. Only plants are equipped with the enzymes allowing conversion of LA to ALA (Leray, 2017).



**Figure 1.** Chemical structure of PUFA (MIC, Linus Pauling Institute, 2020)

For animals and humans, both FA (LA and ALA) are essential since they are unable to be synthesized *de novo*, and they must be obtained through diet. In animals, a series of elongases and desaturases are involved in 7 stages of metabolic conversion of LA and ALA for the biosynthesis of long-chain FA omega-3 EPA or DHA and omega-6 AA (arachidonic acid) (Leray, 2017).



**Figure 2.** Desaturation and elongation of PUFA (MIC, Linus Pauling Institute, 2020)

Moreover, each family cannot be transformed into each other, which leads to competition for the biosynthesis of products from LA and ALA and their metabolic derivatives. For example, an excess in amounts of LA can decrease the synthesis of omega-3 FA, which are already present in low quantities in animal tissues. High levels of EPA and DHA in the blood (over 8%) are found in people with non-westernized eating habits, for example in Scandinavia, and are considered an ideal target for healthy body functioning (Leray, 2017).

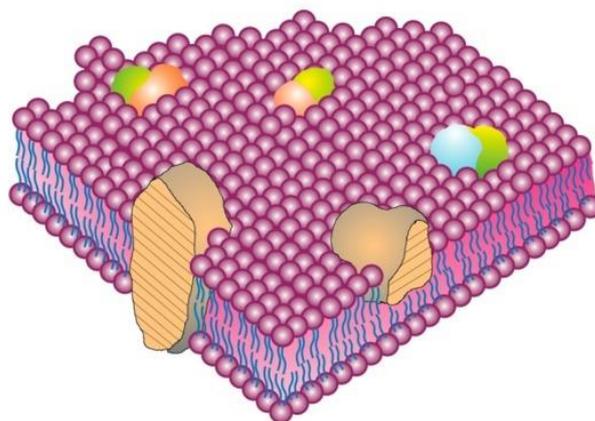
According to the recommendations of FAO / WHO (2008), to avoid ALA deficiency, the minimum amount of ALA required is 0.5-0.6% of the total energy consumed. It is noted that the total content of omega-3 in a healthy diet should be up to 2% of total energy which includes long-chain PUFA (about 0.25 g / day). LA (omega-6) consumption is also recommended in the range of 2-3% of the total energy consumed.

According to various sources, the optimal ratio between consumed omega-6 and omega-3 is 4-5:1, however, in the modern “Western diet” omega-6 consumption is actually much higher,

on the order of a 10-20:1 ratio. Changes in this ratio affects metabolic processes in the liver and can lead to a number of pathologies (Scorletti *et al.*, 2013).

The roles of FA cannot be overestimated. They have been part of the biosphere since the beginning of time, providing a large number of calories for metabolic processes (9 kcal / g), as part of the building blocks for membrane structures, and also as intra- and extracellular signaling molecules (Geiger, 2019). Lipids are now being viewed as novel biomarkers of various diseases that can help in early diagnosis for patients at risk of different lipid-related pathologies, allowing development of more effective treatment strategies, thus significantly improving patients' quality of life (Trostchansky & Rubbo, 2019). Let us consider in more detail the biological functions of lipids of interest.

Lipid membranes, based on phospholipids, are selective barriers that separate cellular functions. They can separate cells from the environment, and separate organelles from each other in the cells. Basically, cell membranes are represented by the Singer and Nicholson liquid mosaic model, in which the liquid phospholipid bilayer contains various types of proteins (Trostchansky & Rubbo, 2019).



**Figure 3.** Singer and Nicholson liquid mosaic cell membrane model

(Singer & Nicolson, 1972; Nicolson, 2013)

In this case, the lipid and protein composition of membranes varies depending on the type of cells and subcellular location. The most common lipid compounds found in mammalian cells are phosphatidylcholine, phosphatidylethanolamine, and cholesterol; some lipids accumulate in specific locations. Also, in cell membranes, lipids are distributed asymmetrically in the membrane bilayer, including glycolipids located on the side of plasma membranes exposed to extracellular fluid, and phospholipids such as phosphatidylserine on the side of these membranes facing the intracellular cytoplasm. There are more complex membrane structures, different from the classical model, that incorporate a high density of transmembrane proteins with heterogeneous thickness. The passage of ions and molecules through molecular membranes is regulated by various mechanisms, including diffusion through lipids, the rate of which depends on the size and hydrophobicity of the transported substance, or the use of transport proteins (for example, for glucose or FA) or receptors for internalization by endocytosis (Trostchansky & Rubbo, 2019).

The liver in mammals plays a major role in the control of energy homeostasis through the metabolism of excess carbohydrates and lipids supplied with food into glycogen or FA. The FA obtained are re-esterified to form triacylglycerides (TAG). TAG, in turn, are secreted by very low-density lipoproteins (VLDL) into the circulation. After hydrolysis of VLDL-TG by endothelial lipoprotein lipase, the FA released are reconverted into TAG in adipocytes for long-term storage in white adipose tissue. Enzymes involved in glycolytic and lipogenic pathways are dynamically regulated at the transcriptional and posttranscriptional levels by various factors (Trostchansky & Rubbo, 2019). These pathways are under tight control by the different factors, and any perturbations, such as a high fat diet, or any other macronutrient can lead to imbalances in lipid homeostasis.

The regulatory system includes a complex signaling chain capable of detecting various stimuli, such as the presence of food, changes in environmental factors and other stress effects to trigger adequate adaptive reactions of the body. At the same time, lipids and their derivatives

perform the function of entering the signaling network, contributing to the genetic control of their own systemic transport, cellular uptake, biosynthesis, storage, and use. This system includes the existence of several nuclear lipid-sensitive receptors that act as transcription factors, regulating the expression of genes involved in several pathways of lipid metabolism. One of these receptors playing a central role is the peroxisome proliferator activated receptor (PPAR). This family of receptors belongs to the sensors of lipophilic molecules that influence their activation. When active, PPARs regulate lipid metabolism and inflammation through complex mechanisms. The ligands of these receptors are a set of lipids, including endogenous FA, products of lipid metabolism (Trostchansky & Rubbo, 2019).

The most obvious biomarker of imbalances in lipid homeostasis is changes in plasma lipid levels (hypercholesterolemia, hypertriglyceridemia or dyslipidemias), which have been shown to be risk factors for pathologies such as atherosclerosis (cardiovascular disease), metabolic syndrome, and more recently, neurodegenerative diseases.

The first observations about the fatty nature of the brain were made in the 17<sup>th</sup> century. Later, several researchers described the nature of brain phospholipids, and a number of simple FA, such as stearic, palmitic and oleic, were identified. In the 1960s and 1970s of the 20<sup>th</sup> century, a high content of DHA (omega-3 FA), discovered in 1942 in fish, was revealed in the brain. The finding of high DHA levels in the brain cell membranes, with the content about 10-15% of all FA in the brain, highlighted the need to study its physiological functions. The first assumptions were that the deficiency of this FA in food at the time of development may prevent the development of the myelin sheath, which insulates the nerves, causing instability of the nervous system. A study in 1971 showed a decrease in DHA in rat retinas after prolonged deficiency of ALA in the diet. Subsequent studies have shown impaired retinal function and effects on vision due to ALA deficiency. A number of studies in the 70s also showed a significant decrease in the learning ability of second-generation rats in the case of FA deficiency in pregnant individuals, but a specific group

of lipids which had effect was not identified. The following experiments showed not only a decrease in learning, but also a decrease in DHA content in a number of brain phospholipids, including data in monkey studies. In 1989 in France, it was confirmed that a diet rich in LA, but in the absence of ALA, caused a significant decrease in learning in rats. These studies have established the importance of omega-3 FA in maintaining brain-related physiological processes including cognitive function. Studies in the mid-1990s in Japan reported increased learning ability of rats, whose mothers received fish oil, but only during gestation, and not during the postpartum period. In addition, it was shown in 1994 in France that ALA deficiency induces important changes in neurotransmission. A hypothesis was proposed that omega-3 FA affects the anatomical and functional development of the brain during its formation, which correlates with anthropological studies of the development of modern humans as a species. There is speculation that human intellectual evolution will depend on the consumption of omega-3 FA (Leray, 2017).

The brain is an organ that is approximately 50% lipids, which represents thousands of biochemical structures that play a number of important functional and structural roles, creating a unique environment. The environment of the brain promotes unique interactions that may not occur in a similar manner in the aquatic environment, including the participation of reactive oxygen species and nitrogen. In addition, lipids are an essential element of the myelin insulating layer around nerves in the brain and spinal cord, and are required for synaptogenesis, neurogenesis, and rapid conduction of spasmodic nerve impulses (Trostchansky & Rubbo, 2019).

The main groups of brain lipids are phospholipids, sphingolipids and cholesterol. Three types of bioactive lipids of various origins play important roles in the physiology and pathologies of the brain: nitrated fatty acids as a result of biochemical modification of dietary fatty acids; cholesterol, which makes up about 25% of total body cholesterol, and endocannabinoids. The first group is supposed to play different physiological roles, modulating NF-kB and Nfr2-dependent signaling cascades and post-translational changes in proteins. These FAs are produced in the

gastric region, are absorbed into the bloodstream, and can cross the blood-brain barrier, providing a means of communication between the gastrointestinal tract and the brain. Cholesterol is synthesized directly in the brain and regulates the biophysical properties of cell membranes, interacts with neurotransmitter receptors and other membrane proteins and enzymes, including those involved in the production and transport of amyloid precursor protein (APP) and A $\beta$  peptide. The third group, endocannabinoids as a class of neuromodulators, are formed from FA, synthesized, and released on demand, and induce cellular reactions by binding to specific membrane receptors. (Trostchansky & Rubbo, 2019).

Today role of lipids, their use as biomarkers is in the point of scientific interest. Lipid science is fast developing, including lipid health studies, nanotechnology use and drug delivery techniques. There are numerous studies reporting that long-chain FA can contribute to the prevention of the development of several diseases, including neurodegenerative changes in the brain during aging. Most studies have focused on the effects of DHA and EPA, and although ALA can be converted to DHA and EPA in the body, the role of ALA itself remains unclear.

## **1.2 PLANT AND SEEDS OF *SALVIA HISPANICA* L. AS SOURCES OF BIOLOGICALLY ACTIVE SUBSTANCES**

Spanish sage (*Salvia hispanica* L.), better known as chia, is an annual plant of the Lamiaceae family. Plants such as perilla, mint, basil and medicinal sage also belong to this family. The name “chia” in the ancient language of Nahua meant “oily” and was a term used by tribes for a variety of plants. The plant was named *Salvia hispanica* in 1753 by the Swedish botanist Karl Linnaeus, which means “Spanish plant for the treatment and preservation / salvation”. Despite the fact that the plant was referred to as Spanish, the birthplace of chia was actually southern Mexico

and Central America including northern Guatemala, Nicaragua and Honduras (Ixtaina *et al.*, 2008; Muñoz *et al.*, 2013; Sosa *et al.*, 2016; da Silva *et al.*, 2017).

Historically, for more than five millennia, chia seeds have been one of the most important cereals in Mesoamerica, including the Aztec and Mayan civilizations. Its cultivation and then the crop itself were banned and forgotten for at least 6 generations due to the Spanish invasion started in 1550. Although much knowledge about chia as an agricultural crop was lost, with the exception of a few local artisanal homesteads, Spanish sage could still be found in several mountainous regions of Mexico. Only in 1932 was there an official mention of chia in agriculture. Acatic Jalisco farmers played an important role in the revival of the crop, and in the 1990s, an active interest in the nutritional characteristics of chia seeds began particularly as a rich source of omega-3 fatty acids (Muñoz *et al.*, 2013; Sosa *et al.*, 2016).



**Figure 4.** Chia seeds variety ORURO France origin

World-wide interest in the plant and seeds of *Salvia hispanica* L. began with a 1991 research project in Argentina aimed at studying Spanish sage in terms of nutritional value,

cultivation and adaptation to climatic conditions in different South American regions including Argentina, Peru, Colombia, Bolivia and Ecuador. Ricardo Ayerza and Wayne Coates were the principal investigators of the project and the founders of the modern scientific interest in chia plant and seeds. The results of the study led to the introduction of Spanish sage culture in agriculture in 14 countries by 2016 and, current trends indicate that the demand for chia continues to grow (Sosa *et al.*, 2016).

Chia refers to plants with short day of flowering with a 12-hour photoperiod. The formation of flowers and the ripening of seeds occurs at certain latitudes and under specific growing conditions, including the absence of frost. In natural conditions, it grows in tropical and subtropical regions (Ixtaina *et al.*, 2008; Sosa *et al.*, 2016; Caruso *et al.*, 2018). The plant *Salvia hispanica* L. with a quadrangular, hairy and ribbed stem grows up to two meters in height, has opposed petiolate dentate leaves with sizes of 4-8 cm in length and 3-5 cm in width. Chia flowers are hermaphrodites and grow in numerous clusters in a spike protected by small bracts with long pointed tips (Muñoz *et al.*, 2013).



(a)



(b)

**Figure 5.** *Salvia hispanica* L. plant: (a) 10 days microgreens; (b) plant about 2-months growth

The color of chia seeds varies from black to white, and there are also spotted and red varieties. Seeds are arranged in groups of four in boxes remaining from the flowers (Muñoz *et al.*, 2013).

A number of studies have been carried out on the influence of the color of the seed coat, which is determined by the genotype, on the physical parameters and chemical composition of the seed. Some studies, according to Ixtaina *et al.* (2008), note small differences in the size of dark and white chia seeds, as well as in protein content and FA composition. The author Ayerza (2010) investigated two chia genotypes - Tzotzol and Iztac, which are black spotted and white domesticated seeds, respectively, growing in 5 different regions of Ecuador. According to the data obtained, the oil content, as well as the total lipid content of the representatives of the two genotypes does not significantly differ regardless of location. In addition, the FA composition, and the ratio of omega-6 FA to omega-3 FA did not show significant differences, due most likely to the low genetic variation between the Tzotzol and Iztac genotypes obtained from one source.

Another study by this author (Ayerza, 2013) also showed the absence of the effect of shell color on the content of protein, dietary fiber, and flavonoids, including myrcetin, quercetin, caffeic and chlorogenic acids, and the amino acid composition of chia seeds.

The study (Ayerza, 2010) revealed a significant difference in a number of indicators between seeds collected in different regions of growth, in particular, on the content of PUFA, which indicates the influence of environmental conditions, such as height of growth, soil composition and climatic parameters, on the chemical composition of chia seeds. The author noted the relationship between a decrease in the height of growth, an increase in the temperature factor and a decrease in the oil content in several crops, including Spanish sage. In addition, warm climates increase the content of SFA. This study suggested that the variability in ALA content cannot be directly attributed to the influence of the height of growth and the percentage of unsaturated FA in the oil. According to the author, there is a relationship up to a certain limit on the growth height, beyond which this factor does not affect the increase in the degree of unsaturation of chia seed oil.

The chia plant is actively cultivated in South and Central America, but today there is growing interest in the cultivation and adaptation of Spanish sage and in other regions of the world. Chia can be grown on light and medium soils, clay and sandy, including even arid soils, with good drainage and low humidity. The plant has moderate tolerance to acidic soils and drought. Chia grows mainly in mountainous areas and has little resistance to abiotic phenomena, such as frost and lack of sun. In its homeland, in Mexico, wild Spanish sage spread through seed dispersal, and was found to grow in juniper, oak, pine and pine-oak forests (Muñoz *et al.*, 2013). A number of countries including the USA, Chile, Argentina are studying agricultural techniques for plant adaptation despite the unfavorable climate for growing Spanish sage. In addition, even in regions where chia is a familiar crop, breeding and selection is performed to develop new varieties with higher yields. Despite their ability to resist drought, maximum seed yield is obtained when grown

in tropical areas, including Ghana, Africa, especially with good irrigation, in the absence of water stress (Sosa *et al.*, 2016).

A number of studies report success in growing chia in European climatic conditions: Italy (Peiretti, 2010; Bochicchio *et al.*, 2015; Raimondi *et al.*, 2015; Caruso *et al.*, 2018; Di Marsico *et al.*, 2018 et 2018b; Lovelli *et al.*, 2019), Greece (Bilalis *et al.*, 2016; Karkanis *et al.*, 2018) and Germany (Ahmed *et al.*, 2014; Grimes *et al.*, 2018 et 2019).

In France, for the first time in Europe, a new genotype of the chia plant, called ORURO, adapted to the climatic conditions of the region was bred in 2017. Studies of this genotype were carried out in the south-west of France in the Toulouse region, to evaluate the compositional analysis including lipid content, FA composition and other components during seed formation and the maturation stage. The authors (Gravé *et al.*, 2019) revealed that the yield of oil extracted with cyclohexane ranged from 23.3 to 28.4% at different stages of seed development and maturation. The maximum oil yield was observed on day 27 after flowering with a further decrease, which may be associated with losses of respiratory processes. It was noted that, in general, the accumulation of oil in oilseeds is regulated by the activity of fatty acid synthase, a system of multifunctional enzymes, which determines the different oil yield at different stages of seed development. When the seed reaches the “plateau” phase, the enzymatic activity decreases, which affects the overall decrease in oil yield (Gravé *et al.*, 2019).

FA composition of the ORURO chia seeds was evaluated in the above study, where the authors reported changes in the major FA levels during seed maturation. Overall FA content in French seeds correlates with the data already available for other chia genotypes. Interestingly, LA content in the ORURO seeds was 15% higher than in previous studies, and the maximum concentration of ALA was observed on the 35th day after flowering. The optimal FA composition of ORURO seeds from the point of view of recommended consumption norms was achieved on

24 days after flowering, which suggests the need to harvest chia seeds before the stage of full maturity, depending on the intended use (Gravé *et al.*, 2019).

### 1.3 COMPOSITION OF CHIA SEEDS

Chia seeds today are an important source of nutrients due to their unique chemical composition and are widely used in foods in the United States of America and Japan, Canada, Australia and New Zealand, and are becoming increasingly popular in Europe (Muñoz *et al.*, 2013; Caruso *et al.*, 2018). In addition, the seeds do not contain gluten, which makes them an excellent alternative in the diet of people with celiac disease, and there are no toxic components and allergic reactions (Muñoz *et al.*, 2013).

The composition of chia seeds from different regions in the world has been studied in extensive detail by the scientific community. Due to the fact that biologically active substances can vary depending on the region of growth, it is of particular interest to compare seed composition for different climatic conditions.

Data of chia seed composition from different suppliers, as well as from some research studies is cumulated by the United States Department of Agriculture. Table 2 presents data of chia seed composition by Food and Nutrient Database for Dietary Studies, a database that provides the nutrient values for foods and beverages reported in What We Eat in America, the dietary intake component of the National Health and Nutrition Examination Survey.

**Table 2.** Chia seeds composition by Survey, Food and Nutrient Database for Dietary Studies  
(USDA, 2020)

<b>Compound name</b>	<b>Amount, 100g of seeds</b>
<b>Nutritional value</b>	
Water, g	6.96
Energy, kcal	534
Protein, g	18.29
Total fat, g	42.16
Carbohydrates, by difference, g	28.88
Total dietary fiber, g	27.3
Sugars, total including NLEA, g	1.55
<b>Mineral content</b>	
Calcium, mg	255
Iron, mg	5.73
Magnesium, mg	392
Phosphorus, mg	642
Potassium, mg	813
Sodium, mg	30
Zinc, mg	4.34
Copper, mg	1.22
Selenium, µg	25.4
<b>Vitamins</b>	
Vitamin C, total ascorbic acid, mg	0.6
Thiamin, mg	1.644
Riboflavin, mg	0.161
Niacin, mg	3.08
Vitamin B6, mg	0.473
Folate, total, µg	87
Folate, food, µg	87
Folate, DFE, µg	87
Choline, total, mg	78.7
Lutein+zeaxantin, µg	651
Vitamin E (alpha-tocopherol), mg	0.31

<b>Compound name</b>	<b>Amount, 100g of seeds</b>
Vitamin K (phylloquinone), $\mu\text{g}$	4.3
<b>FA composition</b>	
SFA, total, g	3.663
C14:0, g	0.008
C16:0, g	2.165
C18:0, g	1.33
MUFA, total, g	7.527
C16:1, g	0.024
C18:1, g	7.359
C20:1, g	0.067
C22:1, g	0.013
PUFA, total, g	28.73
C18:2, g	5.903
C18:3, g	22.813

According to data on chia seeds approved for consumption in the European Union (Commission EU, 2013), the composition of chia seeds should include 4-9% moisture, 20-22% protein, 30-35% of total fat, 25-41% of carbohydrates, including 18-30% of dietary fiber, and 4-6% ash.

A study by da Silva *et al.* (2017) analyzed chia seeds from two regions of Brazil which were found to contain 35% dietary fiber, including 6% soluble fiber, 31% lipids, and an average of about 19% protein, are similar to those for chia seeds growing in other countries and to the guidelines of the EU. Another study of Brazilian seeds revealed a moisture content of about 8%, ash about 4%, 20% crude protein, 18% lipid fraction and 40% carbohydrates (Ganzaroli *et al.*, 2017), according to this data, composition can vary depending on the region of growth even in one country.

Whole chia seeds contain from 34 to 40% dietary fiber, including 5-10% soluble dietary fiber. The fiber content in chia seeds is 1.2 times higher than in flax seeds and more than 3 times

higher than in quinoa and amaranth. (Muñoz *et al.*, 2013). Studies of Chilean chia seeds showed a content of about 25% protein, 30% oil, almost 38% dietary fiber of which 35.07% is insoluble fiber. Dietary fiber is of interest since it plays an important role in maintaining intestinal health and also reduces the risks of heart disease, high blood pressure, diabetes, and obesity (da Silva Marineli *et al.*, 2014).

The protein component in chia seeds is a larger percentage than in traditional cereals and varies from 15 to 23% depending on the region of growth, and, in addition, contains 9 essential amino acids (Muñoz *et al.*, 2013).

The main mineral substances found in chia seeds are potassium and phosphorus, magnesium, calcium, sodium, iron, zinc, and copper. The amount of calcium exceeds the calcium content in milk by about six times, the iron content is also more than 2 times higher than in a traditional source such as meat (da Silva *et al.*, 2017) and 2.4 and 6 times more iron than spinach and liver, respectively (Muñoz *et al.*, 2013). In terms of phosphorus, chia seeds are 11 times higher than milk and 4 times higher in potassium (Muñoz *et al.*, 2013).

Spanish sage seeds can also provide a source of B vitamins, including niacin in the amount of 8.83 mg / 100 g of seeds (Muñoz *et al.*, 2013).

The content of tocopherols in chia seeds is 238-427 mg / kg, which is less than in flax seeds (588.5 mg / kg). Phenols found in Spanish sage seeds have stronger antioxidant properties than traditional ferulic acid and vitamins C and E ( $\alpha$ -tocopherol). Phytosterols, including  $\beta$ -phytosterol, were also found in chia seeds (Muñoz *et al.*, 2013).

The content of vitamin E (total tocopherol) was found to be 8.21 mg / 100 g of seeds in samples from Brazil. Brazilian chia seeds in research by da Silva *et al.* (2017) contained phenolic compounds in amounts of more than 0.9 mg GAE / g seed. The total phenol content in chia seeds of Italian origin was around 1.8 mg GAE / g seed (Caruso *et al.*, 2018). Interestingly, that according to the authors, the antioxidant content in chia seeds can vary from 0.53 to 0.94 mg GAE / g, the

maximum values were 4.79 to 7.89 mg GAE / g in seeds grown in Brazil and Mexico. According to several authors (da Silva *et al.*, 2017; Caruso *et al.*, 2018), antioxidant levels depend not only on the region of growth and plant genotype, but also on the extraction method used and the analysis technique.

Thus, although the composition of chia seeds varies depending on the region of growth, chia seeds represent a promising source of a number of biologically active substances.

In frame of this thesis study, the nutritional and biological value of chia seeds was estimated based on the chemical composition of chia seeds (USDA, 2019). To assess the biological value of the product, the protein, lipid, mineral and vitamin component of chia seeds was estimated using food combinatorics methods (Nadtochii *et al.*, 2019).

The data of the macronutrient composition in terms of 100% solid matter of the product, allow us to highlight the high nutritional value of chia seeds. Carbohydrates (44.71%) are a major constituent, most of which are dietary fiber (36.52%), but chia seeds also contain high amounts of fats (32.63%) and proteins (17.56%). The potential functionality of chia seeds has been demonstrated in terms of the protein component (with a full spectrum of essential amino acids, the amino acid rate which is above 100%), the lipid component (in the content of all groups of fatty acids, most of which are PUFA, in particular, essential omega -3 and omega-6 fatty acids), a carbohydrate component (by the content of a high amount of dietary fiber) and a mineral component (by the content of phosphorus and manganese).

#### **1.4 CHIA SEEDS LIPID FRACTION**

Lipids are a vital element in a healthy diet. Vegetable oils are also a natural source of vitamins, antioxidants, and essential FA. Several FA cannot be synthesized by the body and must be supplied with food. Studies on vegetable oils revealed that the lipid fraction of chia seeds is of

particular interest due to the high content of PUFA (about 80%). These seeds are currently the richest source of  $\alpha$ -linolenic FA known (about 60%), an essential fatty acid and precursor of long chain EPA and DHA fatty acids (Muñoz *et al.*, 2013).

However, the high degree of unsaturation of chia seed oil, according to the researchers, makes it prone to oxidative processes and loss of nutritional value during storage, as well as the possible formation of undesirable toxic compounds. It is oxidative stability that is the most important indicator of the quality of edible vegetable oils (Bodoira *et al.*, 2017; Souza *et al.*, 2017). In addition, as noted earlier, the FA composition of chia seed oil depends on the place of growth. A study (da Silva *et al.*, 2017) reported the influence of temperature of environment on the lipid content in chia seeds. The increase in oil yield correlates with lower ambient temperatures, which, in addition, has a positive effect on the FA composition, increasing the proportion of unsaturated FA in chia seed oil. Therefore, to obtain a complete picture, we considered various regions of the world.

Chia seed oil, which is approved as a food product in the European Union (Commission EU, 2014), should have an ALA content of at least 60%, LA content 15-20% and a peroxide content of not more than 10 mEq O<sub>2</sub> / kg of oil.

The FA composition of chia seeds grown in Brazil was reported in the lipid fraction following extraction by the Bligh & Dyer method (1959) using chloroform, methanol and water in the ratio (2:2:1), with a yield of 27.72% oil. The total content of SFA in chia seed oil was found to be 11.12% containing 6.8% palmitic and 2.7% stearic acids. MUFA levels were 7.40%, with the major MUFA being oleic acid (5.6%). Finally, chia seed oil was found to contain 81.5% PUFA, of which 55.1% and 17.4% were ALA and LA, respectively. The researchers concluded the high nutritional quality of the lipid fraction of chia seeds based on the atherogenicity (0.09) and thrombogenicity (0.054) indices that reflect the potential for stimulating platelet aggregation. Indeed, the lower the levels of these indices, the more anti-atherogenic fatty acids in oil and the

greater the effectiveness in preventing the development of coronary heart disease. Furthermore, the authors reported a hypocholesterolemic / hypercholesterolemic index of 11.41 for chia oil, which considers the specific effect of fatty acids on cholesterol metabolism, also indicative of beneficial health effects from a nutritional point of view. Interestingly, this index for a number of freshwater fish, including dorado, was about 1.5 (Ganzaroli *et al.*, 2017).

A similar FA composition were obtained in chia seed oil from Chile that was obtained by cold pressing, where ALA (62.80%), LA (18.23%), palmitic (7.07%), oleic (7.04%) and stearic (3.36%) were also the major FA identified. According to the authors, the content of ALA was comparable to linseed oil (up to 57%). The ratio of omega-6 to omega-3 FA in Chilean oil was reported as 0.29, which is conducive with a decrease in cardiovascular risks. This is indeed very low, as compared to other seeds rich in MUFA and PUFA, including rapeseed oil (2.26), sunflower oil (30.77), and olive oil (17.86) (da Silva Marineli *et al.*, 2014).

This study analyzed the level of peroxides in chia seeds, which was found to be 2.56 mEq O<sub>2</sub> / kg, consistent with other reports on chia seeds from South America and Argentina. In addition, this indicator is lower than that of linseed oil (da Silva Marineli *et al.*, 2014).

Chia seed oil could contain lipophilic antioxidant compounds, phenolic molecules and other active ingredients, their extractability into the oil is highly dependent upon the extraction method used. The total phenol content was 0.94 mg GAE / g. Myricetin, quercetin, kempferol and chlorogenic acid are some of the phenolic compounds found in chia oil. The authors note that most of the phenolic compounds found in chia seed oil are unique and not present in other oilseeds (da Silva Marineli *et al.*, 2014). Phenolic compounds were also studied in Chilean chia seed oil obtained by cold pressing and showed that there is a decrease of antioxidant compounds in oil compared to chia seeds (Oliveira-Alves *et al.*, 2017). Two major constituents were identified, caffeic and rosmarinic acids, which play important roles in the prevention of neurological disorders and have hepatoprotective properties. For example, according to the authors, caffeic acid

has been studied for its protective mechanisms of memory, and rosmarinic acid plays an important role in immunoregulatory processes and slows down inflammatory reactions. This study used ultrasonic extraction of phenolic components in combination with acid hydrolysis to detect about 30 components. These included: quinic, citric and isocytic acid, hydroxytyrosate acetate, which is also a component of olive oil, a number of hydroxycinnamic acids (caffeic and its derivatives, danshensy, rosmarinic and its derivatives, salvianolic acids C and E; ferulic and caffeoylquinic acids, etc.), as well as carnosol. The overall total phenol content in the crude methanol extract of chia seeds (1) was 1.16 mg GAE / g, fat-free chia flour (2) 1.11 mg GAE / g, and in chia seed oil (3) 0.02 mg GAE / g; and hydrolyzed extracts of (1), (2), (3) contained 2.23, 2.15 and 0.07 mg GAE / g, respectively. In view of these latter results, the authors suggested that, during digestion, the antioxidant activity of chia seeds may increase (Oliveira-Alves et al., 2017).

The presence of an unsaponifiable fraction including squalene in the amount of 0.5 g / kg of oil was reported in a study of the lipid fraction of chia seeds of Mexican origin in two regions, following extraction with hexane using a Soxhlet apparatus. This is considerably lower compared to, for example, olive oil which contains up to 7 g squalene / kg of oil, and amaranth oil which has up to 80 g / kg (Álvarez-Chávez *et al.*, 2008).

Further characterization in the same study revealed the presence of phytosterols (up to 12.6 g / kg of oil), such as stigmasterol (up to 1.83 g / kg),  $\beta$ -sitosterol (up to 7.96 g / kg) and stigmasterol (up to 2.27 g / kg) were found, significantly higher than in olive, sunflower and rapeseed oils.  $\beta$ -sitosterol is the main phytosterol found in unrefined vegetable oils and its content can reach up to 60% of the total number of sterols, while the main loss of sterols occurs at the refining stage. In the lipid fraction of chia seeds, this indicator reached levels of 74%, suggesting that chia seed oil can be a natural source of phytosterols (Álvarez-Chávez *et al.*, 2008).

In the analysis of chia seed oil obtained by cold-pressing,  $\beta$ -sitosterol (60 mg / 100 mg), 4,6-cholestadiene-3 $\beta$ -ol (30 mg / 100 mg), campesterol (9.7 mg / 100 mg) were identified. In

contrast to extraction by Soxhlet, the amount of squalene identified was insignificant (Bodoira *et al.*, 2017).

The effect of storing chia seeds for up to 10 months was reported by Caruso *et al.* (2018). Lipid fractions of seeds from Italy obtained by the Soxhlet method using petroleum ether as solvent were stored in glass containers at room temperature in a dark room. Lipid yield, FA composition, antioxidant content, antioxidant compounds, and resistance to oxidation were evaluated. The oil yield was 32.55%, with the following FA composition: ALA 65.42%, LA 18.71%, oleic acid 5.90%, palmitic acid, 6.47% and stearic acid, 2.74%, and results showed that FA composition of seeds did not significantly change during 10 months of storage. The acid value of chia seed oil was low, with an average value at the beginning of storage of 0.60%. This parameter increased to 1.14% after 10 months of storage of seeds. The peroxide value increased during storage by more than 2 times (from 11.82 to 26.9 mEq O<sub>2</sub> / kg of oil). According to the authors, standard values for chia seeds typically vary from 0.6 to 3.9 mEq O<sub>2</sub> / kg of oil, and the authors suggest that these unusually high peroxide levels may be due to the genotype or characteristics of the growing ecosystem. Chia seed oil was also found to contain  $\gamma$ -tocopherol (393 mg / kg oil),  $\delta$ -tocopherol (16.3 mg / kg oil) and  $\alpha$ -tocopherol (4.52 mg / kg oil).

Bodoira *et al.* (2017) investigated the storage effects on chia seed oil following pressing that was supplemented with natural antioxidants (rosemary, tocopherol and ascorbyl palmitate extract) for 300 days under various conditions. The content of ALA was 61.8%, LA 20.1% and oleic 7.18%. The authors reported that despite a small amount of chlorophyll (4.66 mg / kg of oil) as a photosensitizer, chia seed oil may be subject to photochemical oxidation when stored in a bright room. In addition, the content of carotenoids in oil (5.41 mg / kg) is insufficient for protective functions due to the high unsaturation of the oil. Chia seed oil, which was stored in the dark without the addition of antioxidants as a control sample, showed a similar degree of oxidative degradation as the oil that was stored in the light. At the same time, the amount of tocopherol

decreased by about 30%, but the FA composition and acidity of the oil did not significantly change. This was consistent with other reports that chia seed oil is resistant to hydrolytic degradation of glycerides when exposed to light. The authors concluded that the use of a combination of ascorbyl palmitate and tocopherols (50:50) in an amount of 200 mg / kg of oil each as the most effective natural antioxidant complex for chia seed oil.

In a study by Souza *et al.* (2017), they evaluated the thermal and oxidative stability of chia oil using various methods to assess food safety. Chia oil was obtained using the Soxhlet apparatus with n-hexane as the extractant. In addition, the experiment used natural (including  $\alpha$ -tocopherol, caffeic acid, rosemary extract) and artificial antioxidants as additives. The oil yield was 27%, with the following FA: ALA 64.04%, LA 16.18%, oleic acid 4.80% and about 15% SFA. Interestingly, here, FA determination was carried out using proton magnetic resonance ( $^1\text{H}$  NMR spectroscopy) rather than gas chromatography. In this method, there is esterification of oils is not necessary, which eliminates potential errors or loss in the sample preparation. Chia oil were stable in several tests at 60°C, and even during prolonged tests in the presence of oxygen. The use of tert-butylhydroquinone as effective for vegetable oils has been proposed as an antioxidant supplement, natural antioxidants did not have the desired effect, however, it was mentioned that previous studies have shown the need for higher concentrations. The authors concluded that the use of chia oil for frying is extremely undesirable due to its low thermal stability. Nevertheless, chia oil is at least 3 times less resistant to oxidation than sunflower oil.

The effect of the type of extraction method on oxidative stability of chia seed oil was also studied, comparing extraction using a Soxhlet apparatus with n-hexane or acetone as solvents, supercritical extraction at temperatures of 70°C and 90°C, cold extraction by pressing and hot pressing at 110°C (Dąbrowski *et al.*, 2017). Acetone was the most effective solvent (up to 95% of the total oil content in seeds), especially with respect to extraction of phenolic compounds and carotenoids. This could explain the highest oxidation stability observed in the acetone extracts.

Pressing was found to be the least effective method of obtaining oil, based on the yield (about 70% of the total amount of oil), the content of sterols and phenolic compounds. The quality of the oil obtained by supercritical extraction at 70°C was comparable to the oil obtained by cold pressing; squalene was also found in these extracts. The authors concluded that the levels of lipophilic components, including sterols, tocopherols, squalene, waxes, carotenoids and phenols, vary greatly and depend on the extraction method. Based on their results, the lipid fraction of Spanish sage contains from 4 to 17 g / kg sterols, among which  $\beta$ -sitosterol predominates (more than 50%); from 150 to 500 mg / kg of tocopherols, with a predominance of  $\gamma$ -tocopherol (about 85% and above), and up to 0.5 g squalene. It contains carotenoids from 0.5 to 11.6 mg / kg and up to 700 mg / kg of polyphenols. The extraction methods tested also affected the ratio of omega 6 to omega-3 fatty acids. In addition, regardless of the extraction method, chia seed oil has a low acid (1.4–2.9 mg KOH / g) and a peroxide value (1.0–2.8 mEq O<sub>2</sub> / kg), which is favorable from the point of view of storage stability. According to the authors, the choice of extraction method allowing maximum recovery of antioxidants, is necessary to ensure the oxidative stability of natural chia seed oil (Dąbrowski *et al.*, 2017).

Thus, in recent years, there has been increasing literature on chia seed oil. The FA composition of the oil, its storage stability, the content of antioxidants and other biologically active substances have been extensively studied. However, the phospholipid composition of the lipid fraction of chia seed oil has been little studied, which is of scientific interest.

## **1.5 CHIA SEED OIL COMPARED TO OTHER VEGETABLE OILS RICH IN UNSATURATED FATTY ACIDS**

In terms of nutritional value, there are a number of sources rich in omega-3 FA on the market, including menhaden fish, salmon, a number of algae, flax seeds. Chia seeds have some

advantages including ease in transport as compared with marine sources, and absence of undesirable effects like allergy, and can be consumed in its raw form with no limitations (Sosa *et al.*, 2016).

One of the main potential competitors for chia seed oil as a source of ALA is flaxseed (*Linum usitatissimum*) oil. Also, a number of researchers are studying the oil of the seeds of perilla (*Perilla frutescens*) plant, which belongs to the same family, like the Spanish sage and grows in Southeast Asia.

In a study by Ciftci *et al.* (2012) compares the lipid component of chia, perilla and flax seeds. Lipid extraction was carried out by the Folch method using a mixture of chloroform and methanol (2:1). According to the authors, the oil yield was almost 45% for flax seeds, 40% for perilla seeds and 35% for chia seeds. In general, the oil content of flax, as well as perilla, varies depending on the region and can range from 29% to 46%, on average about 40%, and for perilla from 25% to 52%. The content of ALA was 58.2%, 60.9% and 59.8% for flax, perilla and chia, respectively. It was noted that the amount of ALA in linseed oil can vary from 52% to 64%, in perilla seed oil from 57% to 64%, and in chia seed oil it can reach 65.1%.

Interestingly, in the composition of flax and perilla seeds, more oleic acid was identified (18.1% and 16.21%) than LA (15.3% and 14.72%), and the inverse relationship was observed in chia seeds (10.53 and 20.27%), however, in the other studies mentioned there is no such dependence (Ciftci *et al.*, 2012).

The total content of PUFA in flaxseed oil was 73.6%, in chia and perilla 80.5% and 74.6%, respectively, while the content of MUFA in chia seeds is lower (10.95%) than in other types of seeds (18.50% for flax and 16.57% for perilla). The ratio of omega-6 to omega-3 FA was significantly higher in chia (0.35) and flax seed oil (0.28) than in perilla oil (0.22), but higher values were noted in previous studies by other authors. According to the data obtained in the study,

chia seeds contain significantly fewer total tocopherols and fewer varieties of sterols with a sufficiently high total content (Ciftci *et al.*, 2012).

Another study compared the characteristics, oxidative stability, and antioxidant potential of cold pressed chia seed and flax seeds oils. The authors note that the obtained data on the content of ALA are consistent with previously known data on chia seeds, as the richest source of omega-3 plant origin. In addition, according to the study, chia seed oil is more prone to oxidation due to greater unsaturation, however, it had more antioxidant compounds and the total phenol content in the oil also exceeded this indicator in flaxseed oil (319.12 mg / g and 244.65 mg / g, respectively) (de Souza *et al.*, 2019). According to Saphier *et al.* (2017) chia seeds contain 42% more polyphenols than flax seeds.

Rapeseed oil is currently one of the most important sources of unsaturated fatty acids. Rapeseed oil contains more than 60% oleic, 21% LA and 11% ALA fatty acids, as well as sterols and tocopherols. An analysis of the composition of oils of flax seeds and rapeseed showed that rapeseed oil can be considered a source of MUFA, and linseed oil can be considered a PUFA source (Lewinska *et al.*, 2015).

## **1.6 EFFECT OF CHIA SEED LIPIDS ON HEALTH AND DISEASE**

Neurodegenerative diseases represent a significant socioeconomic problem, due to high medical costs and the care needed for the patient which impacts their quality of life as well as those of their caregivers. Indeed, according to an INSERM study (2019), 900,000 people in France are affected by the neurodegenerative disease, Alzheimer's disease (AD), and this is expected to increase to 1.3 million by 2020. In view of the low efficacy of AD therapeutic treatments that are currently available, preventive measures, in particular dietary intervention, to reduce risk factors of AD may represent a better approach towards reducing neurodegenerative diseases such as AD.

While age is a significant AD risk factor, dyslipidemia and obesity have also been suggested to contribute not only to diseases such as diabetes and cardiovascular disease, but also neurodegenerative processes that could lead to AD. Obesity incidence itself has nearly tripled in the last 40 years worldwide, 23% adults in Europe are considered obese (WHO Non-communicable diseases, 2018), due in part to an increasing sedentary lifestyle and inappropriate dietary habits. Improved quality of diet to minimize these lipid-related disorders may thus contribute towards reducing AD risk.

The brain is in fact a lipid rich organ, where fatty acids and cholesterol play an important role in neuronal function and synaptic plasticity, both as structural components of membranes and myelin, and as signaling factors. PUFA have been of particular interest for prevention of pathological changes associated with the process of aging. Recent studies have revealed significant reduction of changes in unsaturated fatty acid metabolism in brains of AD patients; also, phospholipids are significantly reduced in neural membranes (Hussain *et al.*, 2019).

Studies have shown a decrease in the PUFA, docosahexaenoic acid (DHA) levels in AD patients (Hussain *et al.*, 2019), leading to numerous studies on the potential neuroprotective effects of this omega-3 PUFA. One major source of omega-3 fatty acids is marine products, including fish oil. However, because of many issues with these sources, products of vegetable origin, rich in ALA, are being considered increasingly as alternative and promising sources of PUFA (Lee *et al.*, 2017).

In recent years, *in vivo* investigation on oil and seeds of *Salvia hispanica* L. has increased considerably because of their potential interest in the prevention and treatment of lipid-related disorders, such as dyslipidemias, obesity, cardiovascular diseases. With its high ALA content, the potential use of chia seed oil in treatments is being extensively studied in preclinical research. ALA itself is of interest as a precursor for long-chain FA and its bioconversion, but also for direct physiological effects of this FA.

Han *et al.* (2020) studied the effect of chia seed oil on hyperlipidemia and oxidative stress in mice induced by a high fat diet. Total PUFA content was 89.3% in chia seed oil obtained by Soxhlet extraction using petroleum ether, with a content of ALA of 68.4%. The experiments were carried out on male Kunming mice at the age of 5 weeks. The control group ( $n = 10$ ) received a normal diet with a fat content of 4%, the remaining mice ( $n = 50$ ) received a diet with a high fat content (20%) for 8 weeks to develop hyperlipidemia. After 8 weeks, the mice were randomly divided into 6 groups of 10 animals each - a normal diet, as well as 5 groups with different variations of the high-fat diet and the addition of simvastatin (5 mg / kg body weight) as a positive control, or chia seed oil at different concentrations - 950, 1900 and 3800 mg / kg body weight. The oil or positive control were administered orally by gavage daily for 5 weeks; body weight was monitored on a weekly basis. At the end of the experimental period, animals were fasted 12 h, then sacrificed and blood and tissue samples were taken, including liver, spleen, kidney, and perirenal epididymal fat pad for analyses. Results revealed that chia seed oil supplements reduce body weight, as well as the level of lipids, total and low-density lipoprotein (LDL) cholesterol, and serum triglyceride despite the mice being on a high fat diet. Furthermore, liver index, which is an indication of the amount of fat, as well as spleen and fat index are significantly reduced in mice receiving chia seed oil, as compared to controls. In the groups receiving the 2 higher doses of chia seed oil, total cholesterol levels decreased by 16% and 26%, respectively. The atherosclerosis index, a risk factor for atherosclerosis, calculated as a ratio of LDL and HDL (high-density lipoprotein) cholesterol, was reduced by 47% to 53% in all 3 groups that received chia oil. In addition, chia seed oil increases the activity of 2 enzymes involved in reducing oxidative damage: superoxide dismutase (SOD) and glutathione peroxidase. Serum and liver malondialdehyde levels, markers of oxidative stress, are also reduced in the groups receiving chia oil. For example, in the group that received the maximum amount of chia seed oil, serum glutathione peroxidase activity increased by 21.2%, and in the liver by almost 73%, compared with the group on the high-fat diet. Also, chia seed oil improves liver steatosis and reduces lipid deposits as shown by

histopathological analysis, which is associated with a high content of ALA. The introduction of this oil into the diet significantly inhibited adipocyte hypertrophy, depending on the dose. According to the authors, chia seed oil can improve liver fat metabolism, even if the animal is maintained on a high-fat diet. Moreover, according to the results of Western blotting, chia seed oil increases the expression of the peroxisome proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ ), which plays an important role in lipid metabolism, in the regulation of expression of genes involved in FA transport and their oxidation, including liver carnitine palmitoyltransferase 1a, which is a key enzyme that limits the  $\beta$ -oxidation of FA.

Another study reported effects of chia seed oil can cause browning of white adipose tissue (de Souza *et al.*, 2020), which is the increase of mitochondria and change in characteristics of subcutaneous white adipose tissue that resembles that of brown adipose tissue. In the present study, 28 male Swiss mice were divided into 4 groups - a control diet (13% of energy from fat) and a high-fat diet (45% of energy from fat) for 130 days (18.5 weeks), a high-fat diet supplemented with chia seed oil (1.5% w / w) since the mice were obese (from day 90), and a high fat chia seed oil diet since weaning (from day 21). The data showed that chia seed oil consumption in the 90-day group reduces weight gain. In the group where chia seed oil was present in the diet from day 21, there was a decrease in plasma leptin (a hormone that regulates satiety and energy metabolism), improved glucose metabolism. In both chia groups, the process of browning adipose tissue was activated, which is associated with improved glucose tolerance and reduced insulin resistance and thus a decrease in the negative effects of obesity. This effect was more marked in the group from day 21. Indeed, both chia groups showed higher efficiency of glucose uptake and lower glycemia in tolerance tests. However, only the group from day 21 showed reduced fasting blood glucose levels. In the same study, the authors emphasize that some of the data obtained in the study call into question the relationship between the development of inflammatory processes and obesity. As a conclusion, according to the authors, chia seed oil can be considered as a preventive measure against the development of pathologies associated with obesity.

Study by da Silva Marineli *et al.* (2015) examined the effect of chia seeds and oil obtained by cold pressing (Chile origin) on plasma and liver oxidative status in diet-induced obese rats. For the study, 36 Wistar rats were selected at the age of 21-23 days, and after a 4-week adaptation period, were divided into 6 groups: control group with a standard diet (12% protein); group with a diet high in fat and fructose; two groups on a diet that included ground chia seeds (13.3%, *w:w*) for 6 or 12 weeks; two groups on a diet that included chia seed oil (4%, *w:w*) for 6 or 12 weeks. Animals with a short chia turn-on period were fed a high-fat, high-fructose diet for the first 6 weeks. Chia seeds contained 30.2% oil by Bligh and Dyer (1959) method. Chia diets were designed to maintain an equal dose of oil and ALA among the groups and replacing soybean oil in the diet. The diet has also been adjusted for protein and fiber in chia seeds. The animals were weighed weekly. The experiment was carried out for 12 weeks, with weekly monitoring of body weight. After a 12h fasting period, animals were sacrificed for tissue sampling for analyses of biomarkers of lipid peroxidation in plasma and liver, endogenous enzymatic and non-enzymatic antioxidant systems and antioxidant capacity. The high-fat, high-fructose diet group was characterized by increased weight, as well as an increase in oxidative markers in the plasma and liver of animals. The authors note that this is consistent with previous studies showing that a high proportion of fructose in the diet leads to an increased susceptibility of lipids to peroxidation due to its influence on the balance between antioxidant defense and free radical production, a decrease in total glutathione and suppression of the activity of antioxidant liver enzymes. In this study, in contrast to previous studies cited above, neither chia seed nor oil consumption had any effect on weight gain in rats. However, compared to the high-fat diet group, the chia oil or chia seed group showed lower oxidative stress, demonstrated by an increase in plasma reduced thiol (GSH) levels, plasma catalase (CAT) and glutathione peroxidase (GPx) activities. The group that received chia seeds for 12 weeks also showed a 28% increase in GSH in the liver. In addition, glutathione reductase (GRd) activity was increased at the liver level in the chia group, which the authors suggest may occur to restore glutathione levels required in the glutathione cycle. This

demonstrates the complexity of the system of enzyme interactions, that may lead to unexpected changes, or lack thereof. In this study, no changes in SOD activity were observed between the chia and high fat diet groups in either liver or plasma. Although consumption of chia seeds and oil does not affect liver lipid peroxidation, plasma but not liver levels of the reactive substances thiobarbituric acid (TBARS) and 8-isoprostane are reduced. In addition, plasma, and liver antioxidant capacity increase by 35% and 47% in the groups receiving chia seed and chia seed oil in the diet, respectively, compared with the high-fat diet. The authors conclude that chia seed or chia seed oil improve endogenous antioxidant protection, even in the presence of a high fat diet. However, this antioxidant effect was independent of the duration of treatment or the dose of chia seed or oil in the diet. It is necessary to take into account a whole range of influencing factors, which presents certain difficulties in *in vivo* studies. The authors hypothesize that ALA in the body can reduce the formation of hydroperoxides by scavenging free radicals and peroxidation of PUFA. In addition, the authors were unable to eliminate the possible effect of antioxidants already present in chia seed oil. Thus, additional research is required to distinguish effects of ALA and those of antioxidants that are in the chia oil. Although there was no significant difference in experimental results between the consumption of seeds and chia seed oil, the authors point out that the results may depend on the biomarkers used or the tissues or methods used for analysis. Also highlighted, that there is a lack of studies evaluating the antioxidant potential of chia seeds and oil *in vivo*, determination of biologically active compounds and their main *in vivo* metabolites in plasma, urine and tissues.

A study by the same authors above (da Silva Marineli *et al*, 2015b) investigated the effect of dietary chia seeds and seed oil (Chilean origin) on the stress proteins, heat shock proteins (HSP) and related factors in obese rats. For the experiment used 48 male Wistar rats obtained at the age of 21-23 days. After a 4-week adaptation period, the animals were divided into 6 groups with different diets - control (standard diet 12% protein), high-fat with a high fructose content (HFF, 4% soybean oil, 31% lard and 20% fructose), as well as 2 groups receiving short-term (6 weeks

with HFF, then 6 weeks with HFF including 13.3% chia seeds or 4% chia seed oil) or long-term (12 weeks HFF and chia seeds or chia seed oil). Soybean oil in diets was replaced by chia seeds or oil (cold pressing), and protein and fiber dietary content was adjusted for the chia seed supplementation. The groups were placed on a high-fat diet for the first 6 weeks with increased fructose, followed by weekly monitoring of body mass, and analyses of plasma indicators of glucose tolerance and liver damage, the expression of antioxidant enzymes in skeletal muscle and of proteins that control oxidative energy metabolism. Groups consuming chia seeds or oil did not exhibit a difference in body weight or the accumulation of fatty tissue in the abdominal area, nor did food intake change. Although hyperinsulinemia was not modified, glucose and insulin tolerance were improved in both groups receiving chia seeds or oil. Expression of HSP70 and HSP25 in skeletal muscle as well as SOD and glutathione peroxidase were restored levels similar to those of control group in both short and long-term experiments with chia seed oil. Short-term dietary intake of chia seeds led to only an increase in HSP70 expression and decreased HSP60. Here, the authors conclude that chia seeds can improve glucose tolerance through mechanisms other than induction of HSP, that involve the insulin signaling pathway. The groups on long term dietary intake of chia seeds or short-term diet of chia seed oil showed increased expression of the peroxisome proliferator-activated gamma receptor co-activator 1a (PGC-1a). This protein is involved in the control of genes involved in energy homeostasis, thermogenesis. It is also involved in the control of glucose metabolism and transport without changes in insulin sensitivity, as well as in the regulation of biogenesis and mitochondrial respiration in skeletal muscle. Insulin degrading enzyme involved in the breakdown of insulin was increased in the short-term groups receiving chia seed or oil. Despite this potentially beneficial change, hyperinsulinemia remained unchanged in the groups. Chia seed oil reduces the oxidative stress induced by the HFF, more so than chia seeds, which may be due to the different content of phenolic compounds, although this was not demonstrated in this study. All chia groups showed lower levels of aspartate and alanine

aminotransferase to values similar to that of baseline of the control group, with the exception of the group on short-term intake of chia seeds.

The biological mechanisms that explain the effect of flavonoids on the activation of PPAR- $\alpha$  gene expression and its decrease for SREBP-1c (sterol regulatory element-binding protein 1), as well as the increase in the plasma concentration of long-chain omega-3 upon their consumption, are not fully understood (Gallegos *et al.*, 2018). In research, authors studied the effect of consumption of ALA from chia seed oil in combination with anthocyanins from purple corn on the aforementioned genes, desaturase  $\delta$ -5 and  $\delta$ -6, their activity in the liver and changes in its lipid profile. The studies were carried out on 36 female Sprague-Dawley rats, about 24 days old, weaned. Animals received an isocaloric and isoproteic diet in the form of corn and soy flour. At 28 days of age, animals were divided into 4 groups of 9 rats, supplemented with olive oil / chia seed oil / olive oil combined with purple corn extract or chia seed oil combined with purple corn extract. Chia seed oil was cold pressed. Purple corn extract was introduced into the diet in an amount of 1% (equivalent to 401 mg of anthocyanins per kg of food). Olive oil and chia seed oil accounted for about 20% of dietary energy and were administered via cannulas. Upon reaching 10 weeks of age, 3 rats from one group were mated. Between the fourth and fifth postpartum day the litter size was homogenized to 6 pups. On the 16<sup>th</sup> day, the mothers were withdrawn from the experiment, followed by extraction, weighing, and freezing of the liver. The authors noted an increased activity of the PPAR- $\alpha$  gene expression when consuming chia seed oil, as well as chia seed oil in combination with purple corn extract. Chia seed oil consumption also increased the expression of the SREBP-1c gene and  $\delta$ -5 desaturase enzymes. The activities of both  $\delta$ -5 and  $\delta$ -6 enzymes were also increased when dietary supplementation of chia seed oil with purple corn extract was compared to pure chia seed oil, as explained by the authors in terms of the protective effect of anthocyanins against high doses of ALA. The content of ALA and long-chain FA in the liver increased with the consumption of chia seed oil, decreasing the level of arachidonic FA and

long-chain omega-6. No effects on body / liver weight were found in rats fed this diet. According to the authors, the combination of ALA and anthocyanins shows an anti-adipogenic effect.

Santos-López *et al.* (2017) reported that chia seed oil can prevent oxidative damage and alter the inflammatory response by regulating the antioxidant system. This study investigated the effect of chia seed oil added to restructured pork (152.2 g / kg fresh matter) in an aged rat model of non-alcoholic steatohepatitis, which is associated with red meat consumption and can progress to non-alcoholic fatty liver disease, one of the most common liver diseases. Wistar rats aged 1 year, considered as an equivalent to 60-years in humans received this chia seed oil enriched diet for 8 weeks. The authors noted that in the chia group, the hypercholesterol effect was reduced almost to the control level, and the redox index was improved. In addition, there was a decrease in reactive substances with thiobarbituric acid (TBARS) - lipid peroxidation, as well as sufficient protein levels of SOD to maintain activity and to minimize reduce oxidative damage. Chia seed oil promoted the activation of Nrf2 (a key component involved in attenuating oxidative stress processes), which contributed to the limited pro-oxidant effect of cholesterol and age and increased the body's antioxidant defense system. Chia also partially blocked the increase in tumor necrosis factor alpha (TNF- $\alpha$ ).

The potential anti-oxidative effects of chia seed oil have also been studied in animal models of arthritis. Mohamed *et al.* (2020) investigated the effect of chia seed oil and mucilage as anti-inflammatory nutraceuticals on obesity and arthritis. Chia seed oil (Egypt origin) was obtained by cold pressing (yield 33%, ALA content 58.14%), and mucilage was used after lyophilization and re-dissolved in water. For the study, 48 male Sprague-Dawley rats were used in 8 groups of 6 animals. The experiment involved: 1<sup>st</sup> control group on a balanced diet; 2<sup>nd</sup> control group with arthritis; Group 3 with arthritis, and the introduction of chia seed oil (300 mg / kg body weight) into the diet; Group 4 with arthritis and the introduction of chia seed mucilage into the diet at a dosage of 100 mg / kg of animal weight; 5<sup>th</sup> control group with obesity; 6<sup>th</sup> control group with obesity and arthritis; 7<sup>th</sup> group with obesity, arthritis and the introduction of chia seed oil in the

diet; and group 8 with obesity, arthritis and chia seed mucilage. Some groups (5-8) were kept on a diet high in fat and sucrose (30%, w:v sucrose water) for 8 weeks to develop obesity. At week 9, certain groups of rats (3,4,7,8) were introduced into the diet with oil (in the form of an emulsion) or chia seed mucilage (dissolved in water) for 21 days. Also, at the beginning of week 9, several groups of rats (2-4,6-8) were injected with Freund's complete adjuvant to induce arthritis. During the experiment, the paws were measured periodically, and animals were weighed on a weekly basis. After 11 weeks from the beginning of the experiment, after overnight fasting, the animals were sacrificed, and tissue samples were taken for analyses. Analyses including plasma levels of TNF- $\alpha$  and cachectin, plasma lipid profile, markers of liver and kidney functions, serum lipid peroxidation and erythrocyte catalase activity. According to the obtained results, chia seed oil and mucilage (mainly oil) reduced the degree of paw swelling in all groups of rats with arthritis. Starting from the 7<sup>th</sup> day of the introduction of chia products into the diet, less significant paw swelling, and a reduced inflammatory process were observed and increased inhibitory activity in all groups of arthritic rats. In addition, there was a decrease in TNF- $\alpha$  and an improvement in lipid profile.

Several studies have been published on the effects of chia seed oil on body, tissue, and serum FA composition. In study of Ayerza & Coates (2007) examined the effect of various forms of chia seeds or chia seed oil on the plasma composition of Wistar rats. The experiment was carried out for 30 days, rats (4 groups of 8 rats) were kept on an isocaloric diet (4 kcal / g diet, 20% protein) supplemented as an energy source - corn oil (1), whole chia seeds 16% (2), ground chia seeds 16% (3) or 5.34% chia seed oil (4), three chia diets contained equal amounts of ALA. At the end of the experiment, the animals were fasted for 4 hours. The group that received chia seed oil in the diet had reduced food intake. The authors noted that chia decreased the triacylglycerol content of the whole chia seed diet (2) and increased HDL in the chia seed diet (3) compared to the control diet. In addition, all diets showed an increase in plasma ALA, EPA and DHA and an improvement in the n-6 / n-3 ratio in rats compared to the control group.

Valenzuela *et al.* (2014) reported the accumulation of ALA, EPA, DHA in phospholipids extracted from erythrocytes, liver, kidneys, small intestine, heart, quadriceps muscle and brain in rats with different diets. 26 male Wistar rats 3 weeks age were selected for the experiment. 5 experimental groups of 12 individuals received an isocaloric diet for 21 days, consisting of 10% fat, 20% protein and 70% carbohydrates. The fat content was provided by vegetable oils with different ALA content - sunflower (1%), canola oil (10%), Rose canina (33%), Sacha Inchi (48%) and chia seed oil (63%) on 100 g diet. After overnight fasting, animals were sacrificed, and tissue samples taken. The final weight of the animals showed no significant differences. The groups in the diet containing 49 and 64% ALA showed a higher accumulation of ALA, EPA and a decrease in the content of arachidonic FA in all tissues except the brain, suggesting that that excess ALA consumption is not converted into long-chain FA and is not subjected to beta-oxidation, but accumulates in membrane phospholipids. The chia and Sacha Inchi groups also increased DHA levels in liver, small intestine, and brain tissue. The content of the main FA groups in the brain did not change regardless of the fatty component of the diet. The increased DHA content in the brain is explained by the authors by two possible factors: selective transport of these FA from the liver using lysophospholipids or the effective bioconversion of FA supplied with food into long-chain FA, which occurs in glial astrocytes.

Another research Fonte-Faria *et al.* (2019) determined the effect of chia seed oil on body composition and insulin signaling in the skeletal muscle of obese mice. In the experiments, male C57 BL / 6 N mice were used, 3 groups of 8 animals with diets - control and high-fat (135 days), high-fat diet with the introduction of chia seed oil (1.5 g - about 12.8%) (from 90 – to 135 days). After weaning (21 day), the animals received a high-fat diet (up to day 90). Changes in body composition, such as decreased fat accumulation and increased muscle / lean mass were observed in animals receiving chia seed oil. Moreover, obese mice treated with chia seed oil showed improved insulin pathway signaling in skeletal muscle, higher levels of tyrosine Tyr989 phosphorylation of insulin receptor substrate-1, activation of protein kinase B, and increased

translocation of glucose transporter type 4 GLUT4 (glucose transport via cell membranes) into skeletal muscle tissue in response to insulin. Chia seed oil improved glucose levels and insulin tolerance, and also decreased fasting serum insulin levels, compared to the high-fat group. There was also a decrease in serum leptin and triacylglycerides, but no change in total cholesterol. Serum HDL cholesterol was higher than in the high-fat diet group. In the chia group, increased accumulation of EPA and DHA in the liver (8.74% DHA compared to 7.44% in control and 7.88% in HFD) was observed. ALA is the most abundant omega-3 FA in adipose tissue (about 1%) along with only traces of EPA and DHA and has its own value in the human body. In obesity, it is possible that ALA promotes the redistribution of lipids in the abdominal area with an improvement in glucose tolerance, insulin sensitivity, cardiovascular system parameters, as well as a decrease in dyslipidemia. Despite these similarities with effects of EPA and DHA, they do not affect glucose tolerance. Inflammatory markers in adipose tissue and plasma did not change in animals on a high-fat diet, which may be coherent with recent data suggesting that insulin resistance caused by a high-fat diet leads to inflammation, and not the contrary.

Studies have been conducted on the individual role of omega-3 FA, where Poudyal *et al.* (2013) compared the effect of individual FA (ALA, EPA, and DHA) on the cardiovascular system, liver, and metabolism. The experiment used rats with metabolic syndrome induced by a high-fat diet, 96 male Wistar rats aged 9-10 weeks. The animals were divided into 8 groups of 12 animals with diets based on - corn starch (1), corn starch with added chia seed oil (2), corn starch with added EPA oil (3), corn starch with added DHA oil (4), high-fat, high-carbohydrate diet (5), high-fat, high-carbohydrate diet with added chia seed oil (6), high-fat, high-carbohydrate diet with added EPA oil (7), high-fat, high-carbohydrate diet with added DHA oil (8). Oil in a dosage of 30 ml (3%) was added to the main diet instead of an equivalent amount of water. Diets supplemented with omega-3 were introduced for 8 weeks, after 8 weeks of a starch-based diet or a high-fat, high-carbohydrate (25% fructose) diet, respectively. The FA composition of plasma, adipose tissue, liver, heart, and skeletal muscles was studied. The authors noted that the same doses

of these FA have a different effect on reducing the risks of metabolic syndrome. Including ALA did not reduce total body fat but caused a redistribution of lipids in the abdomen and improved glucose tolerance, insulin sensitivity, dyslipidemia, hypertension and left ventricular size, contractility, volume, and stiffness. In addition, there was an increase in DHA concentration. EPA and DHA increased sympathetic nervous system activation, decreased abdominal obesity and total body fat, decreased insulin sensitivity, hypertension, and left ventricular stiffness, but had no effect on glucose tolerance. All three FA reduced inflammation in the heart and liver, as well as cardiac fibrosis and hepatic steatosis. These effects are interrelated with the suppression of the activity of stearoyl-CoA desaturase 1. Thus, according to the authors, ALA has a physiological role of its own, unrelated to its conversion to DHA.

A systematic list of the above mentioned studies can be found in the table below (Table 3).

**Table 3.** Chia (*Salvia hispanica* L.) seeds and chia seed oil *in vivo* research: summary

Source	Study purposes	Experimental design	Application form and doze of chia	Study duration	Main experimental results
Han <i>et al.</i> (2020)	The effect of chia seed oil on hyperlipidemia and oxidative stress in mice induced by a high fat diet	Male Kunming mice  6 groups (of 10 animals):  - control with normal diet - fat content 4% - 50 animals with a high fat diet (20%); in 8 weeks divided for 5 groups: the same diet + 1 group with simvastatin as positive control (5 mg/kg) or chia seed oil in different concentrations	Chia seed oil via gavage, different concentrations – 950, 1900 and 3800 mg/kg	8 weeks of preparation + 5 weeks of treatment	Chia seed oil supplements ↓ body weight, the level of lipids, total and LDL cholesterol, and serum triglyceride  ↓ liver index, spleen and fat index  ↓ atherosclerosis index  ↑ the activity of 2 enzymes involved in reducing oxidative damage  ! Chia seed oil can improve liver fat metabolism, even if the animal is maintained on a high-fat diet
de Souza <i>et al.</i> (2020)	The effect of chia seed oil on browning of white adipose tissue	Male Swiss mice  4 groups (of 7 animals):  - control with 13% fat in diet - high fat diet (45% fat), from day 90 or day 21 + chia seed oil	Chia seed oil (1.5%, w:w)	130 days (18.5 weeks)	↓ weight gain  In group day 21 - ↓ plasma leptin, ↑ glucose metabolism  ! In both chia groups process of browning adipose tissue was activated - > ↓ insulin resistance, ↑ glucose tolerance
da Silva Marineli <i>et al.</i> (2015)	The effect of chia seeds and oil on plasma and liver oxidative status in diet-induced obese rats	Wistar rats  6 groups (of 6 animals):  - control with standard diet (12% protein) - group with high fat-high fructose diet - 2 chia seed groups (6 or 12 weeks) - 2 chia oil groups (6 or 12 weeks)	Ground chia seeds (13.3%, w:w) or chia oil (4%, w:w)	12 weeks	Neither chia seed nor oil consumption had any effect on weight gain  Chia oil or chia seed group showed ↓ oxidative stress  Chia seed or chia seed oil ↑ endogenous antioxidant protection

Source	Study purposes	Experimental design	Application form and doze of chia	Study duration	Main experimental results
		For groups chia 6 weeks first 6 weeks animals received high fat-high fructose diet			
da Silva Marineli <i>et al.</i> (2015b)	The effect of chia seeds and seed oil on the stress proteins, heat shock proteins (HSP) and related factors in obese rats	<p>Wistar rats</p> <p>6 groups (of 8 animals):</p> <ul style="list-style-type: none"> <li>- control with standard diet (12% protein)</li> <li>- group with high fat-high fructose diet</li> <li>- 2 chia seed groups (6 or 12 weeks)</li> <li>- 2 chia oil groups (6 or 12 weeks)</li> </ul> <p>For groups chia 6 weeks first 6 weeks animals received high fat-high fructose diet</p>	Ground chia seeds (13.3%, w:w) or chia oil (4%, w:w)	12 weeks	<p>No difference in body weight or the accumulation of fatty tissue in the abdominal area in chia groups</p> <p>↑ glucose and insulin tolerance</p> <p>In chia oil groups expression of HSP70 and HSP25 in skeletal muscle as well as SOD and glutathione peroxidase were restored</p> <p>6 weeks chia seed group led to only ↑ in HSP70 expression and ↓ HSP60</p> <p>12 weeks chia seeds or 6 weeks chia seed oil showed ↑ expression of the PGC-1α</p> <p>Hyperinsulinemia remained unchanged</p> <p>Chia seed oil ↓ the oxidative stress induced by the HFF diet, more so than chia seeds</p>
Gallegos <i>et al.</i> (2018)	the effect of consumption of ALA from chia seed oil in combination with anthocyanins from purple corn on the PPAR-α and SREBP-1c gene expression, desaturase δ-5 and δ-6, their activity in the liver	<p>Female Sprague-Dawley rats</p> <p>4 groups (of 9 animals):</p> <p>(isocaloric and isoproteic diet)</p> <ul style="list-style-type: none"> <li>- control with olive oil</li> <li>- group with chia seed oil</li> <li>- group with olive oil combined with purple corn extract</li> </ul>	Chia oil (20% of dietary energy) via cannulas	-	<p>↑ activity of the PPAR-α gene expression when consuming chia seed oil, as well as chia seed oil in combination with purple corn extract</p> <p>↑ expression of the SREBP-1c gene and δ-5 desaturase enzymes in chia seed oil group</p>

Source	Study purposes	Experimental design	Application form and doze of chia	Study duration	Main experimental results
	and changes in its lipid profile	- group with chia seed oil combined with purple corn extract			↑ content of ALA and long-chain FA in the liver in chia seed oil group
Santos-López <i>et al.</i> (2017)	The effect of chia seed oil in an aged rat model of non-alcoholic steatohepatitis	Wistar rats (1 y.o)  4 groups (of 6 animals):  - control restructured pork, high-saturated fat with no added cholesterol - cholesterol-enriched high saturated fat/high cholesterol control restructured pork diet - chia- enriched high saturated fat/high cholesterol diet - hydroxytyrosol cholesterol- enriched high saturated fat/high cholesterol diet	Chia seed oil added to restructured pork (152.2 g / kg fresh matter)	8 weeks	Chia seed oil can prevent oxidative damage and alter the inflammatory response by regulating the antioxidant system
Mohamed <i>et al.</i> (2020)	The effect of chia seed oil and mucilage as anti-inflammatory nutraceuticals on obesity and arthritis	Male Sprague-Dawley rats  8 groups (of 6 animals):  - control group on a balanced diet - control group with arthritis - group with arthritis, and the introduction of chia seed oil - group with arthritis and the introduction of chia seed mucilage - control group with obesity - control group with obesity and arthritis - group with obesity, arthritis and the introduction of chia seed oil - group with obesity, arthritis and chia seed mucilage	Chia seed oil (300 mg / kg) in the form of emulsion or chia seed mucilage (100 mg / kg) dissolved in water	11 weeks	Chia seed oil and mucilage (mainly oil) ↓ the degree of paw swelling in all groups of rats with arthritis  ↓ TNF-α  ↑ lipid profile
Ayerza & Coates (2007)	The effect of various forms of chia seeds or	Wistar rats	Whole chia seeds 16%, ground chia	30 days	↓ food intake in chia oil group

Source	Study purposes	Experimental design	Application form and doze of chia	Study duration	Main experimental results
	chia seed oil on the plasma composition	4 groups (of 8 animals):  (isocaloric diet)  - corn oil group - whole chia seeds group - ground chia seeds group - chia seed oil group	seeds 16% or 5.34% chia seed oil		↓ TAG content in whole chia group  ↑ HDL in ground chia diet All chia diets showed ↑ in plasma ALA, EPA and DHA and an improvement in the n-6 / n-3 ratio in rats compared to the control group
Valenzuela <i>et al.</i> (2014)	The study of accumulation of ALA, EPA, DHA in phospholipids extracted from erythrocytes, liver, kidneys, small intestine, heart, quadriceps muscle and brain in rats with different diets	Male Wistar rats  5 groups (of 12 animals):  (isocaloric diet)  - sunflower oil group - canola oil group - Rose canina oil group - Sacha Inchi oil group - chia seed oil group	ALA content: sunflower (1%), canola oil (10%), Rose canina (33%), Sacha Inchi (48%) and chia seed oil (63%) on 100 g diet	21 days	The groups with the highest ALA content in the diet showed a higher accumulation of ALA, EPA and a decrease in the content of arachidonic FA in all tissues except the brain  The chia and Sacha Inchi groups also ↑ DHA levels in liver, small intestine, and brain tissue
Fonte-Faria <i>et al.</i> (2019)	The effect of chia seed oil on body composition and insulin signaling in the skeletal muscle of obese mice	Male C57 BL / 6 N mice  3 groups (of 8 animals):  - control diet group - high-fat diet group - high-fat diet with the introduction of chia seed oil group (from day 90)	Chia seed oil (1.5 g - about 12.8%)	135 days	↓ fat accumulation  ↑ muscle / lean mass  ↑ glucose levels and insulin tolerance  ↓ fasting serum insulin levels  ↑ accumulation of EPA and DHA in the liver
Poudyal <i>et al.</i> (2013)	The effect of individual FA (ALA, EPA, and DHA) on the cardiovascular system, liver, and metabolism	Male Wistar rats  8 groups (of 12 animals):  - corn starch group	Oil (30 ml or 3%)	16 weeks	ALA: No reduction in total body fat  Caused a redistribution of lipids in the abdomen

Source	Study purposes	Experimental design	Application form and doze of chia	Study duration	Main experimental results
		<ul style="list-style-type: none"> <li>- corn starch with added chia seed oil group</li> <li>- corn starch with added EPA oil group</li> <li>- corn starch with added DHA oil group</li> <li>- high-fat, high-carbohydrate diet</li> <li>- high-fat, high-carbohydrate diet with added chia seed oil group</li> <li>- high-fat, high-carbohydrate diet with added EPA oil group</li> <li>- high-fat, high-carbohydrate diet with added DHA oil group</li> </ul>			<p>↑ glucose tolerance, insulin sensitivity, dyslipidemia, hypertension and left ventricular size, contractility, volume, and stiffness.</p> <p>↑ in DHA concentration</p> <p>EPA and DHA:</p> <p>↑ sympathetic nervous system activation</p> <p>↓ abdominal obesity and total body fat</p> <p>↓ insulin sensitivity, hypertension, and left ventricular stiffness</p> <p>No effect on glucose tolerance</p> <p>All three FA ↓ inflammation in the heart and liver, as well as cardiac fibrosis and hepatic steatosis</p>

Little information is available on potential neuroprotective effects of chia seeds. It has been reported that risk of dementia correlated with low ALA concentration in plasma (Hussain *et al.*, 2019), and long-term ALA consumption improved cognitive function (Lee *et al.*, 2017).

Two studies using either chia seed powder or ground chia seeds as dietary supplements either did not detect any significant effect on cognitive in an accelerated aging mouse model (Rui *et al.*, 2018) or observed detrimental effects in an aluminum chloride-induced AD mouse model (Bilgic *et al.*, 2018). However, these studies either lacked the appropriate control conditions, or did not use chia seed oils or extracts.

Human consumption of chia seeds does lead to increasing ALA levels in plasma of postmenopausal women and overweight adults (Rui *et al.*, 2018). Interestingly, a recent study by Lee *et al.* (2017) demonstrated that ALA from oil derived from the perilla plant, which is from the same family as chia, reduced effects of the toxic A $\beta$ -peptide potentially due to anti-oxidative effects and modifications in its production, which is involved in AD development.

As previously reported, chia seeds also rich in tocopherols and polyphenols, which may be useful to maintain in chia-derived dietary products because of their anti-oxidative properties which are potentially of interest in reducing AD risk.

Thus, chia-derived ALA-rich products may represent a useful ingredient in the prevention or reducing risk of neurodegenerative disorders and other lipid-related pathologies.

## **1.7 METHODS OF CHIA SEED LIPIDS EXTRACTION**

Today, the most common methods for extracting vegetable oils are cold and hot pressing, and extraction with organic solvents. Interestingly, the Spanish sage seed oil (*Salvia hispanica* L.) was admitted to the European market as a food product only in 2014 (chia seeds entered the EU market in 2009 and permission was extended in 2013), with the caveat that the oil can be obtained only cold pressing (Commission EU, 2014). Thus, many researchers use cold-pressing method to

obtain chia seed oil (Ixtaina *et al.*, 2011; Martínez *et al.*, 2012; Noshe & Al-Bayyar, 2017; Özcan *et al.*, 2019 et 2019b).

Even though the oil obtained by pressing, especially cold oil, is considered to be of the highest quality, from the point of view of industrial production the main difficulty is the low oil yield, so extraction with organic solvents remains one of the most common methods (Ixtaina *et al.*, 2011; Martínez *et al.*, 2012; Silva *et al.*, 2016; Arişanu *et al.*, 2017; Noshe & Al-Bayyar, 2017). In several industries, oil is obtained from oilseeds in two stages where the raw materials are pressed, followed by extraction of the obtained meal with organic solvents.

One of the most used methods in obtaining the lipid fraction from chia seeds is extraction using a Soxhlet apparatus using various solvents, such as n-hexane, petroleum ether and others (Ixtaina *et al.*, 2011; Noshe & Al-Bayyar, 2017; Özcan *et al.*, 2019b). Conventional Soxhlet extraction, known since 1879, is based on recirculation of extractant through the sample. This system has some advantages: the sample is in multiple contact with fresh portions of extractant; no filtration is needed; low cost of the basic equipment and simple methodology. Long extraction times, large amounts of solvent and possible thermal decomposition of samples because of temperature factor over time, solvent limitations and difficult automatization process are the major drawbacks of this classical technique (De Castro & Priego-Capote, 2010).

Another commonly used lipid extraction method is the Folch method (Folch *et al.*, 1957), which uses a 2:1 mixture of chloroform and methanol. However, little information is available on this solvent mixture for chia seeds (Ciftci *et al.*, 2012; Ganzaroli *et al.*, 2017; Souza *et al.*, 2017b), despite a number of advantages regarding the more complete extraction of polar lipids relative to other methods.

The study of various techniques for producing and extracting chia seed oil and its biologically active components, such as vitamins, antioxidants, sterols, and others has been a subject of interest in recent years. The main trend in the oil and fat industry today is promoting

a “green economy”, in which the extraction solvents and methods used must be effective but have minimal impact on the environment. To obtain chia seed oil, different technologies were evaluated and compared including ultrasonic assisted extraction, or liquid extraction under pressure (Rosas-Mendoza *et al.*, 2017; Scapin *et al.*, 2017; Fernandes *et al.*, 2019; Villanueva-Bermejo *et al.*, 2019).

The use of organic solvents on an industrial scale, despite their widespread use in production, also has several disadvantages, including the difficulty of removing solvent at the end of the extraction process, the use of non-renewable resources, the need for precautions related to the high volatility and flammability of reagents, their toxicity, and utilization of processed products and possible impact on the composition of the final product.

Of great interest today are methods of supercritical CO<sub>2</sub> extraction, that has been used to obtain the lipid fraction of Spanish sage seeds (Ixtaina *et al.*, 2010; Uribe *et al.*, 2011; Labanca *et al.*, 2017; Calvo *et al.*, 2020; Villanueva-Bermejo *et al.*, 2020). Supercritical extraction is considered as “green technology”, since it does not have the restrictions imposed on the residues of organic solvents in the finished product. In addition, it is considered non-toxic with fewer by-products, lower energy costs compared to solvent extraction, and the ability to obtain products with special properties (Knez *et al.*, 2019).

However, methods based on liquid extraction also have a number of disadvantages, including the low efficiency and complexity of the extraction, the possible modification of the initial composition of the studied substances due to solvent impurities and possible losses, and in some cases, a high environmental load due to pollution of large volumes of water and low selectivity (Glazkov *et al.*, 2006). The use of supercritical extraction, despite a number of advantages, also has its drawbacks, including the selective extraction of components, the complexity of the equipment used under high pressure, and high investment costs, as well as in some cases quite high temperatures (up to 90°C), which are critical when working with the lipid fraction. When using this method with high-oil seeds, a fairly good yield is noted, however, with

lower taste and aromatic quality in comparison with traditional methods. In addition, the cost of the obtained supercritical extracts is quite high, and it is also difficult to organize continuous processing of large volumes in production (Kasyanov *et al.*, 2005; Arişanu *et al.*, 2017; Knez *et al.*, 2019).

## **1.8 LIPID-BASED MICRO- AND NANOVESICLES AND DRUG DELIVERY**

The high levels of unsaturated FA and the peculiarity of the chemical structure of PUFA contribute to the instability of chia seed oil to external stress factors. Today, an urgent issue is both the preservation of the properties of vegetable oils, as well as the development of systems for delivery of essential lipid components while maintaining their quality.

According to a review by Timilsena *et al.* (2017 and 2020), today 2 main approaches are used to minimize the processes of deterioration of vegetable oils - the introduction of natural or synthetic antioxidant systems or microencapsulation of oils to minimize direct contact with environmental stress factors. In the latter approach, wall materials used include proteins, lipids (including phospholipids) and polysaccharides, or a combination of these components. In addition, with this method, characteristics of the encapsulated substance can be hidden such as an undesirable taste, color, or odor. The type of encapsulation can be used to ensure controlled delivery and/or to increase bioavailability of the molecule of interest. The first successful encapsulation was reported in 1927, using acacia gum and spray drying. The main methods for obtaining microcapsules include spray and freeze drying, fluidized bed coating, centrifugal extrusion, inclusion complexation, complex coacervation, ionotropic gelation, electrospray, and liposomal capture.

The use of these and other techniques has been studied in recent years in relation to chia seed oil. An example of this is a recent study by Lehn *et al.* (2018), chia seed oil was

microencapsulated using spray drying. The emulsions were formed using different wall materials, including pure whey, whey permeate, gum arabic and soy lecithin. The resulting emulsions were dried, and he obtained microcapsules of chia seed oil had different sizes particles with hemispherical shapes without agglomeration with sizes ranging from 15 to 50  $\mu\text{m}$ . According to the authors, the effectiveness of encapsulation depends on the type of oil used and is also related to the degree of unsaturation of the oil. For chia seed oil, the figure ranged from 43% to almost 57%, depending on the wall material used. With higher encapsulation values, the amount of free oil is reduced, which has a positive effect on storage, as indicated by lower peroxide levels even after 30 days of storage at room temperature (25°C).

Microparticles with chia seed oil have been obtained using Maillard reaction products with different protein / carbohydrate ratios (Copado *et al.*, 2017). In this study, the particles were obtained by freeze-drying an oil-in-water emulsion containing sodium caseinate and lactose and chia seed oil at various concentrations. A number of emulsions were heated to 60°C at the stage of preparing a protein-carbohydrate mixture to obtain the products of the Maillard reaction. The encapsulation efficiency in the study ranged from 41 to 84%; the particle size varied depending on the method used from 0.1 to 10  $\mu\text{m}$  in the case of heating and up to 239  $\mu\text{m}$  without preliminary heat treatment. Heat treatment of the samples increased the oxidative stability. In addition, the microcapsules were similar in composition to the untreated oil. It is noted that all the formulations obtained have high rehydration properties.

In a study by Encalada *et al.* (2020) considered a method for preparing an emulsion based on chia seed oil and using a pectin-rich food additive from carrots as a source of antioxidants (carotenoids) and a gelling agent. The resulting emulsion showed high storage and oxidative stability at 25°C for 45 days.

Conventional oil-in-water emulsions were one of the first systems believed to deliver biologically active lipids due to their relative ease of preparation and low cost. The question of

formulating microemulsions using the lipid fraction of Spanish sage seeds and various materials as an emulsifier and microcapsule wall material, as well as several techniques used, has been studied quite extensively (Julio *et al.*, 2015; González *et al.*, 2016; Julio *et al.*, 2016; Timilsena *et al.*, 2017b; Guimarães-Inácio *et al.*, 2018; Julio *et al.*, 2018; Us-Medina *et al.*, 2018; Alcântara *et al.*, 2019). However, the use of chia seed oil for particles of nanoscale remains yet almost unexplored (de Campo *et al.*, 2017; Teng *et al.*, 2018), despite the increased interest in nanotechnology in recent years.

Research by de Campo *et al.* (2017) focused on chia seed oil nanocapsules using chia seed mucilage as wall material. Nanoparticles with high stability for 28 days at 40°C were obtained using the surfactant Tween 80 and subsequent homogenization. Interestingly, the experiment used unrefined mucilage, which also included minor components such as proteins, lipids, and inorganic elements. The authors believe that the presence of proteins in the composition facilitate the emulsification process, and the high percentage of dietary fiber in the mucilage increases the efficiency of encapsulation and the stability of the system. The average particle size of these particles was 205 nm and the charge was in the range of -12 mV, with an encapsulation efficiency of about 83%. The authors note that the resulting nanoparticles showed good thermal stability at 300°C, and a higher oxidation stability as compared to unencapsulated chia seed oil.

In the industry, the use of nanoemulsions with a particle size of less than 1 µm as delivery systems for the necessary lipophilic substances is of interest particularly with the possibility of enrichment and increasing the biological value of food products without negatively affecting traditional organoleptic characteristics. In addition, nanoemulsions can be added to translucent and transparent products, as they often have a neutral color and high transparency. They have high stability to gravitational changes due to nanoscale (Teng *et al.*, 2018).

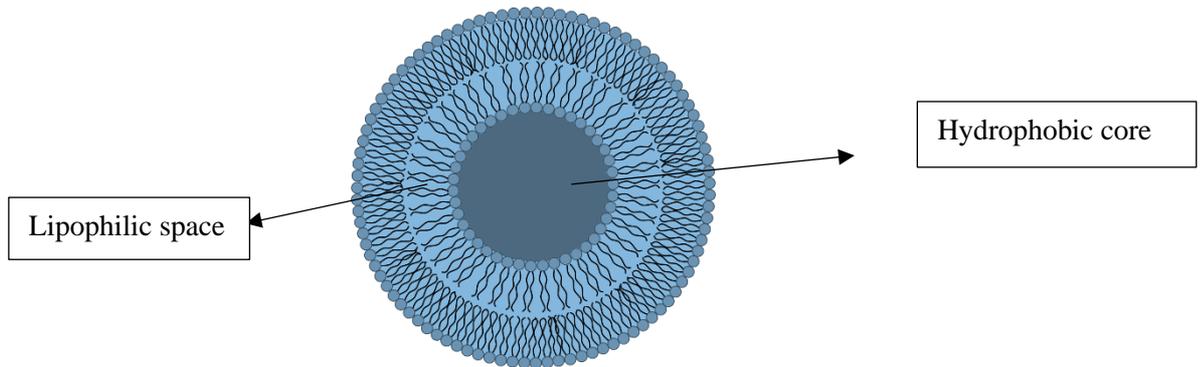
Despite a number of advantages compared to traditional vegetable oils, the use of emulsions has a significant drawback due to the fact that only lipophilic molecules can be

encapsulated in this delivery system. This factor significantly limits the use of micro- and nanoemulsions as vector systems and leads to the need for additional research and the complexity of the technology using the variability of techniques and components to wide their properties.

Besides emulsification, other methods have been used to obtain microparticles from chia seed oil as delivery systems for essential PUFA. Di Marco *et al.* (2020) studied the properties of amylose-lipid complexes with a high content of omega-6 and omega-3 FA (about 3%) based on corn starch and chia seed oil as potential PUFA delivery systems. The study used PUFA obtained by enzymatic hydrolysis of chia seed oil.

Gañan *et al.* (2020) used soy protein as a wall material to encapsulate chia seed oil. In this study, the authors relied on a method to impregnate microparticles with soy protein-based chia seed oil using supercritical carbon dioxide as a green technology. The resulting chia seed oil particles were 1 to 10  $\mu\text{m}$  in size with a uniform oil distribution. According to the results of experiments, the oil quality did not change using this technology, and the results of stability studies for 120 days showed a high protective effect of microencapsulation against oxidative damage compared to oil without treatment. In addition, as part of an *in vitro* experiment, it was determined that up to 96% of the oil was released in a system mimicking gastrointestinal conditions, mainly at the intestinal stage, while remaining available for absorption.

Today, an alternative way for delivery of PUFA while preserving these FA from oxidation may be through the use of liposomes. Liposomes are artificial lipid particles with a lipid membrane that can encapsulate, protect, and deliver active molecules such as proteins, nucleic acids, and other biological active and drug substances. Liposomes can be formed from various lipid classes; however, phospholipids are the main ingredient for their formation. These particles are biocompatibility, which can be influenced by their composition. Their ability to encapsulate both hydrophilic and lipophilic types of active molecules makes them ideal vectors for the delivery of drugs and other biologically active substances.



**Figure 6.** Nanoliposome approximate structure

Nanosized liposomes are multilamellar and have a large surface area, thus increased potential for solubility, bioavailability, improved controlled release, and more accurate targeting (Danaei *et al.*, 2018).

Currently, studies about nanoliposomes from chia seed lipids are lacking. We have in our laboratory developed the technology enabling the preparation of PUFA-rich nanoliposomes prepared from phospholipid rich extracts (Arab-Tehrany *et al.*, 2012) that can be used as vectors for delivery and applied for chia seed-derived lipids.

## 1.9 MAIN OBJECTIVE AND SPECIFIC AIMS OF THESIS RESEARCH

In view of the above detailed study of existing research in the field of chia seeds and chia seed lipids, as well as identifying possible niches for research, and based on the scientific expertise of ITMO University, LIBio and QUALIVIE-URAFPA, we established the following objective and specific aims for this thesis project “Development and characterization of chia (*Salvia hispanica* L.) plant-derived products as natural bioactive compounds”:

**The main objective** of the thesis is to evaluate bioavailability of chia seed lipid (ChSL) extracts in different forms and to assess their bioactivity focusing on potential treatment of lipid-related disorders and associated diseases.

For this project, we chose to use chia seeds (*Salvia hispanica* L.) of the ORURO variety that have been adapted for growth and cultivation in France. Due to the fact that biologically active substances can vary depending on the region of growth, it is of particular importance to evaluate chia seed, particularly lipid composition for this variety.

**The specific aims** for this thesis are:

**Aim 1** – Extraction and characterization of ChSL;

**Aim 2** – Preparation and physicochemical characterization of NE and NL using ChSL;

**Aim 3** – Studies of bioavailability and bioactivity of chia seed-derived NE and NL using cell models (hepatoma cells, astrocytes, and neurons).

In accordance with the main objective and specific aims set, materials and research methods were selected, which you will find in the next chapter.

## CHAPTER II. MATERIALS AND METHODS

### 2.1 MATERIALS

We chose to study the ORURO variety of chia seeds, which is a new genotype bred in France in 2017, adapted to the climatic conditions of the region (Gravé *et al.*, 2019). ORURO chia seeds (*Salvia hispanica* L.) were purchased from the producer Agrofün, Occitania, France.

Purified rapeseed lecithin (Solae Europe SA society, Geneva, Switzerland), rapeseeds (*Brassica napus* L.) (NATUR-Haus Jurgen Kaiser, NK-Wiebelskirchen, Germany) and flax seeds (*Linum usitatissimum* L.) (Graines de Folie, La E Penell, Normandy, France) were used as for oil extractions (seeds) or NL preparation (rapeseed lecithin).

Ammonium molybdate, internal standard nonadecanoic acid (C19), boron trifluoride diethyl etherate and methanol were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Formic acid and toluene were obtained from Fluka (Charlotte, NC, USA) and VWR International (Villers-lès-Nancy, France), respectively. Chloroform, hexane, diethyl and petroleum ethers were purchased from Biosolve (Dieuze, France).

#### 2.1.1 CELLS

Hepa 1-6 mouse hepatoma cell line was obtained from ECACC (European Collection of Authenticated Cell Cultures, Sigma Aldrich). Hepa 1-6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v:v) decomplexed fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin. Plating conditions were 300 000 cells/well for 6-well plates, and 60 000 cells/well for 24-well plates.

Murine astrocytes expressing human isoforms ApoE2, E3, and E4, were a kind gift courtesy of Dr. D. Holtzman (Morikawa *et al.*, 2005). Cells were grown in advanced DMEM containing 10% (v:v) decomplexed FBS, 1 mM sodium pyruvate and 0.2 mg/mL geneticin.

Primary cultures of rat embryonic cortical neurons were prepared as previously described (Malaplate *et al.*, 2019).

Cell culture media, FBS, DMEM, advanced DMEM, geneticin, were obtained from Gibco Life Technologies (Thermo Fisher Scientific). FBS was decomplexed by incubating at 56°C for 30 min.

## **2.2 METHODS**

### **2.2.1 PHYSICO-CHEMICAL ANALYSIS OF ORURO CHIA SEEDS**

#### **2.2.1.1 MOISTURE CONTENT**

The moisture mass fraction in samples was determined by drying method at a constant temperature of  $105 \pm 1^\circ\text{C}$  for 24 h (Wang *et al.*, 2007).

#### **2.2.1.2 ASH CONTENT**

In the ashing process, porcelain crucibles were heated at a constant temperature of  $103^\circ\text{C}$  to constant mass, cooled in a desiccator and pre-weighed. A sample of ground chia seeds (3 g) was weighed into crucibles. Prepared samples were placed in a muffle furnace heated to  $500^\circ\text{C}$  for 12 h. The furnace was turned off until completely cooled, followed by reheating the crucibles

with samples at a temperature of 103°C to ensure the removal of any residual moisture, cooled in a desiccator and weighed.

### 2.2.1.3 PROTEIN CONTENT

The total nitrogen content in samples was determined by the Kjeldahl arbitration method (Bradstreet, 1954). This method is based on the process of converting organic nitrogen to inorganic in the ammonia form  $(\text{NH}_4)_2\text{SO}_4$  during oxidation by boiling sulfuric acid in the presence of a catalyst.

Determination of total nitrogen in samples was carried out in 3 stages. Ground samples (0.2 and 0.5 g) were added into Gerhardt tubes. Next, 2 mL of concentrated sulfuric acid (96%) in the presence of a PanReac catalyst (Cu-Se) was added to the samples using an automated pump. Samples were placed in a Kjeldahltherm system (Gerhardt, Les Essarts le Roi, France) for mineralization for 2.5 h at 400°C. Then the system was turned off and after 20 min mineralized samples containing mainly ammonium sulfate were saved for distillation and titration steps.

The second step was preliminary titration of a control empty Gerhardt tube on a Vapodest 50 distillation apparatus (Gerhardt, Les Essarts le Roi, France) in automatic mode.

In the distillation system, sodium hydroxide (32%) was added to the tubes in an amount equal to the volume of sulfuric acid used for mineralization, to initiate the following reaction:



Next, the released ammonia combines with water steam to form ammonium hydroxide ( $\text{NH}_4\text{OH}$ ), which is captured by steam and transported to a solution of boric acid (3%) for formation of ammonium borate ( $\text{NH}_4)_3\text{BO}_3$ .

In the third step, the solution is automatically titrated with 0.01 N sulfuric acid. The volume of sulfuric acid (mL) required for titration, corresponds to the amount of ammonia contained in the flask at the beginning, which represents the amount of nitrogen in the mineralized fraction.

The control sample was titrated until three consecutive ( $\pm 0.1$  mL) volumes of sulfuric acid were obtained to evaluate the average volume. Then, titration of the samples was carried out. The mass fraction of protein (%) in 100 g of sample was calculated by the following formula:

$$\text{Protein content (\%)} = \frac{(V_1 - V_0) \times 0.14 \times 100}{m \times 1000} \times 6.25,$$

where  $V_1$  – volume of 0.01N sulfuric acid, obtained after titration of the sample, mL;

$V_0$  – average volume of 0.01N sulfuric acid, obtained after titration of control samples, mL;

$m$  – mass of the sample, g;

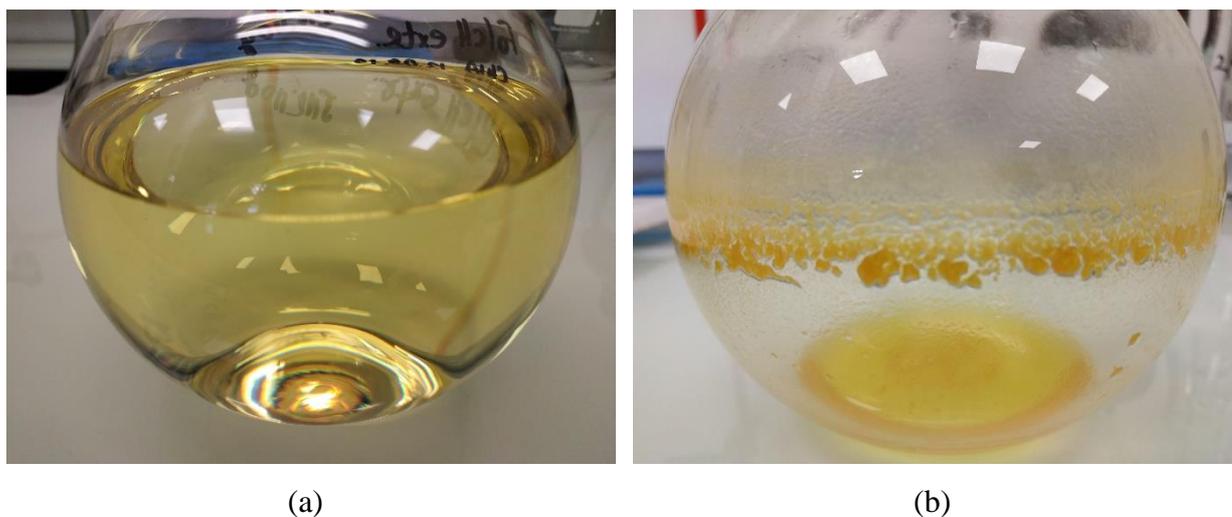
6.25 – conversion factor for total nitrogen to protein content.

#### **2.2.1.4 LIPID CONTENT**

Seed samples were ground before lipid extraction using a portable grinder (Bosh MKM 6003, BSH Electroménager, Saint-Ouen, France).

## ORGANIC SOLVENT EXTRACTION. FOLCH-BASED METHOD

Lipids were extracted using the modified Folch method (1957). Chloroform:methanol mixture (600 mL, 2:1, v:v) was added to ground chia seeds (100 g) and incubated for 30 min under constant stirring at room temperature. The resulting mixture was vacuum filtered through a funnel with a N°4 Schott filter (poresize 10-16  $\mu\text{m}$ ), while washing the resulting defatted cake with an additional amount (300 mL) of chloroform:methanol (2:1). The filtered lipid extract was added to a pre-weighed round-bottomed flask, and the solvent was removed using BÜCHI Rotavapor R-144 rotary evaporator (BÜCHI Sarl, Villebin-sur-Yvette, France) at 50°C until the solvent was completely removed. The vacuum system was interrupted by introducing N<sub>2</sub>(g) (inert atmosphere). Figure 7 shows obtained chia seed lipids before and after solvent removal.

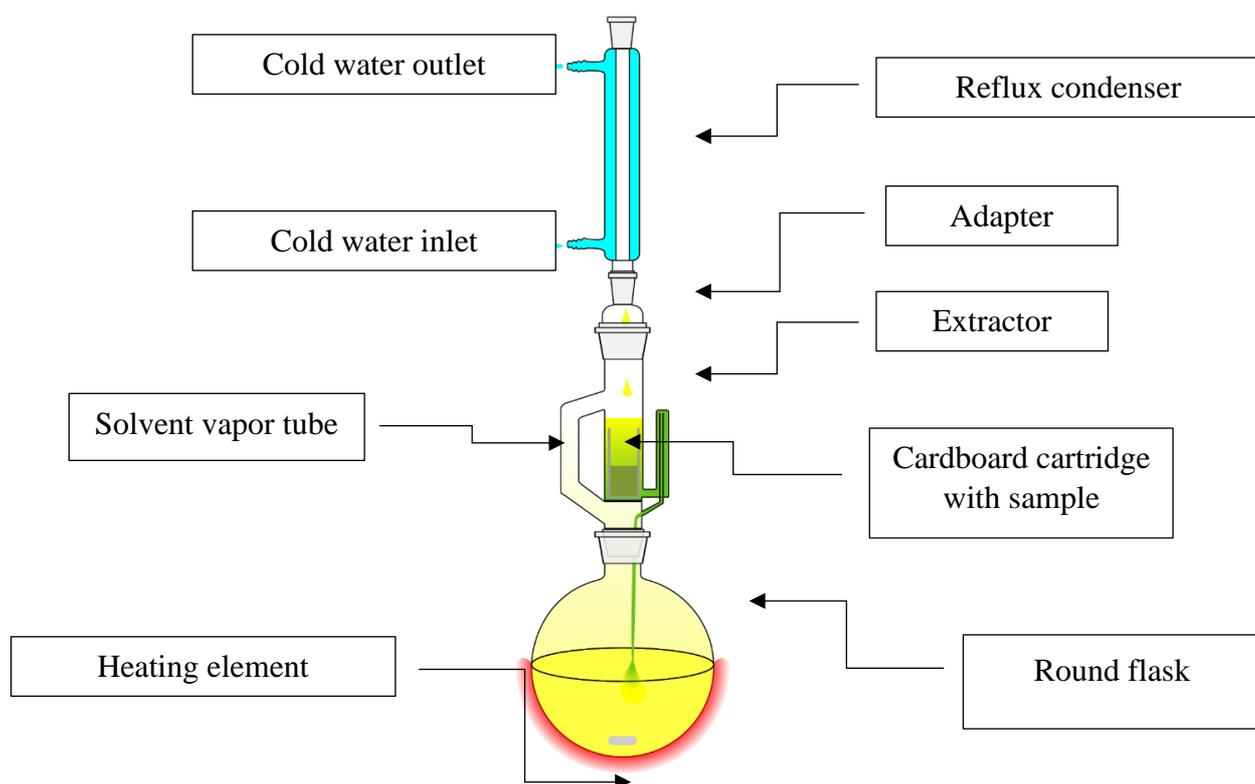


**Figure 7.** Chia seed lipids obtained by modified Folch method: (a) before solvent removal; (b) after solvent removal and residue formation

The residue remaining as deposits on the flask wall was collected with a spatula after the removal of the oil fraction. Oil and residue yields were calculated and expressed based on the initial mass of seeds and lipid mass, respectively.

## SOXHLET METHOD

Extraction of samples (10 g) was carried out using 300 mL of diethyl ether:petroleum ether mixture (1:1, v:v) (Guillemin, 2006) in Soxhlet apparatus (VWR international, Fontenay-sous-Bois, France) for 6 h. The structure of the Soxhlet apparatus is shown in Figure 8.



**Figure 8.** The structure of the Soxhlet apparatus

Extraction was performed in a fume hood at room temperature. Sample was placed in cellulose extraction thimble, grade 501, 25×100 mm (VWR International, Fontenay-sous-Bois,

France), covered with cotton and inserted in extractor. Solvent mixture heating was realized by dry bath to keep intensive boiling in the round distillation flask. The temperature of extraction was at the boiling point of the solvent. Time of extraction started from the first solvent drop failed from reflux condenser.

The solvent was removed using BÜCHI Rotavapor R-144 rotary evaporator (BÜCHI Sarl, Villebon-sur-Yvette, France) at 40°C. The vacuum was interrupted by introducing N<sub>2</sub>(g) (inert atmosphere) to avoid oxidative damage to lipids caused by exposure to air.

#### **2.2.1.5 CARBOHYDRATES CONTENT**

Mass fraction of carbohydrates was determined by the following formula (Ganzaroli *et al.*, 2017):

Carbohydrates content (%) = 100 – (moisture content + ash content + lipid content + protein content).

#### **2.2.1.6 TOTAL DIETARY FIBER CONTENT**

Total dietary fiber (TDF) content in chia solid residue obtained after Folch-based extraction were determined using a Megazyme Total Dietary Fiber Kit according to detailed manufacturer's instructions. Due to this manual, assay procedure based on AOAC 991.43, AOAC 985.29, AACC 32-07.01 methods. Used technique is applicable to cereal grains, fruit and vegetables, cereal and fruit products and foods. Determination of TDF was implemented by few steps. Main principle describes quantification on duplicate samples of dried and defatted material. In our case we used chia lipid solid residue for analysis directly. Briefly, samples (1 g) (completely dispersed in earlier prepared Megazyme buffer (pH 8.2)) and blanks (control) (with buffer) were maintained

with 50  $\mu\text{L}$  of heat-stable  $\alpha$ -amylase 30 min at  $100^\circ\text{C}$  in water bath to provide gelatinization, hydrolysis and depolymerization of starch. Then they were incubated 30 min at  $60^\circ\text{C}$  in water bath with 100  $\mu\text{L}$  protease to solubilize and depolymerize proteins. After this step 5 mL of 0.561N HCl was added and pH checked (4.1-4.8), pH was adjusted if needed using 5% NaOH or 5% HCl. Then 200  $\mu\text{L}$  amyloglucosidase were added to hydrolyze starch fragments to glucose and incubated 30 min at  $60^\circ\text{C}$  in water bath. At the next step samples were treated with 4 volumes (225 mL) of pre-heated  $60^\circ\text{C}$  ethanol during 60 min at room temperature to precipitate soluble fiber and remove depolymerized protein and glucose from starch. The rest was vacuum filtered (Pyrex N2), washed with 78% ethanol, 95% ethanol and acetone, dried ( $103^\circ\text{C}$ , 12 h) and weighted. One duplicate was analyzed for protein content by Kjeldahl method as described in section 2.2.1.3 and another one was analyzed for ash content using 5 h incubation at  $525^\circ\text{C}$ . Calculation of TDF was done by using the Megazyme Mega-Calc<sup>TM</sup>, downloadable from where the product appears on the Megazyme web site ([www.megazyme.com](http://www.megazyme.com)). In general, TDF is the weight of filtered and dried rest excepting ash and protein content.

### **2.2.1.7 TOTAL ENERGY VALUE**

The total energy value was calculated according to a conversion factor equal to 4 kcal / g for proteins and carbohydrates, and 9 kcal / g for fats (da Silva *et al.*, 2017).

### **2.2.2 CHARACTERIZATION OF CHIA SEED LIPID FRACTION**

#### **2.2.2.1 FATTY ACIDS COMPOSITION AND CHARACTERIZATION OF LIPID CLASSES**

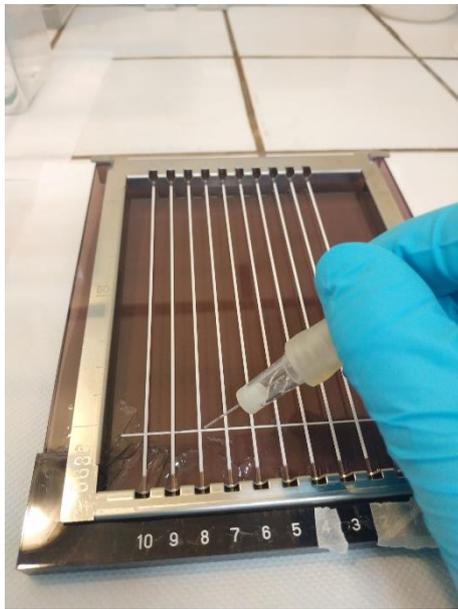
Fatty acid composition and lipid classes were characterized as described previously (Gbogouri *et al.*, 2011; Arab-Tehrany *et al.*, 2012; Hasan *et al.*, 2014; Hasan *et al.*, 2019).

Briefly, fatty acid methyl esters (FAMES) were prepared by the Ackman method (1998). First, nonadecanoic acid (19:0) internal standard solution with concentration 1 mg/mL was prepared using chloroform as solvent. Before samples esterification 1 mL of this solution was added in a glass tube as an internal standard (IS) for quantification of FA and totally evaporated by N<sub>2</sub>(g). For FAME preparation, tested sample (100 mg) was weighted in glass tube with pre-added IS, with following addition of 1.5 mL hexane and 1.5 mL of boron trifluoride (14% in methanol). Samples were vortexed 60 s and incubated 60 min at 100°C under N<sub>2</sub>(g). After tube cooling, 1 mL of hexane and 2 mL of distilled water were added, vortexed 60 s and incubated 1-2 min for phases separation. Supernatant was transferred to the second glass tube by Pasteur pipette. The rest in fist tube was washed with 1 mL of hexane, vortexed, supernatant transferred, and washing repeated one more time. Next, second tube washed with 2 mL of water, vortexed and supernatant transferred to the third glass tube, which was pre-weighted with the lid. All steps were implemented under N<sub>2</sub>(g) to preserve lipids degradation. Liquid part in third tube was evaporated during 5-10 min by N<sub>2</sub>(g). The rest FAMES were weighted, hexane was added in proportion 100 mg FAMES to 1000 µL of hexane, samples stored under N<sub>2</sub>(g) at 4°C, covered with aluminum. Before analysis samples were diluted 10 times using hexane.

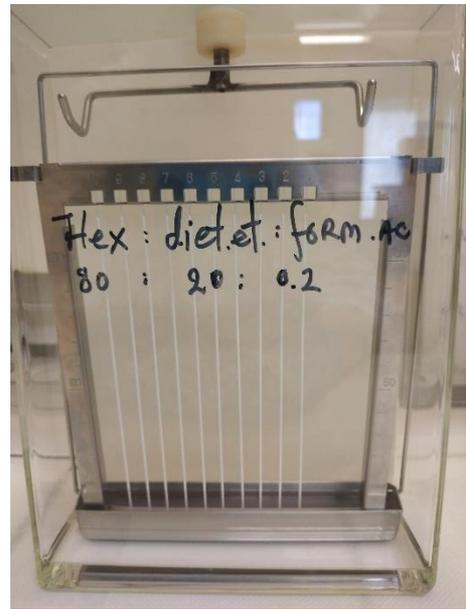
The separation of FAMES was carried out by gas chromatography on a Shimadzu GS 2010-Plus (Perichrom, Saulx-lès-Chartreux, France) equipped with a flame-ionization detector. A fused silica capillary column was used (60 m, 0.25 mm i.d. × 0.2 µm film thicknesses, SP<sup>TM</sup>2380 Supelco, Bellefonte, PA, USA). The column temperature was set initially at 120°C for 3 min, then increased to 180°C at a rate of 2°C min<sup>-1</sup> and maintained at 220°C for 25 min. Injection and detection temperatures were set at 250°C. Standard mixtures (PUFA1 from marine source and PUFA2 from vegetable source; Supelco, Sigma–Aldrich, Bellefonte, PA, USA) were used to

identify fatty acids in the elution profile. The results are shown as means of triplicate determinations.

Lipid classes of chia seed, rapeseed and flax seed extracts were determined by thin layer chromatography (Iatroscan MK-5 TLC-FID, Iatron Laboratories Inc., Tokyo, Japan) as shown on the Figure 9.



(a)



(b)



(c)

**Figure 9.** Chia lipid classes evaluation by thin layer chromatography method: (a) deposit of 1  $\mu\text{L}$  of sample on silica coated quartz rod; (b) separation of lipids by migration in hexane/diethyl ether/formic acid (80:20:0.2,  $v:v:v$ ) mixture; (c) Iatroscan MK-5 TLC-FID analyzer

Samples (1  $\mu\text{L}$ ) were applied onto Chromarod S-III silica coated quartz rods. Lipids were separated by migration in hexane:diethyl ether:formic acid (80:20:0.2,  $v:v:v$ ) for 25 min, oven dried for 1 min at 100°C and then scanned in the Iatroscan analyzer. The Iatroscan settings were set with a hydrogen flow rate of 160  $\text{mL min}^{-1}$  and air flow rate of 2  $\text{L min}^{-1}$ . The recording and integration of the peaks were obtained using ChromStar software.

#### 2.2.2.2 PHOSPHOLIPID ANALYSIS

Qualitative and semi-quantitative analyses of chia seeds phospholipids were performed on an HPLC-MS system consisting of an UltiMate 3000<sup>TM</sup> quaternary solvent delivery pump connected to a linear ion trap mass spectrometer (LTQ) equipped with an atmospheric pressure ionization interface operating in electrospray negative ion mode (ESI<sup>-</sup>) (ThermoFisher Scientific, San Jose, CA, USA).

Extracts (16  $\mu\text{L}$ ) were injected on a LiChroCART (250mm  $\times$  4mm-5 $\mu\text{m}$ ) LiChrospher 100 DIOL column (Merck, Darmstadt, Germany). The flow rate was set at 300 $\mu\text{L}\cdot\text{min}^{-1}$  and the column temperature at 30°C. Mobile phase A consisted of methanol with 0.1% ( $v:v$ ) formic acid, ammonia added to pH 5.3 (approx. 0.05%,  $v:v$  of ammonia) and 0.05% ( $v:v$ ) triethylamine; mobile phase B was chloroform. Lipids were eluted using a first linear gradient from 95% to 70% of B for 11 min, a second linear gradient to 20% of B for 3 min and an isocratic step at 20% B for 4 min. Mass spectrometric conditions were as follows for ESI<sup>-</sup> mode: spray voltage was set at -4.5 kV; source

gases were set (in arbitrary units  $\text{min}^{-1}$ ) for sheath gas, auxiliary gas and sweep gas at 40, 5 and 5, respectively; capillary temperature was set at  $230^{\circ}\text{C}$ ; capillary voltage at  $-36\text{ V}$ ; tube lens, split lens and front lens voltages at  $-133\text{ V}$ ,  $70\text{ V}$  and  $6.25\text{ V}$ , respectively. Ion optics parameters were optimized by automatic tuning using a standard solution of a phospholipids mixture at  $0.1\text{ g L}^{-1}$  infused in mobile phase (A/B: 5/95) at a flow rate of  $5\text{ }\mu\text{L min}^{-1}$ .

Full scan MS spectra (500 to 2000  $m/z$ ) allowed us to detect parent ions of general form  $[\text{M-H}]^{-}$  for all the phospholipids of interest except for phosphatidylcholine (PC) and lysophosphatidyl-choline (lyso-PC) classes for which the parent ion is  $[\text{M}+\text{HCOO}]^{-}$  due to ionic complex formation between choline group and formiate.

Data dependent  $\text{MS}^2$  and  $\text{MS}^3$  scans were carried out automatically in order to obtain fatty acid composition for phospholipid classes phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylglycerol (PG), PC and lyso-PC. In the case of PC and lyso-PC phospholipids,  $\text{MS}^3$  scans were necessary to obtain the same level of information because  $\text{MS}^2$  events were only informative of their class (through characteristic neutral loss of 60 corresponding to simultaneous departure of formiate and a methyl group of choline).

A semi-quantitative analysis of the samples was carried out for each class of phospholipids using MS screening by grouping together the main species identified in the class.

Two phospholipid standard solutions were used ( $1\text{ g/L}$ ). Phosphatidylcholine and phosphatidylglycerol were selected as standards. Since the phospholipids PG, PA, PI and PE give rise to a parent ion  $[\text{M-H}]^{-}$  of similar structure, we estimated that the MS response of these four classes was similar and we used the standard phospholipid PG for their semi-quantitative analysis. The same approach was used for the phospholipids PC and lyso-PC classes, both giving rise to a similar parent ion of structure  $[\text{M}+\text{HCOO}]^{-}$  due to the presence of the choline group. The standard phospholipid PC was therefore used for semi-quantitative analysis of these phospholipids.

Table 4 shows the results of HPLC-MS analysis of standards used to carry out a semi-quantitative analysis of data for all classes of phospholipids present in the lipid fraction of chia seeds ORURO variety.

**Table 4.** Results of chromatographic analysis of phospholipid standards

<b>Standard, 1g/L</b>	<b>Retention time, min</b>	<b>MA square, arbitrary units</b>	<b>M (molecular mass), g mol</b>
Phosphatidylcholine (PC)	7.45	13 345 558	760
Phosphatidylglycerol (PG)	9.89	13 075 849	749

For the quantitative assessment of the phospholipids mass fraction in the total lipid content of chia seeds, a standard peak area of 13,000,000 units was set.

Since the first sample was used for analysis before removing the solvent, its concentration was estimated based on the mass of chia seed oil obtained after solvent evaporation and the solvent volume used for extraction.

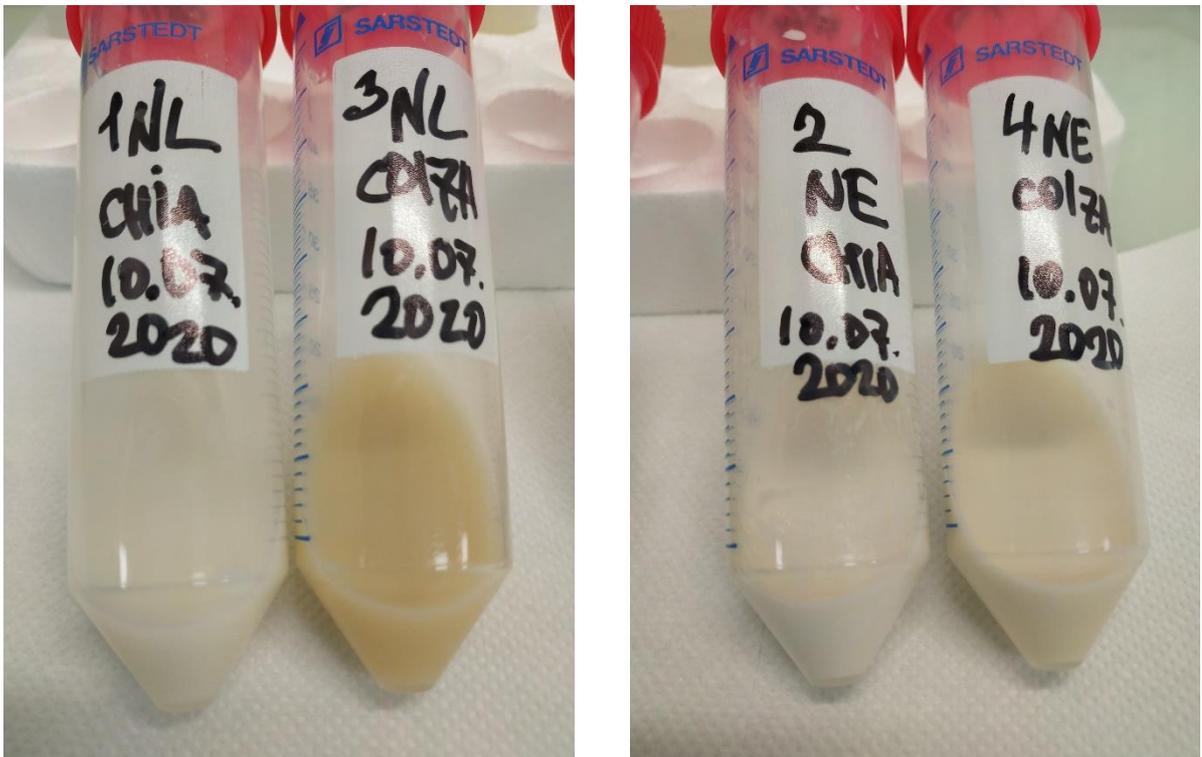
The mass resolution was 0.2 Da. Raw data were processed using the XCALIBUR software program (version 2.1, ThermoFisher Scientific, San Jose, CA, USA).

### **2.2.3 NANOPARTICLES PREPARATION**

Nanoparticles were prepared as described by Arab-Tehrany *et al.* (2012) and Hasan (2015) with slight modifications.

For nanoemulsions (NE), a 10% (*w:v*) oil-in-water stock solution was prepared. A blend of oil with phospholipids as an emulsifier was used (0.233 g of phospholipids or residue and 0.766 g

of oil and 10 mL distilled water). For nanoliposomes (NL), a 2% stock solution (*w:v*) was prepared (0.2 g of phospholipids or residue and 10 mL distilled water). Suspensions were mixed for 4 h under agitation under N<sub>2</sub>(g). The mixtures were then sonicated at 20 kHz at 40% amplitude for 4 min (1 s on and 1 s off) on ice to obtain the homogeneous solutions. Samples (Figure 10) were stored in sterile 50 mL plastic tubes under N<sub>2</sub>(g) in the dark at 4°C.



(a)

(b)

**Figure 10.** Prepared nanoparticles from left to right:

(a) chia NL; rapeseed NL; (b) chia NE; rapeseed NE

#### **2.2.4 NANOPARTICLES SIZE, POLYDISPERSITY INDEX AND ZETA-POTENTIAL MEASUREMENTS**

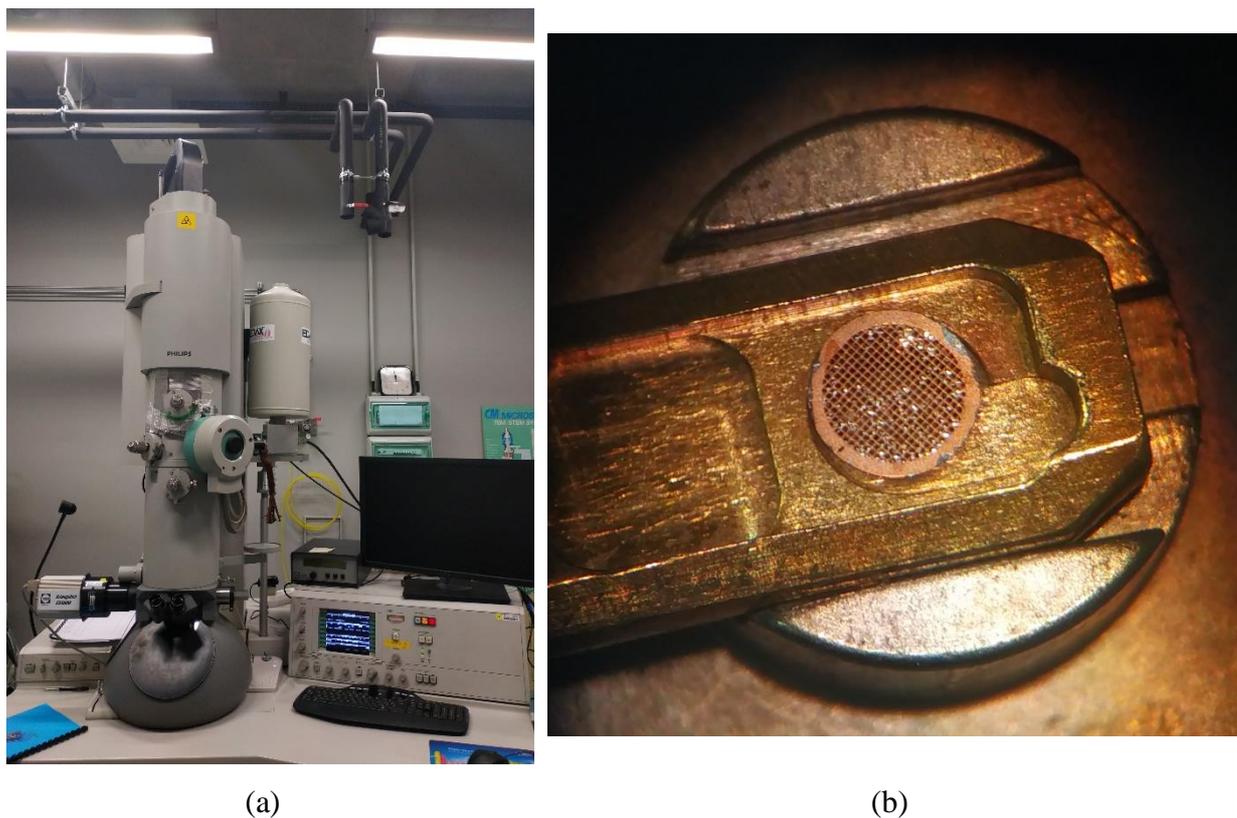
The size, polydispersity index (PDI), and zeta-potential of nanoparticles were measured by DLS (Malvern Zetasizer Nano ZS, UK) (Hasan *et al.*, 2019). Samples were diluted (1:200) using distilled water and then filtered (0.22  $\mu\text{m}$ ). Samples were analyzed in standard capillary electrophoresis cells to measure their zeta-potential. Size and zeta-potential measurements were studied with an absorbance of 0.01, a fixed scattering angle of  $173^\circ$ , a refractive index of 1.4650 (for rapeseeds-based particles) or 1.4804 (for chia-based particles). Analyses were performed at  $25^\circ\text{C}$ .

In some studies, if PDI was  $>0.3$ , to control sample sizes the nanoparticle tracking analysis were used. Samples were analyzed by NanoSight LM10 HSGF T14 machine (Malvern Panalytical, UK). Before analysis, samples were gradually diluted to 1:10.000 using ultra-pure water. After dilution 0.7 mL of sample was injected to the viewing unit from apparatus by 1 mL plastic syringe. Data measurements and analysis were done by Nanosight NTA v3.4 software with the following study parameters: number of captures - 5; capture duration – 30 s; target temperature -  $25^\circ\text{C}$ ; dilution 10.000.

#### **2.2.5 TRANSMISSION ELECTRON MICROSCOPY**

Transmission electron microscopy (TEM) was performed to monitor the microstructure of the nanoparticle samples using negative staining as previously described (Arab-Tehrany *et al.*, 2012; Hasan *et al.*, 2014). The samples were diluted 30-fold with distilled water. An equal volume of 2% ammonium molybdate solution was added to the diluted sample, followed by incubation for 3 min at room temperature. A drop of this solution was placed on a carbon face of EMS CF200-CU type carbon-copper grid (200 mesh). The excess liquid was removed by filter paper, and the

grid was left to dry (room temperature, 5 min). Micrographs were taken using a Philips / FEI CM200 transmission electron microscope (ThermoFisher Scientific, Mérignac, France) operating at 200 kV and recorded using an Olympus TEM CCD camera.



(a)

(b)

**Figure 11.** (a) TEM; (b) carbon-copper grid with a sample

## 2.2.6 STABILITY OF NANOPARTICLES

The aliquots of diluted and filtered nanoparticles prepared at the first day were stored at 4°C. Mean particle size, charge and PDI of all formulations were analyzed every day during the first 5 days. The same protocol described above was used for each analysis (Section 2.2.4).

### **2.2.7 LONG-TERM STUDIES OF OBTAINED NANOPARTICLES**

Some preliminary long-term tests of soft nanoparticles were done. For this test we used the protocol for stability studies (Section 2.2.6).

To investigate FA composition of NL and NE, freeze-dry procedure was used (applied to stock solution of NL and NE). Freeze-dried samples were methylated by Morisson & Smith (1964) method. FAMES preparation was done by following procedure. In glass tube with pre-added IS as described in section 2.2.2.1, 2-3 mg of sample were weighted. After addition of 1 mL of boron trifluoride (14% in methanol) and 1 mL of toluene samples were vortexed 60 s and incubated 60 min at 100°C under N<sub>2</sub>(g). When tube get cold, 1mL of cyclohexane and the same quantity of distilled water and N<sub>2</sub>(g) were added. Next, tubes were centrifuged 4500 rpm during 10 min at 20°C. Supernatant was collected and injected to GC. Gas chromatography studies were conducted as described in section 2.2.2.1.

### **2.2.8 PHOSPHOLIPID DETERMINATION IN NANOLIPOSOMES AND NANOEMULSIONS**

Choline-containing phospholipid content of NL and NE were determined using an enzymatic Wako Phospholipids C assay (LabAssay™ Phospholipids, Fujifilm Wako Chemicals, Neuss, Germany) according to manufacturer's detailed instructions. Chosen method based on spectrophotometrical analysis which measures the absorbance of a blue color produced during enzymatic test. The main principle is described by the following reactions. Choline-containing phospholipids (lecithin, lysolecithin, sphingomyelin) from the sample are hydrolyzed by phospholipase D when sample added to the Color reagent from the kit. Choline released during the reaction is oxidized by choline oxidase to betaine and hydrogen peroxide. In the presence of

released hydrogen peroxide, N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (DAOS) and 4-aminoantipyrine undergo a quantitative oxidative condensation catalyzed by peroxidase (POD), producing a blue pigment, which can be measured spectrophotometrically.

According to the method, samples and blank (control) added in duplicate or triplicate in 96-well microplate. Blank, standard, and sample consists of 2  $\mu\text{L}$  of Saline (0,15M NaCl) or PBS (1x, nonsterile) for blank; 2  $\mu\text{L}$  of phospholipid standard for standard; 2  $\mu\text{L}$  of sample for analyzed sample. Then 200  $\mu\text{L}$  of reagent (phospholipid) solution previously prepared from the kit were added to each well. Cover strip was placed on a plate, solutions were mixed briefly on shaker and incubated for 5 min at 37°C. After microplate was read on a Fluostar Galaxy plate reader (BMG Labtech, Champigny-sur-Marne, France) at 600 nm absorbance. Choline-containing phospholipid concentration in sample was calculated by the following formula:

$$\text{Sample's concentration} = \frac{ABS_{sample} - ABS_{blank}}{ABS_{standard} - ABS_{blank}} \times n \times f_d$$

where ABS – absorbance measured, nm;

n – standard concentration (for phospholipid, 300 mg/dL);

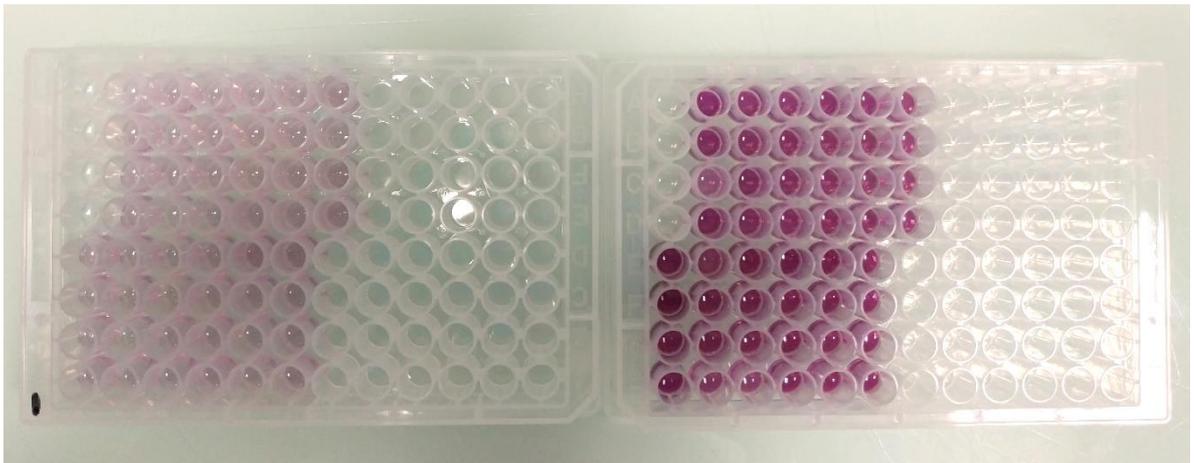
$f_d$  – dilution factor.

## 2.2.9 CYTOTOXICITY OF OBTAINED CHIA-BASED NANOLIPOSOMES AND NANOEMULSIONS

Cells were plated in 24-well plates. Cell lines were treated with NL or NE at 80% confluence), or at Day 4 after plating for primary cultures of rat cortical neurons. On each plate, a group of control cells were present that did not receive NL or NE treatment. After 24 h incubation

at 37°C in the 95% air and 5% CO<sub>2</sub> incubator, cytotoxicity of the soft nanoparticles was evaluated using the MTT-test (Mosmann, 1983) which is a colorimetric method using the pale-yellow tetrazolium dye 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl-tetrazolium bromide (MTT).

MTT was added to the medium (30 µL of 1 mg/mL MTT in DPBS for cell lines, 30 µL of 4 mg/mL MTT in DBS for primary cultures of neurons). When incubated with living metabolically active cells, MTT is cleaved and forms, with the help of mitochondrial succinate dehydrogenase, dark blue-violet formazan. The amount of formed formazan directly depends on the number of living cells; growing cells produce more formazan than living cells in the sedentary phase. After 1 h or 3 h incubation at 37°C in the 95% air and 5% CO<sub>2</sub> incubator, media was removed, and 150 µL DMSO was added to solubilize the formazan in the cells. Plates were incubated an additional 10 min while gently agitating, then 100 µL of medium was recovered into 96-well plates for colorimetric analysis on a Fluostar Galaxy plate reader (BMG Labtech, Champigny-sur-Marne, France). The color intensity of the purple solution is dependent on the activity and viability of the cells.



**Figure 12.** Cell samples transferred into 96-well plates for colorimetric analysis:

from left to right – 1 h incubation and 3 h incubation of Hepa 1-6 with MTT

### **2.2.10 ANTIOXIDANT STUDIES OF CHIA-DERIVED NANOPARTICLES**

Reactive oxygen species (ROS) production can be measured by fluorimetry using 2',7'-dichlorofluorescein-diacetate (DCFH-DA, Sigma). DCFH-DA is transformed into 2',7'-dichlorofluorescein (DCF), a highly fluorescent compound, under the action of ROS.

Hepa 1-6 cells were plated between 250.000 and 300.000 cells/well in 6-well plates and grown in DMEM containing 10% FBS and 1% penicillin/streptomycin. After reaching 70-80% confluence, media was replaced with complete media containing 150 µg/mL chia NL or chia NE (filtered 0.22 µm). After 24 h incubation at 37°C in 95% air and 5% CO<sub>2</sub>, cells were rinsed 2 times in Hank's buffered salt solution (HBSS), and incubated 1 h at 37°C in HBSS containing 100 µM DCFH-DA in ethanol 1% no more 10 ml/1ml. H<sub>2</sub>O<sub>2</sub> was added for a final concentration of 10 mM, and cells were incubated an additional 1 h at 37°C. Cells were rinsed 2 times with HBSS, cell lysis buffer (RIPA) was added (200 µL/well), and cells were scraped and recovered into Eppendorf tubes. Lysates were vortexed 10 s, tubes were centrifuged at 4°C, 14 000 rpm for 5 min. Supernatants (100 µL) were transferred to a 96-well plate to measure fluorescence (485 nm excitation/525 nm emission).

### **2.2.11 STATISTICAL ANALYSIS**

Statistical analyses were carried out using the Statview® 4.5 statistical package (Abacus Concepts, Inc., NJ, USA).

Results are shown as means ( $\pm$  SEM) and statistical significance was set at  $p < 0.05$ . In the figures statistical differences are shown as: \* -  $p < 0.05$ ; \*\* -  $p < 0.01$ ; \*\*\* -  $p < 0.001$ .

**CHAPTER III. NANOLIPOSOMES AND NANOEMULSIONS BASED ON CHIA SEED  
LIPIDS: PREPARATION AND CHARACTERIZATION\***

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**ABSTRACT**

Polyunsaturated fatty acids (PUFA) are important in reducing the risk for cardiovascular, metabolic and neurodegenerative diseases. Chia (*Salvia hispanica* L.) seeds contain high levels of

omega-3 PUFA,  $\alpha$ -linolenic acid (ALA) in particular, and are a potential source for development of omega-3 PUFA-based products. Our objective was to obtain and characterize chia seed lipids, focusing on phospholipid fraction, and to investigate their use in the formulation of nanoemulsions (NE) and nanoliposomes (NL). Solvent-based lipid extraction was performed on the ORURO variety of chia seeds, followed by lipid composition analysis using GC and LC-MS and physico-chemical characterization of chia NL and NE. Folch extraction led to a slightly higher yield of ALA as compared to Soxhlet extraction. Lipid, phospholipid, and fatty acid composition analysis of the oil and residue revealed that the residue was rich in phospholipids; these were used to prepare NE and NL. Physico-chemical characterization showed that NE and NL were generally spherical (transmission electron microscopy), with a size of  $< 120$  nm under hydrated conditions that remained stable over 5 days. In conclusion, chia oil and phospholipid-rich residue can be used to obtain stable NL or NE using a simple method that involves spontaneous emulsification during lipid hydration, which may be useful in cosmetics, pharmaceutical, and other health applications.

**Keywords:** chia seeds; PUFA; nanoliposome; nanoemulsion

### 3.1 INTRODUCTION

Omega-3 polyunsaturated fatty acids (PUFA) are important in nutrition and health, playing an integral part in lipid homeostasis by virtue of their functions in modulating cell membrane fluidity and function, in cell signaling, and in the transcriptional regulation of gene expression. Numerous studies have demonstrated the relationship between PUFA deficiencies and risk for diseases including metabolic syndrome, insulin resistance, cardiovascular and neurodegenerative diseases (Scorletti *et al.*, 2013; Bodoira *et al.*, 2017). Investigations have focused on their use in both preventive, as nutritional supplements, as well as in curative strategies as potential therapeutics of these lipid-related disorders. Omega-3 fatty acids are found in both marine

organisms and plants. Because the former can be limited in supply or present a problem due to its allergenicity, interest has become focused on plant-based products as an interesting alternative and promising source of PUFA (Lee *et al.*, 2017). Seed oils are particularly rich in  $\alpha$ -linolenic acid (ALA), which is an essential fatty acid and precursor of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). ALA itself also displays anti-inflammatory and neuroprotective properties, and therefore is of potential interest as a risk-reducing agent of related diseases.

Chia (*Salvia hispanica* L.) seeds are the richest ALA plant source known today. This plant was originally native to southern Mexico and northern Guatemala, and is now cultivated all over the world, including in Europe. Both the seeds and oil are EU-approved for consumption (Commission EU, 2009, 2013 et 2014), and studies have highlighted their prophylactic properties for metabolic syndrome, hypertension, and cardiovascular diseases. Studies of the composition of chia seeds revealed a content of around 65% ALA of total fat, and up to 20% of linoleic acid (da Silva Marineli *et al.*, 2014; Bodoira *et al.*, 2017; Ganzaroli *et al.*, 2017; Souza *et al.*, 2017; Caruso *et al.*, 2018). In addition, chia seed oil also contains antioxidants (Álvarez-Chávez *et al.*, 2008; da Silva Marineli *et al.*, 2014; Bodoira *et al.*, 2017; Dąbrowski *et al.*, 2017; Caruso *et al.*, 2018), which could also contribute to the interest of this product in nutrition and health.

However, the high PUFA levels in chia seed oil (about 80%) also makes it prone to oxidation which could lead to a loss of nutritional value during storage, as well as the possible formation of undesirable toxic products. The oxidative stability is the most important indicator of edible vegetable oil quality (Bodoira *et al.*, 2017; Souza *et al.*, 2017). Thus, improving the stability of chia seed oil and preserving PUFA from degradation is of particular importance for its use in nutrition and health applications, such as functional foods or in drug development.

The use of nanotechnology makes it possible to change the traditional characteristics of edible oils, improve their quality and safety, increase bioavailability, and expand the scope of applications (Ferreira *et al.*, 2019). Nanoemulsions prepared from oils are of particular interest for

encapsulation, preservation, and elaboration of nanocarriers of sensitive biologically active compounds. The properties of delivery systems based on nanoemulsions depend on the surfactants that coat the lipid droplets and are simple to manufacture displaying high bioavailability due to particle size, as well as gravitational stability (McClements *et al.*, 2013; Teng *et al.*, 2018). However, conventional nanoemulsions are limited as a potential drug delivery system in that only lipophilic molecules can be encapsulated, which significantly limits its use. This can be overcome by the use of nanoliposomes, which by virtue of their structural morphology, permit encapsulation or incorporation of both hydrophilic and lipophilic molecules. This immediately widens the range of their applications for use in functional foods, cosmetics, and pharmaceuticals. Nanosized liposomes are multilamellar and have a large surface area, thus increased potential for solubility, bioavailability, improved controlled release, and more accurate targeting (Danaei *et al.*, 2018). We have, in our laboratory, developed the technology enabling the preparation of PUFA-rich nanoliposomes prepared from phospholipid rich extracts, and have recently shown neurotrophic effects of nanoliposomes prepared from lecithin-rich extracts of fish by-products (Malaplate *et al.*, 2019) that can be used as vectors for delivery of anti-inflammatory drugs such as curcumin (Hasan *et al.*, 2018).

In view of the interest of PUFA-rich chia seeds and their bioactivities, our goal in this study was to obtain and characterize chia seed lipids, focusing on phospholipid fraction, and to investigate their use in the formulation of nanoemulsions (NE) and nanoliposomes (NL). Studies have reported the use of chia seed lipid fraction for the formulation of microemulsions using various materials as emulsifier and different microcapsule wall techniques (Julio *et al.*, 2015; González *et al.*, 2016; Julio *et al.*, 2016; Timilsena *et al.*, 2017b; Guimarães-Inácio *et al.*, 2018; Julio *et al.*, 2018; Us-Medina *et al.*, 2018; Alcântara *et al.*, 2019), but little information is available on the use of ALA-rich chia seed lipids in nanosized particles (Teng *et al.*, 2018; de Campo *et al.*, 2017). Here, we demonstrate the feasibility of using chia seed lipids for preparing stable NL and NE, nanocarriers that have potential uses in both prevention and therapeutic applications.

## 3.2 RESULTS

### 3.2.1 CHIA SEED LIPID EXTRACTION

For this study, we selected the chia ORURO variety, which has been specifically adapted for cultivation in Europe. Since oil yield and fatty acid composition of plants is variety-dependent (Ayerza, 2010 et 2013; da Silva *et al.*, 2017), our first goal was to assess these parameters in the oil extracted from these seeds.

Lipid extraction by modified Folch method was chosen as the target extraction method to be used for chia seeds. This method uses a mixture chloroform:methanol (2:1, *v/v*) which allows the extraction of polar lipids, including phospholipids for the preparation of nanoliposomes. Oil yield was found to be  $29.37 \pm 0.97\%$  ( $n = 10$ ) of total seed mass. This was 17% lower than that obtained when compared to the method using the Soxhlet apparatus and a diethyl ether/petroleum ether mixture (1:1, *v/v*), which led to an oil yield of  $35.25 \pm 1.42\%$  ( $n = 5$ ,  $p < 0.05$ ) of total seed mass.

We compared oil yield obtained after Folch extraction of chia and two other seed types, flax and rapeseed, known to be rich in unsaturated fatty acids. Oil yield from chia seeds using the Folch-based method was significantly lower as compared to that of flax seed ( $38.63 \pm 1.33\%$ ,  $n = 3$ ,  $p < 0.05$ ) or rapeseed ( $42.83 \pm 1.33\%$ ,  $n = 3$ ,  $p < 0.05$ ); the oil yields of flax seed and of rapeseed were not statistically significantly different.

### 3.2.2 CHARACTERIZATION OF CHIA SEED LIPID FRACTION

### 3.2.2.1 FATTY ACID COMPOSITION AND CHARACTERIZATION OF LIPID CLASSES

Fatty acid (FA) composition of the lipid fraction of chia seeds was next analyzed using gas chromatography after extraction using the Soxhlet and Folch-based methods (Table 5).

**Table 5.** Comparison of fatty acid composition of chia seeds lipid fraction obtained by Soxhlet or Folch extraction methods

Index	Soxhlet extraction, %	Folch-based extraction, %
C16:0	7.77 ± 0.16	7.28 ± 0.07*
C18:0	3.56 ± 0.09	3.36 ± 0.01
SFA	11.33	10.64
C16:1	0.24 ± 5.8 × 10 <sup>-5</sup>	0.24 ± 0.003
C18:1 n-9	6.87 ± 0.01	6.77 ± 0.03*
C18:1 n-7	0.79 ± 0.01	0.82 ± 0.01
C18:1 n-5	0.14 ± 0.01	0.13 ± 0.01
MUFA	8.04	7.96
C18:2 n-6	19.16 ± 0.05	18.99 ± 0.06
C18:3 n-6	0.27 ± 0.01	0.25 ± 0.001
C18:3 n-4	0.27 ± 0.004	0.26 ± 0.003*
C18:3 n-3	60.94 ± 0.20	61.92 ± 0.16*
PUFA	80.64	81.42
SFA/MUFA	1.41	1.34
SFA/PUFA	0.14	0.13
Ratio n-6/n-3	0.32	0.31

\*p < 0.05 (Student's t-test); Soxhlet extraction (*n* = 3), Folch extraction (*n* = 3).

Analysis of the chromatograms revealed 10 FA species, with the highest levels being ALA, linoleic, palmitic, oleic and stearic acids, in descending order. For the Folch method, there was a

small but significantly lower amount of palmitic acid. On the other hand, % ALA was slightly but significantly higher with the Folch as compared with the Soxhlet methods. Analysis of polar lipid content of oil by thin-layer chromatography (Iatroskan) revealed no significant difference between that obtained after Folch ( $2.21 \pm 0.33\%$ ,  $n = 6$ ) or Soxhlet extraction ( $2.34 \pm 0.61\%$ ,  $n = 3$ ).

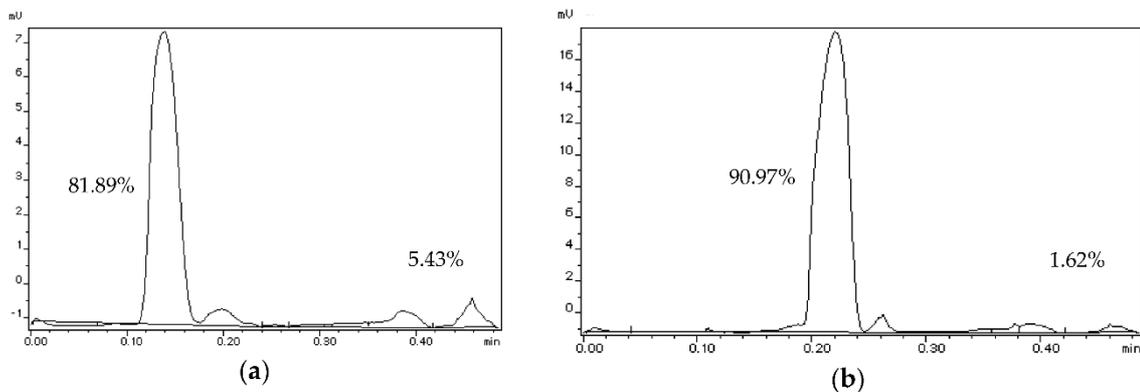
The evaporation step of the solvent after lipid extraction by the Folch-based method revealed the presence of a solid residue on the walls of the flask, which could not be completely re-dissolved in chloroform:methanol (2:1,  $v/v$ ). This represented a yield of  $13.97 \pm 0.25\%$  ( $n = 5$ ) of oil mass, and we hypothesized that a complex, including phospholipids as heavy phase, had formed with other components of the chia seeds during the evaporation step. To test this, the composition of this solid residue obtained was analyzed, and found to contain moisture ( $10.13 \pm 2.6\%$ ), protein ( $3.59 \pm 0.15\%$ ), ash ( $2.61 \pm 0.56\%$ ), dietary fiber (1.79%) relative to the total mass. Results showed that free sugars (glucose and fructose) were only a minor component ( $< 0.05\%$ ).

Analyses of the oil phase obtained by the modified Folch method were also carried out before and after solvent evaporation to determine if this step could affect the recovery of polar lipids. Results in Table 6 showed significant decreases in polar lipid content after solvent removal for the chia and rapeseed oil fractions. Interestingly, solid residue was found on the wall of the flask for all seed types. Polar lipid content was not statistically significant difference between the different seed types, either before or after the solvent evaporation step.

**Table 6.** Polar lipid content before and after solvent removal in three seed types  
by Folch-based extraction

Seed type	Before solvent evaporation	After solvent evaporation
	% total lipids	
Chia seed lipids	4.57 ± 0.69	2.21 ± 0.33
Flax seed lipids	3.70 ± 0.31	2.25 ± 0.53
Rapeseed lipids	3.00 ± 0.70	1.55 ± 0.58

An example of this is shown in Figure 13, following Folch extraction of chia seeds. Polar lipid content of the lipid extract before evaporation of the solvent was decreased two-fold after evaporation and solid residue formation.



**Figure 13.** Separation for lipid classes by IATROSCAN:

(a) chia lipids before solvent removal; (b) chia lipids after solvent removal

Based on these analyses, we would therefore propose that this solid residue consists of a polar lipid-rich natural complex containing moisture, protein and fiber, and other components as described above, which could explain the significant loss of the polar lipid content in the oily phase following evaporation after Folch extraction.

### 3.2.2.2 PHOSPHOLIPID ANALYSIS

Since there is little information in the literature on chia seed phospholipid composition, phospholipid composition was analyzed before and after solvent removal using high performance liquid chromatography with mass spectrometry (LC-MS).

Analysis revealed that total phospholipid content in chia seed oil was 7.49% before solvent removal and 0.26% in the liquid phase of the lipid fraction after solvent evaporation. This confirms the significant loss of phospholipids in oil following this step, most likely found in the residue.

Six phospholipid classes were identified. In the sample before evaporation, PL composition was revealed to be 30.87% phosphatidylinositol (PI), 20.20% phosphatidic acid, 19.15% phosphatidylethanolamine (PE), 12.66% phosphatidylglycerol (PG), 11.82% phosphatidylcholine and 5.3% lysophosphatidyl-choline (Lyso-PC).

Data analysis was next performed to determine the FA composition of the chia seed phospholipid classes. This is presented in Table 7 by phospholipid class for the sample before evaporation.

**Table 7.** Molecular species composition of chia seeds PL determined by liquid chromatography with mass spectrometry (LC-MS)

FA fragments	PA	PI	PG	PE	PC	Lyso-PC
Relative composition (%)						
16:0	-	-	-	-	-	12.68
16:0/18:2	15.09	21.50	24.07	22.56	19.05	-
16:0/18:3	13.73	32.57	20.57	15.40	13.35	-
18:0	-	-	-	-	-	17.18
18:0/18:2+18:1/18:1	-	-	-	6.65	-	-
18:0/18:2	-	9.98	-	-	6.89	-

<b>FA fragments</b>	<b>PA</b>	<b>PI</b>	<b>PG</b>	<b>PE</b>	<b>PC</b>	<b>Lyso-PC</b>
Relative composition (%)						
18:0/18:3	5.40	20.52	6.33	4.47	4.55	-
18:1/18:1+18:0/18:2	6.19	-	7.46	-	-	-
18:1/18:2	5.40	-	4.59	5.68	5.13	-
18:1/18:3	4.24	-	3.36	-	3.91	-
18:2	-	-	-	-	-	38.76
18:2/18:2	15.03	-	10.64	19.02	11.13	-
18:2/18:3	23.35	9.49	15.63	19.15	15.80	-
18:3	-	-	-	-	-	31.37
18:3/18:3	11.57	5.94	7.35	7.07	7.48	-
n/a	-	-	-	-	6.23	-
n/a	-	-	-	-	6.49	-

The amount of PL containing PUFA ALA was found to range between 45 and 68% in the different PL classes, with the highest amount found in PI, and the lowest in PC.

### **3.2.3 PHYSICOCHEMICAL CHARACTERIZATION OF NANOEMULSIONS AND NANOLIPOSOMES**

We next prepared NE from the chia seed oil and phospholipid-rich solid residue, and NL from the phospholipid-rich solid residue. NL and NE were also prepared from rapeseed oil and commercial rapeseed phospholipids, which we have previously characterized (Arab-Tehrany *et al.*, 2012), as a reference. In general, the average particle size and distribution in the volume of emulsion or suspension depends on the lipid composition and the method of their preparation. The parameters for assessing the quality of NL and NE preparations are the particle sizes and their homogeneity in the total mass. Polydispersity index which is a measure of the heterogeneity of a

sample based on size (Danaei *et al.*, 2018; Mudalige *et al.*, 2019), size and zeta-potential were determined, and the results are shown in Table 8.

**Table 8.** Characterization of nanoparticles after preparation

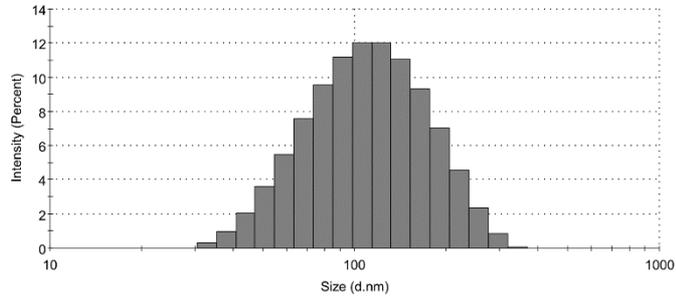
Source	Particle	PDI	Size, nm	Zeta-potential, mV
Chia	NL	0.208 ± 0.02	117.65 ± 5.88	-31.775 ± 3.88
Rapeseed	NL	0.214 ± 0.01	116.85 ± 2.96	-28.95 ± 2.76
Chia	NE	0.205 ± 0.01	103.80 ± 2.70	-34.10 ± 7.23
Rapeseed	NE	0.194 ± 0.02	131.90 ± 4.69*	-30.075 ± 2.39
Chia/rapeseed	Mixed NE	0.138 ± 0.01*	127.30 ± 1.50*	-27.33 ± 0.58

\*p < 0.05 (Fisher's test), for each NL or NE (*n* = 4), mixed NE (*n* = 3).

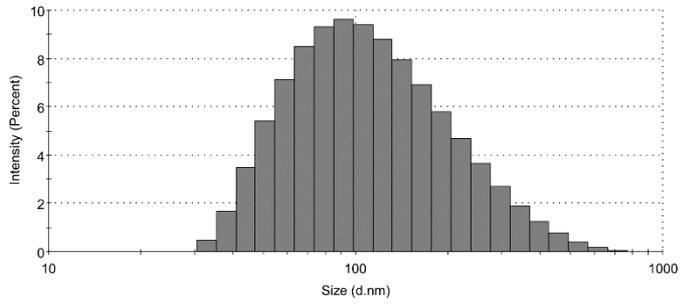
PDI values of all particles were found to be < 0.3, indicating monodisperse solutions with controlled size distribution and narrow dispersity (Hasan *et al.*, 2019). Statistical analysis was performed comparing data obtained for NL and for NE. Size was determined using DLS (dynamic light scattering), and found to be slightly larger than 100 nm, very similar to NL prepared from rapeseed phospholipids. There were no statistically significant differences in either size or charge between chia- or rapeseed-based NL.

On the other hand, the size of chia-based NE was significantly smaller as compared to rapeseed NE and the NE using chia oil and rapeseed PL. However, this difference was not significant when comparing NE using rapeseed PL as emulsifier (rapeseed NE and mixed NE). Interestingly, the PDI was much lower in mixed NE as compared to the other NE particles.

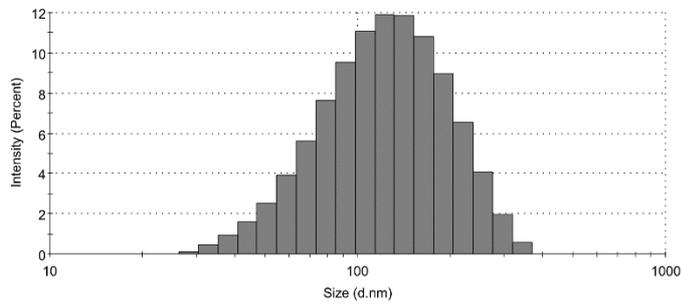
Representative histograms showing size distribution versus intensity of prepared nanoparticles are shown in Figure 14.



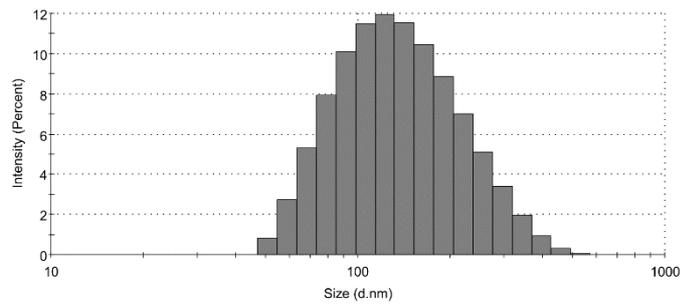
(a)



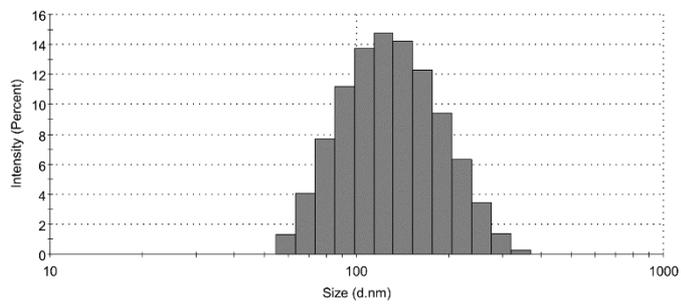
(b)



(c)



(d)

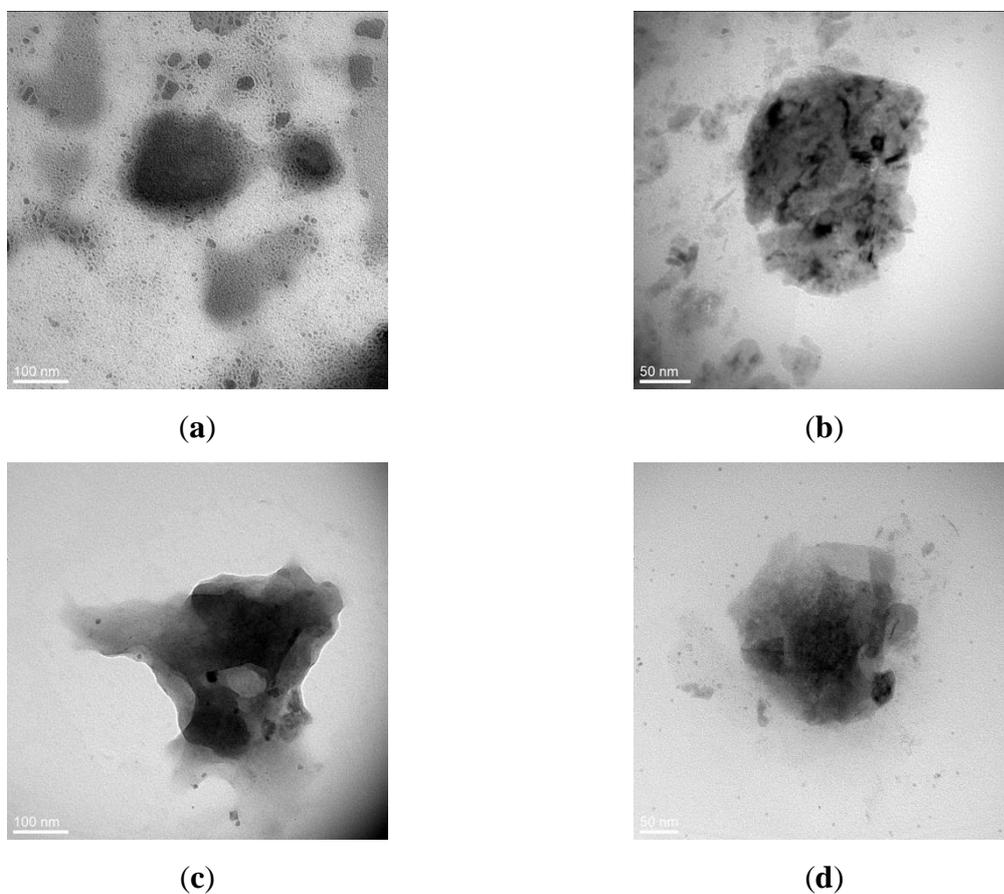


(e)

**Figure 14.** Particle size distribution after preparation: (a) chia NL; (b) rapeseed NL; (c) chia NE; (d) rapeseed NE; (e) mixed NE

We measured the stability of NL and NE by measuring the particle size every day over 5 days after preparation, while stored at 4°C under N<sub>2</sub>(g) in dark vials. No significant variation was observed during this time period.

TEM was performed to assess the morphological properties of the particles (Figure 15).



**Figure 15.** Plant-based nanoparticles by TEM: (a) chia-based NL; (b) chia-based NE; (c) rapeseed-based NL; (d) rapeseed-based NE

Images showed spherical morphology of chia NL and NE preparations. The structure of the obtained particles was not homogenous, with darker and lighter areas, which suggests the presence of multilayers. Indeed, previous studies in our laboratory, using the similar technique of nanoparticle preparation, revealed the form of multilamellar vesicles because of the sonication step (Arab-Tehrany *et al.*, 2012).

The rapeseed-based NL appeared slightly larger, but some discrepancies may be expected since the DLS results reflect the hydrodynamic size of the particle, whereas TEM is performed *in vacuo*.

Finally, we sought to verify the presence of phospholipids in chia NL and NE preparations. Analysis using an enzymatic kit revealed a content of  $3.4 \pm 1.04$  mg/mL and  $3.8 \pm 1.26$  mg/mL choline-containing phospholipids in NL and NE, respectively, confirming the presence of phospholipids in the residue used to prepare these nanoparticles.

### 3.3 DISCUSSION

In this study, we report the composition of chia seed lipids including phospholipids, and the use of chia oil and phospholipid-rich residue for the preparation of NL and NE. Here, we used chia seeds of the ORURO variety that is cultivated in France. Since the composition of chia seeds, including oil yield and PUFA content depends on the climate and region of origin, fatty acid composition was determined, and showed a low n-6/n-3 fatty acid ratio. The only study on this variety reported differences in omega-6 and omega-3 content depending on the maturity of the chia grain. Levels of PUFA increased from 74 to 80% up to fruit development, with a similar trend for omega-3, while those of omega-6 PUFA, although higher in earlier stages, decreased when the fruit was close to full maturity (Gravé *et al.*, 2019). This study therefore demonstrates the interest

in using chia seeds before maturation, allowing the recovery of lipid fraction with a low n-6 / n-3 fatty acid ratio, which is in agreement with the results obtained here.

The type of solvent extraction method used can affect oil yield from chia seeds ORURO variety, as well as fatty acid composition. Extraction with organic solvents is still one of the most common methods for extracting vegetable oils. Even if oil obtained by cold or hot pressing is considered to be of the highest quality (especially with cold pressing), the main difficulty from the point of view of industrial production is the low oil yield. Therefore, extraction with organic solvents remains one of the preferred methods (Arişanu *et al.*, 2017).

A higher yield of chia seed oil was obtained using the Soxhlet apparatus and a mixture of ethers similar to those described previously using petroleum ether (Caruso *et al.*, 2018), as compared to the chloroform and methanol mixture used in the Folch-based procedure (Ganzaroli *et al.*, 2017). Nevertheless, oil yields with both methods ranged between 29 and 35%, which are comparable to those values provided by the EU commission (2013) (between 30 and 35%). We chose to continue the Folch-based extraction, which is the preferred method for extraction of polar lipids, including phospholipids needed for the preparation of NL. Indeed, little information is available on the use of chloroform-methanol mixture for the extraction of chia seed oil (Ciftci *et al.*, 2012; Ganzaroli *et al.*, 2017; Souza *et al.*, 2017b). Although the Bligh-Dyer method (1959) can also be used for polar lipid extraction, it is less effective when the sample contains more than 2% lipids as can be found in chia or other oily seeds (Iverson *et al.*, 2001).

Chia oil fatty acid composition reported here for the ORURO variety using Folch method is similar to those reported by other investigators using other solvents. In general, literature review shows that the major FA in chia seed oil from different growing regions are ALA (up to 65%), linoleic (up to 20%), palmitic (about 7%), oleic (about 5-7%), and stearic (about 3-4%) acids. Higher ALA content (65.4%) as compared to this study was reported using Soxhlet extraction in petroleum ether of a genotype G8 of chia seeds originating from America and grown in southern

Italy (Caruso *et al.*, 2018). In the study on the chia ORURO variety (Gravé *et al.*, 2019), oil yield from chia by extraction with cyclohexane was slightly lower as compared to the results of this study, ranging from 23.3 to 28.4% from seeds at different stages of development. The content of ALA, according to the authors, varied from 52.4 to 60%, linoleic acid from 19.9 to 22.0%, oleic acid from 0.9 to 11.3%, palmitic acid from 8.2 to 9.2%, and stearic acid from 3.4 to 4%. In our study of chia ORURO, even if the solvent extraction method was different, the profile of MUFA and PUFA is very close to seeds at early stages of maturation.

LC-MS analysis revealed 7.5% phospholipid content in the oil after Folch extraction, which was higher than the values of total polar lipid content obtained using thin-layer chromatography (Iatroscan). This difference may be due to lower sensitivity and detection limit of the latter method, which could contribute to large variations observed following analysis by Iatroscan (for example, we obtained values between 1.86% and 6.94% of polar lipids from an individual sample). Chia PL composition analysis revealed six major PL classes including PI > PA = PE > PG = PC > lyso-PC. The same classes were also found by Calvo *et al.* (2020) in chia seeds (Mexican origin), with the exception of lyso-PC. Using pressurized liquid or supercritical fluid extraction methods with ethanol as co-solvent, they reported a chia PL yield varying between, 0.1% to 16.5%. Recovery was method-dependent, since higher PC levels were obtained following supercritical fluid extraction, while similar amounts of PC and PI were found using pressurized liquid extraction.

Iatroscan and LC-MS analyses revealed a significant loss of polar lipids in the oil after evaporation, which we postulated to be in the solid residue that formed following evaporation of the chloroform-methanol solvent after lipid extraction. The presence of PC and lyso-PC in NL and NE prepared from the residue was confirmed by analyses using an enzymatic kit for detection of these two choline-containing PL. Our results also showed that this residue was a complex of not only lipids, but also moisture, protein, and dietary fiber, with glucose and fructose only being

minor components. Similar solid residues were observed after evaporation in the flax and rapeseeds, and we suggest that these residues may be due to the presence a wide range of possible compounds, including polysaccharides and polyphenols associated with the plant cell membranes (Oliveira-Alves *et al.*, 2017), leading to the formation of complexes with the phospholipids difficult to re-solubilize after evaporation.

Our results show that chia seed oil and the phospholipid-rich solid residue can be used to prepare stable NL and NE. Indeed, we have previously used PL from other agrosources, including salmon, rapeseeds, or soya, to prepare stable nanoparticles at a temperature of either 4°C or 37°C (Arab-Tehrany *et al.*, 2012; Hasan *et al.*, 2014; Hasan *et al.*, 2019). The amphiphilic nature of phospholipids provides the means to prepare stable multilamellar liposomes. Physicochemical characterization revealed both NL and NE particle type to be stable, with a low PDI (< 0.3). In drug delivery applications using lipid-based carriers, such as liposome and nanoliposome formulations, a PDI of 0.3 and below is considered to be acceptable and indicates a homogenous population of phospholipid vesicles (Danaei *et al.*, 2018). The stability of the NL and NE was also demonstrated by their low negative charge (zeta-potential). High value negative/positive charges of these systems are more stable due to repulsive forces. TEM revealed that chia NL and NE were in the 100 nm range, consistent with that measured by DLS, and appeared spherical. Since these particles are PL-based, we would suggest that they are multilamellar in nature, as has been previously demonstrated for other PL-based NL (Arab-Tehrany *et al.*, 2012). There is little information in the literature on the use of chia lipid fractions in nanotechnology (de Campo *et al.*, 2017; Teng *et al.*, 2018). Here, we show the possibility of preparing chia NE using only chia-derived lipids and PL residue as emulsifier. Stable NE, stored at 4°C or ambient temperature, have been reported using chia oil, but using exogenous emulsifiers (Tween 80, Span 80 and others) (Teng *et al.*, 2018). However, no information has been found in the literature concerning the preparation of NL from chia seed lipids. This is therefore the first report on the preparation and characterization of NL and NE from pure chia seed compounds.

### 3.4 CONCLUSIONS

Here, we report for the first time fatty acid composition, phospholipid classes and the phospholipid fatty acid profile of chia seeds of the ORURO variety following Folch extraction. We also demonstrate that stable NE and NL can be obtained from chia oil or chia phospholipid-rich residue fractions using a simple method that involves spontaneous emulsification during lipid hydration. Indeed, such an NL and NE preparation method is a low-energy procedure and is inexpensive, requiring a single sonication step, which can be advantageous for industrial-level production (Arab-Tehrany *et al.*, 2012; Teng *et al.*, 2018). Furthermore, the use of the phospholipid-rich residues for the preparation of NL and NE allows the transformation of a by-product following lipid extraction into potentially useful products as vesicles. While we used a solvent-based method in this initial study to extract chia lipids for composition analysis, chia lipids obtained using sustainable “green” lipid extraction methods merit investigation, which would be of particular interest in ensuring the compatibility of chia-based NE and NL, particularly in applications concerning nutrition and health. In conclusion, we propose that chia lipids represent an interesting source of omega-3 PUFA and phospholipids that can be used in developing NL and NE as nanocarriers for numerous health-based applications including cosmetics and pharmaceuticals.

**CHAPTER IV. CYTOTOXICITY STUDIES OF NANOLIPOSOMES  
AND NANOEMULSIONS OBTAINED FROM CHIA SEED LIPIDS  
IN LIVER AND BRAIN CELLS**

**4.1 INTRODUCTION**

ALA is a precursor of DHA, but its direct role in the organism remains unclear and currently is of interest in a number of studies. It is believed that conversion of ALA to DHA is relatively low in humans, what makes an issue to scale animal studies. A recent review by Domenichiello *et al.* (2015) suggests that it is a mistake to assess only oral intake of ALA and its bioconversion, however, it is necessary to consider the total synthesis of DHA in the body, as well as specific conditions that may affect the rate of synthesis, including diet, genetics, age, injury and disease. The change in approach suggests that sufficient DHA synthesis for the brain needs of healthy adults could exist but needs additional research.

To better understand bioavailability of chia derived nanoparticles rich in ALA in the entire organism, this needs to be evaluated not only in the central nervous system (CNS), but also in the peripheral compartment, notably the liver. The choice of liver cells as an experimental *in vitro* model can be justified by the functions that the liver plays in the human body. The liver is the main regulator of lipid metabolism, in terms of secretion, synthesis, and catabolism of FA. In addition, it plays an important role in lipid homeostasis and plasma lipid levels (Huang *et al.*, 2011). One of the most important metabolic processes in the liver is the conversion of ALA and LA into long-chain AA, EPA and DHA, which play an important role in the processes occurring in the brain. Moreover, changes in the balance of omega-6 and omega-3 in the diet can lead to metabolic disorders in the liver and several pathologies (Scorletti *et al.*, 2013). Interestingly, a study by

Rapoport *et al.* (2007) suggests that there is a certain relationship between bioconversion occurring in the liver and processes occurring in the brain.

Because of this, we decided to evaluate cytotoxicity of cha-derived NE and NL in different cell lines from the different tissues including the liver and the CNS. For this study, we used Hepa 1-6, a murine hepatoma cell line which has been previously used by UR AFPA QUALIVIE for studies on the regulation of lipoprotein receptors and lipid metabolism (Yen *et al.*, 2008, Stenger *et al.*, 2010, Ahmad *et al.*, 2012, Layeghkhavidaki *et al.*, 2014).

For the CNS, there are different cell types, including neurons which serve for the transmission of electrical signals via synapses, and glial cells which provide an important support system. Neurons acquire nutrients including lipids from glial cells, primarily astrocytes. ApoE is a ligand for lipoprotein receptors and plays an important role in cholesterol transport in both peripheral tissues and the brain (Hauser *et al.*, 2011). It is produced by astrocytes, secreted in the form of cholesterol-containing lipoproteins for uptake by neurons. ApoE exists as 3 isoforms, apoE2, E3, and E4, where apoE2 is a risk factor for hyperlipidemia (more specifically, hypertriglyceridemia, so high plasma levels of triglycerides), while apoE4 is a major risk factor of Alzheimer's disease. Here, we use astrocyte cell lines prepared from mice expressing the different isoforms of human apolipoprotein E (apoE) as multiple apoE isoforms do not exist for mice (Morikawa *et al.*, 2005).

Primary cultures of cortical neurons from rat embryos were used in this study. These cells have been previously shown to be a model for studying the pro-apoptotic effects of the oligomeric soluble form of amyloid- $\beta$  peptide, the toxic agent that contributes to the pathology of Alzheimer's disease (Garcia *et al.*, 2010; Colin *et al.*, 2016). The neurotrophic effects of fish lecithin-based nanoliposomes was demonstrated in this cell model in our laboratory (Malaplate *et al.*, 2019).

The objective of this study was to evaluate the cytotoxicity of the naturally obtained NL and NE derived from chia oil and chia phospholipid-rich residue in different cell lines from the liver and the brain.

To evaluate cytotoxicity of the nanoparticles, we used the MTT ((3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay (Riss *et al.*, 2016), where MTT is a water-soluble tetrazolium dye MTT that can be converted to purple formazan crystals by mitochondrial dehydrogenases. These crystals are resolubilized in DMSO, and absorbance is then measured on a plate reader or spectrophotometer at 570 nm. Since only metabolically active cells can convert MTT to formazan, the intensity of the formazan color is directly related to the number of viable cells.

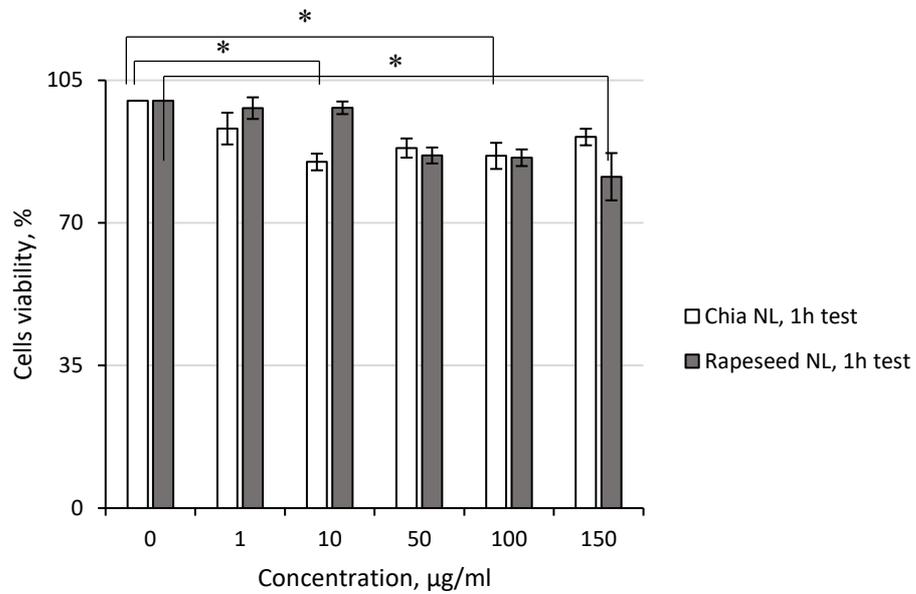
## **4.2 RESULTS AND DISCUSSION**

Cells were incubated at concentrations from 0 to 150  $\mu\text{g}$  of NL or NE/mL culture media, based on the amount of oil and/or residue used in the preparation of the nanoparticles.

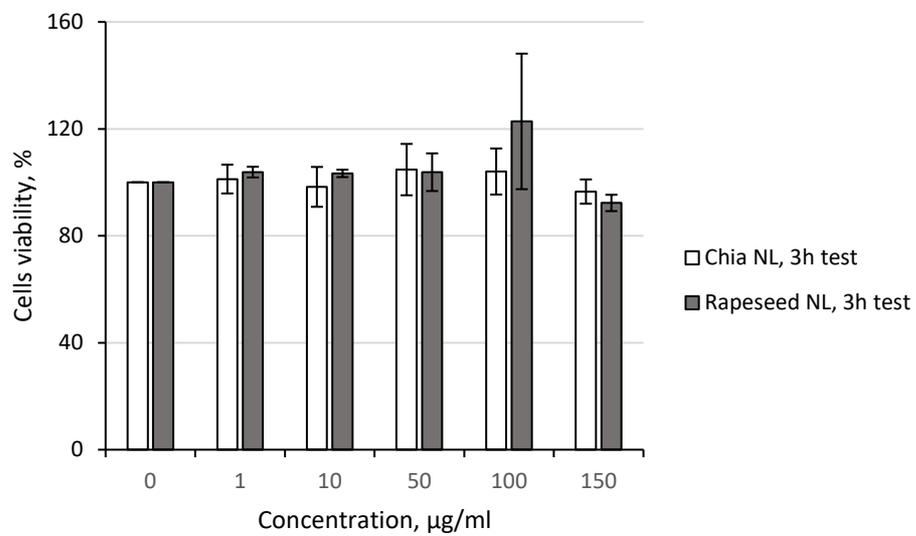
### **4.2.1 HEPA 1-6 CELL LINE**

No cytotoxicity observed with Hepa 1-6 cells, no significant decrease in all nanoparticle types, with increasing concentrations even up to 150  $\mu\text{g}/\text{mL}$  of NL or NE, whether it was derived from chia or rapeseed oil.

Figure 16 demonstrates result of Hepa 1-6 cell line viability tests, for chia and rapeseed NL treatments.



(a)



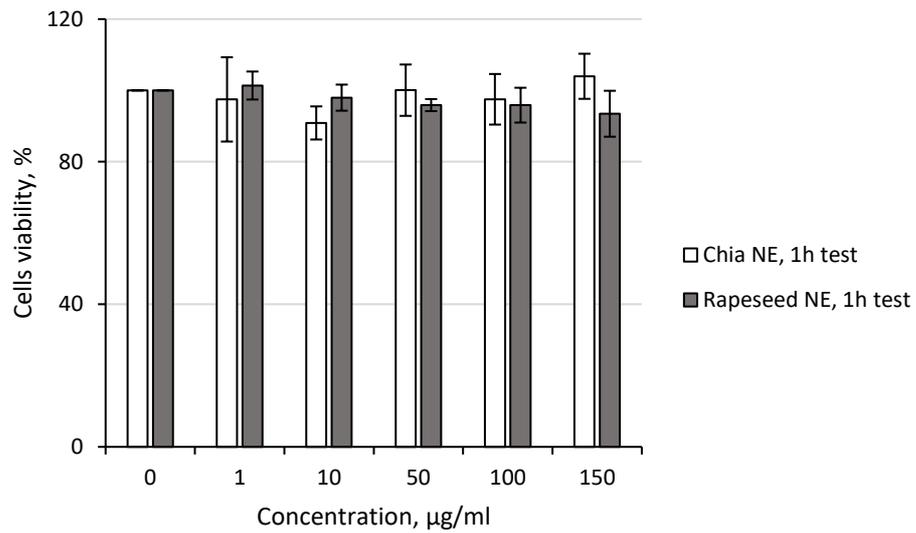
(b)

**Figure 16.** Hepa 1-6 cell line viability, NL: (a) 1 h test; (b) 3 h test

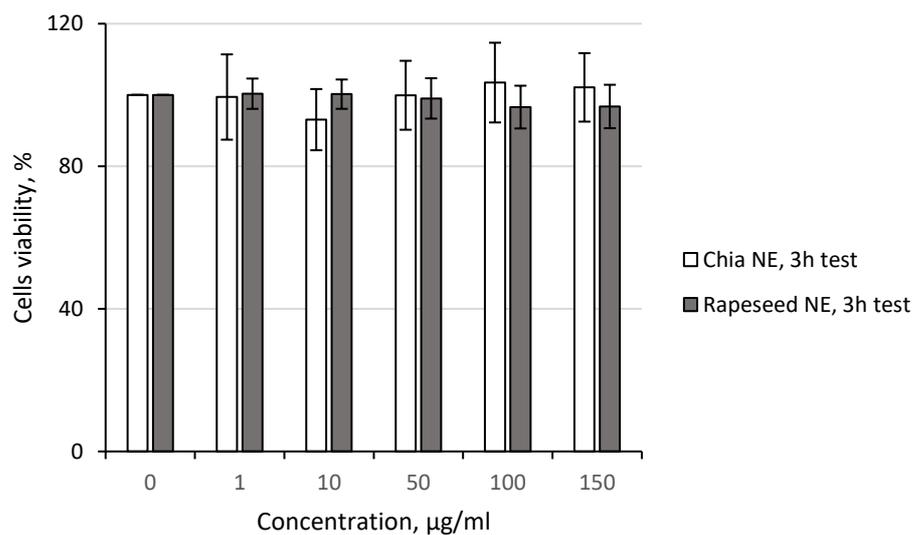
We observed some significant differences for 1h tests with both types of NL ( $p < 0.05$ , Dunn's test,  $n = 3$ ). 10 and 100 µg/mL concentrations showed decrease of viability with chia NL treatments, and 150 µg/mL with rapeseed NL treatments, which may be due to the uneven cell

distribution in the wells or other external factors. However, viability was higher than 80% in all samples.

Figure 17 shows result of Hepa 1-6 cell line viability tests, for chia and rapeseed NE treatments.



(a)



(b)

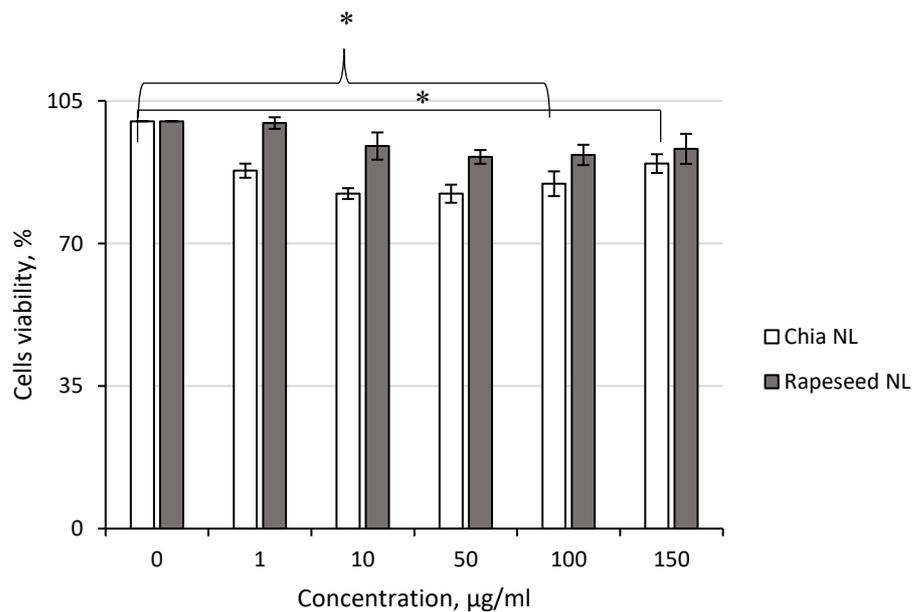
**Figure 17.** Hepa 1-6 cell line viability, NE: (a) 1 h test; (b) 3 h test

No significant difference was observed by these tests between control and treated cell samples.

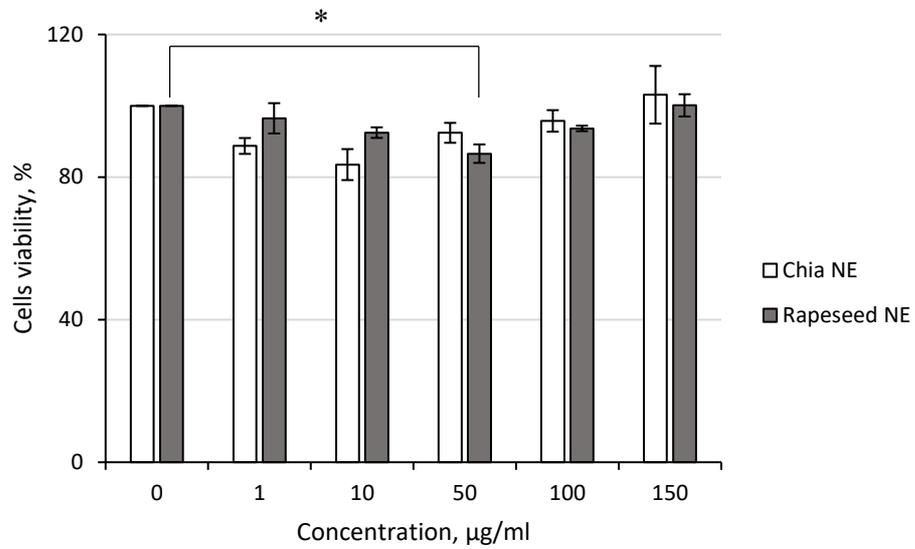
#### 4.2.2 ASTROCYTES CELL LINES

In astrocytes, a general tendency towards a lower number of viable cells was observed with increasing concentrations of NL or NE at intermediate concentrations (between 10 and 50  $\mu\text{g}/\text{mL}$ ), as compared to lower or higher concentrations of the nanoparticles. Interestingly, chia-derived NL had a statistically significant effect on astrocytes expressing the human apoE2 isoform, with a 20% loss of viable cells at even 10  $\mu\text{g}/\text{mL}$ . Although similar trends were observed in the other astrocyte cell lines expressing E3 or E4, these did not achieve statistical significance.

Figure 18 demonstrates result of apoE2 cell viability tests, for chia and rapeseed NL and NE treatments.



(a)

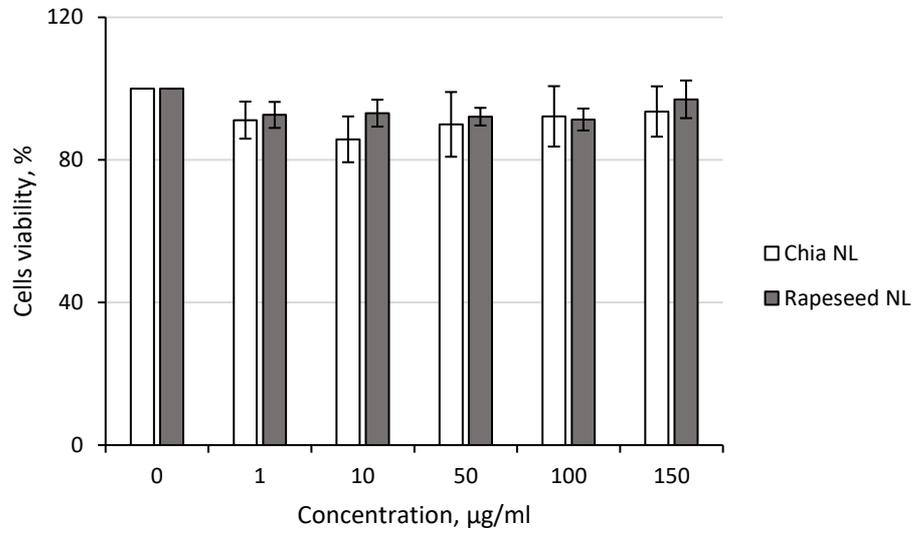


(b)

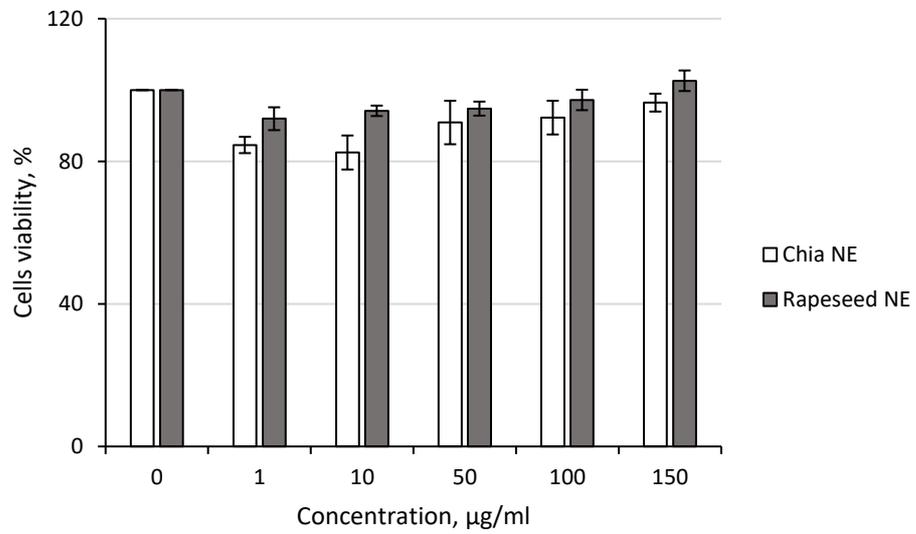
**Figure 18.** ApoE2 cell viability: (a) NL test (b) NE test

We observed significant differences for all test apoE2 concentrations of chia NL ( $p < 0.05$ , Dunn's test,  $n = 4$ ), and only concentration 50 µg/mL on rapeseed NE reached significance level compare to control ( $p < 0.05$ , Dunn's test,  $n = 3$ ) with viability higher than 80%.

Figure 19 shows result of apoE3 cell viability tests, for chia and rapeseed NL and NE treatments.



(a)

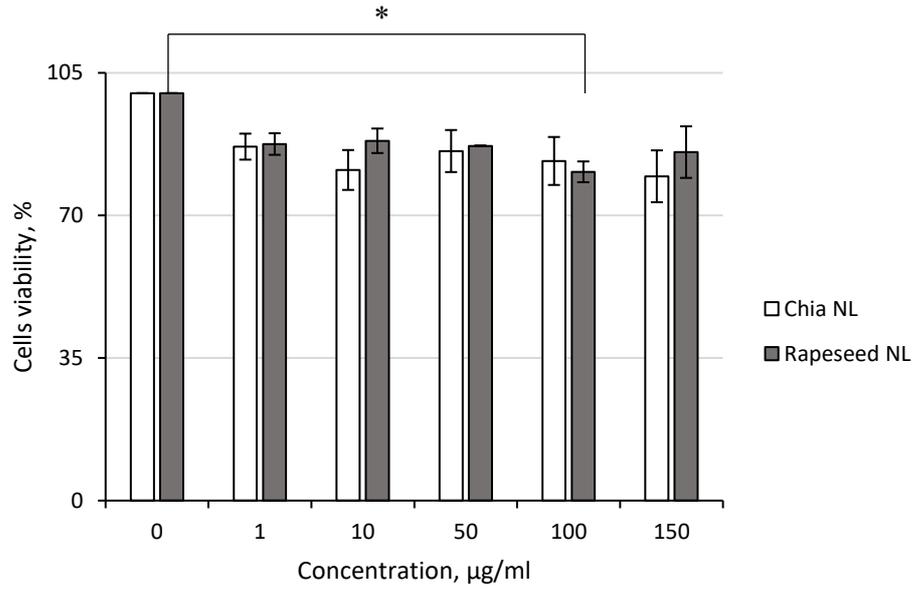


(b)

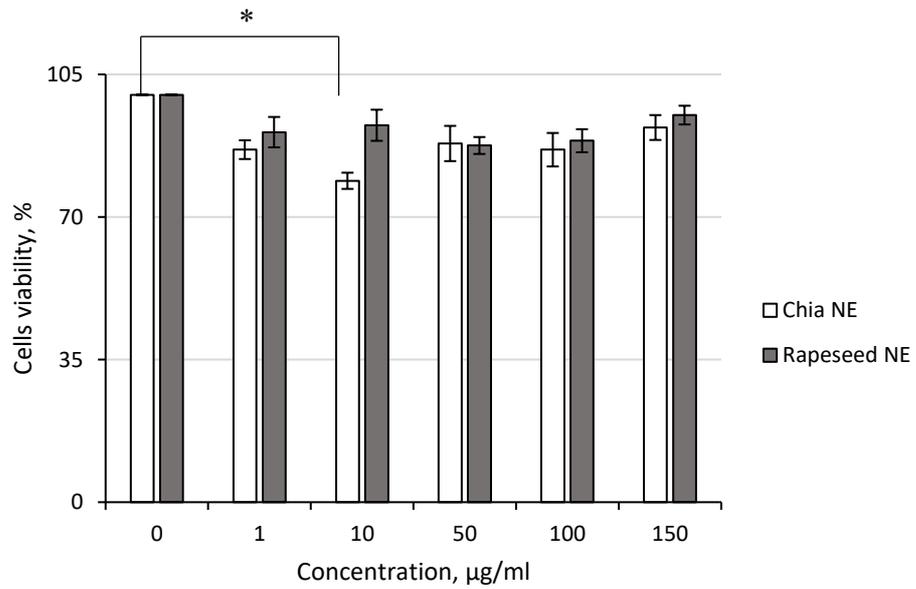
**Figure 19.** ApoE3 cell viability: (a) NL test (b) NE test

No significant difference was observed by these tests between control and treated cell samples.

Figure 20 demonstrates result of apoE4 cell viability tests, for chia and rapeseed NL and NE treatments.



(a)



(b)

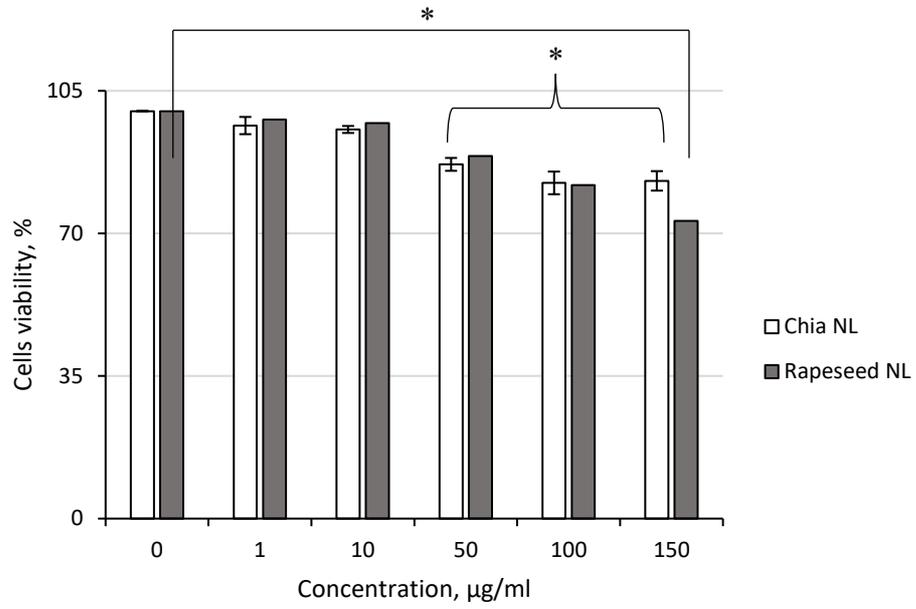
**Figure 20.** ApoE4 cell viability: (a) NL test (b) NE test

Significance level was reached in sample rapeseed NL, concentration 100  $\mu\text{g}/\text{mL}$  ( $p < 0.05$ , Dunn's test,  $n = 3$ ) and chia-derived NE, concentration 10  $\mu\text{g}/\text{mL}$  ( $p < 0.05$ , Dunn's test,  $n = 4$ ), with viability  $80.70 \pm 2.55\%$  and  $78.89 \pm 1.99\%$  respectively.

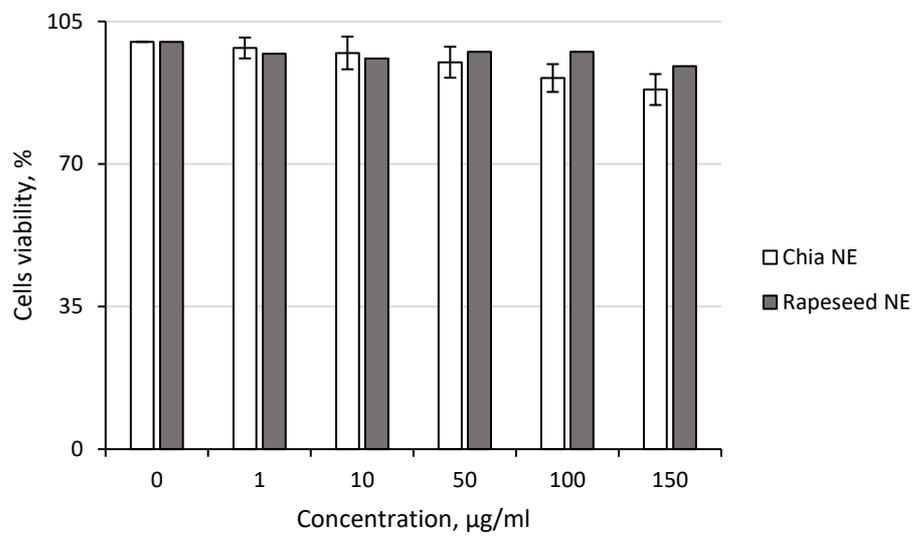
The apoE2 isoform is a risk factor for hypertriglyceridemia in the periphery and has been reported to be a protective factor in Alzheimer's disease as compared to E4, which is a high-risk factor for this neurodegenerative disease (Mahley, 1988; Huang & Mahley, 2014; Mahley, 2016). While apoE2 affinity for lipoproteins and lipoprotein receptors has been studied extensively in the peripheral tissues, there is no current literature on its effect on lipid metabolism in astrocytes. Instead, studies have focused instead on apoE4 as a risk factor of Alzheimer's disease. The results here indicate that the effects of chia-derived NL may be apoE-isoform dependent, and it would therefore be interesting to study the differences in lipid metabolism of these different cell lines in response to different types of nanoparticles.

#### **4.2.3 PRIMARY CULTURE OF CORTICAL NEURONS**

Interestingly, less effects of chia-based NL were observed on cellular metabolic activity of primary cultures of rat embryonic cortical neurons (Figure 21).



(a)



(b)

**Figure 21.** Primary culture neurons cell viability: (a) NL test (b) NE test

Statistically significant decrease in cell viability of neurons treated with chia derived NL were observed only in concentrations  $\geq 50 \mu\text{g/mL}$  ( $p < 0.05$ , Dunn's test,  $n = 3$ ), while that of neurons treated with rapeseed-derived NL were seen at concentration of  $150 \mu\text{g/mL}$ . This is similar to that observed for fish lecithin derived NL (Malaplate et al, 2019).

Since astrocytes also serve a role in detoxification to maintain extracellular homeostasis, it may be that this effect could be due to increased uptake of the nanoparticles, which may lead to perturbations in cellular lipid homeostasis leading to cell death. It would be interesting to quantify the uptake of fluorescently-labelled NL or NE to test this hypothesis, and to determine the changes in lipid status (fatty acid and PL composition, for example) following incubation of NL or NE with astrocytes, as compared to neurons.

### 4.3 CONCLUSIONS

During this study, the toxicity of obtained NL and NE based on the chia seed lipids was evaluated *in vitro*.

The MTT cell viability assay showed little to no or low cell toxicity following 24 h incubation with concentrations from 1 to  $150 \mu\text{g/mL}$  NL or NE in Hepa 1-6 (mouse hepatoma), mouse astrocytes expressing 3 isoforms of human apolipoprotein E (apoE2, E3, and E4), and primary cultures of rat embryonic cortical neurons.

A significant decrease in cell viability was noted during a 24-hour incubation of astrocytes expressing the apoE2. Some significant effects of NL or NE were observed in neurons.

## CHAPTER V. ADDITIONAL STUDIES

### 5.1 LONG-TERM STORAGE OF CHIA-DERIVED NANOLIPOSOMES AND NANOEMULSIONS

The 5 days storage period chosen in chapter III demonstrates the stability of NE and NL needed for *in vitro* studies. We also performed some long-term tests (Table 9). Data obtained showed good stability of soft nanoparticles in 1, 3 or 6 months with PDI, size and charge (zeta-potential) on same levels as the first day of studies.

**Table 9.** Preliminary characterization of nanoparticles on the day 150

Source	Particle	PDI	Size, nm	Zeta-potential, mV
Chia	NL	0.202	124.2	-29.4
Rapeseed	NL	0.284	127.5	-26.9
Chia	NE	0.238	106.0	-29.0
Rapeseed	NE	0.181	133.4	-27.9

The PDI at day 150 was 0.202 and 0.298 for chia-derived NL and NE, respectively, which reflects of lack of soft nanoparticles aggregation. Size of chia NL was 124 nm, for NE - 106 nm, and charge values -29.4 mV and -29.0 mV what is in accordance with previous data.

To evaluate composition of chia- derived NL and NE, we carried out some preliminary FA tests on different days of storage – day 1 (day of preparation), and also particles stored at 4°C for about 5 - 6 months and more. For these tests we used stock solutions of NL and NE. Samples were lyophilized and methylated before gas chromatography analysis.

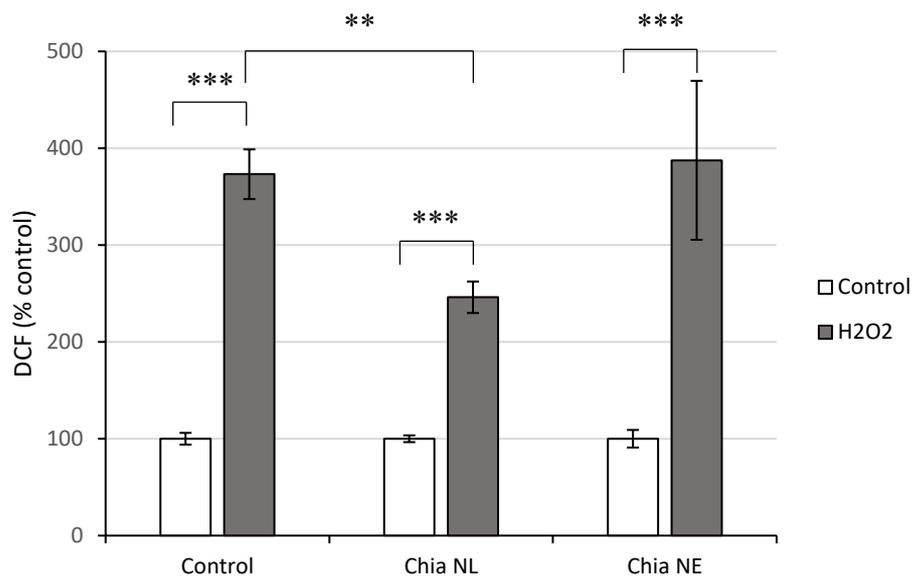
The studies of NL and NE FA composition showed that the major FA which were observed previously in chia seed lipids (Chapter III, Table 5), are still present, including high level of ALA. In addition, the relative proportion of FA as compared to that of chia oil appeared to be maintained during whole storage period. While these are only preliminary results, this supports our hypothesis that FA composition of chia-seed derived NL and NE remain stable during storage, without significant loss of ALA. Additional tests are required to support conclusions above.

## **5.2 ANTIOXIDATIVE PROPERTIES OF NANOLIPOSOMES AND NANOEMULSIONS OBTAINED FROM CHIA SEED LIPIDS**

Reactive oxygen species (ROS) are essential intermediates in oxidative metabolism. However, the production of excess ROS can damage cells by peroxidizing lipids and disrupting the structure of proteins, enzymes, and nucleic acids. Excess ROS can result from a number of different cellular stress mechanisms such as inflammatory processes that is involved in a number of pathologies. Because of this, antioxidants have been actively studied as potential therapeutics and as anti-inflammatory agents. Plants are a rich source of these molecules, including chia seeds. For example, caffeic and rosmarinic acids have been identified in chia, and have been shown to play important roles in the prevention of neurological disorders and to have hepatoprotective properties (Oliveira-Alves *et al.*, 2017).

As described in chapter III, we prepared and characterized chia NL using the phospholipid-rich solid residue obtained after Folch extraction. This same residue was also used as emulsifier to prepare NE with chia oil. Composition analysis revealed that this residue contained other chia seed components, including fiber and protein. Since antioxidant compounds has been found in chia seeds and oil extracts, we questioned if antioxidants were also present in chia-based NL and NE. We tested this using the Hepa 1-6 cell model. Hepa 1-6 cells were incubated 24 h with 150

$\mu\text{g/mL}$  chia NL or NE, which was shown to have no significant effect on cell viability (see previous chapter). After inducing oxidative stress with  $\text{H}_2\text{O}_2$ , ROS production was measured fluorimetrically using 2',7'-dichlorofluorescein-diacetate (DCFH-DA), which is transformed into 2',7'-dichlorofluorescein (DCF), a highly fluorescent compound by ROS (Cathcart *et al.*, 1983; LeBel *et al.*, 1992).



**Figure 22.** ROS production in Hepa1-6 cells incubated 24h with chia-based NL or NE and treated with  $\text{H}_2\text{O}_2$

Results show that there was a significantly lower production of ROS in Hepa 1-6 cells that had been incubated 24 h in the presence of chia NL. Results with chia NE were quite variable and thus did not achieve statistical significance. While these are only preliminary results, it suggests the presence of antioxidants in chia NL, indicating an added beneficial effect of these nanoparticles that may not only contribute to the stability of NL, but also provide useful for preventive or therapeutic purposes.

The method used to study antioxidative activities has some limitations, such as high concentration of hydrogen peroxide used, and possible entrapment of these molecules by lipid nanoparticles which could appear as an anti-oxidative property of the NL. Also, we cannot rule out potential NL-mediated inhibition of cholesterol esterases converting DCFH-DA to DCFH, which could contribute to lower DCF levels. Other tests to measure anti-oxidative activities should also be performed to confirm these observations.

If this anti-oxidative activity is confirmed in NL, further investigation is required to identify the antioxidant molecule(s) that are present in the chia NL using detection methods such as HPLC. It would be of interest to assess the antioxidative properties in other cell types, including neurons and astrocytes, and by other methods, as mentioned above.

## CHAPTER VI. THESIS RESEARCH CONCLUSIONS AND PERSPECTIVES

This research project “Development and characterization of chia (*Salvia hispanica* L.) plant-derived products as natural bioactive compounds” is multidisciplinary work realized in “*co-tutelle*” conditions between two universities and three departments. Expertise of these departments lie in the fields from food biotechnology and safety (ITMO University) to lipid nanoparticles scientific knowledge in the “Laboratory of Biomolecule Engineering” (LIBio) and interest of omega-3 role on health and disease in the Quality of Diet and Aging team (QUALIVIE) of the Research Unit “Animals and Functionality of Animal Products” (UR AFPA).

Final objective of present study was to evaluate bioavailability of chia seed lipid extracts in different forms and to assess their bioactivity focusing on potential treatment of lipid-related disorders and associated diseases.

For this study, we used the chia ORURO variety, which has been specifically adapted for cultivation in Europe, France in 2017. We characterized oil yield and fatty acid composition of this chia seeds by different extraction methods at the first step as it is variety-dependent parameters.

The type of solvent extraction method used can affect oil yield from chia seeds ORURO variety, as well as fatty acid composition. Lipid extraction by modified Folch method was chosen as the target extraction method to be used for chia seeds which allows the extraction of polar lipids, including phospholipids for the preparation of nanoliposomes. Oil yield was found to be close to 30% of total seed mass. Analysis of the chromatograms revealed ten FA species, with the highest levels being ALA, linoleic, palmitic, oleic, and stearic acids, in descending order. % ALA was slightly but significantly higher with the Folch ( $61.92 \pm 0.16$  %) as compared with the Soxhlet methods ( $60.94 \pm 0.20$ %).

The evaporation step of the solvent after lipid extraction by the Folch-based method revealed the presence of a solid residue on the walls of the flask (a yield of  $13.97 \pm 0.25\%$  of oil mass), and we hypothesized that it is a complex, including phospholipids. Further analyses showed that this complex consists of phospholipids, moisture, protein and fiber, and other components.

Phospholipid analysis of chia seed lipids revealed that total phospholipid content in chia seed oil was 7.49% before solvent removal. Six phospholipid classes were identified. In the sample before evaporation, PL composition was revealed to be 30.87% phosphatidylinositol (PI), 20.20% phosphatidic acid, 19.15% phosphatidylethanolamine (PE), 12.66% phosphatidylglycerol (PG), 11.82% phosphatidylcholine and 5.3% lysophosphatidyl-choline (Lyso-PC). Also the FA composition of the chia seed phospholipid classes was determined.

We next prepared NE from the chia seed oil and phospholipid-rich solid residue, and NL from the phospholipid-rich solid residue. The results of this thesis project demonstrate the feasibility of using chia lipids for the preparation of stable spherical NL and NE with high negative charge and size in 100 nm range. NL represent high scientific interest compared to NE which can be explained by their possibility to encapsulate both hydrophobic and hydrophilic molecules instead of only hydrophobic in NE, and more controlled drug release. As a rich source of omega-3 PUFA, development of chia-derived products is of high interest in health and nutrition, and this work has revealed the potential of using chia oil and lipids.

Indeed, “green” extraction methods, such as press extraction and aqueous enzymatic extraction were also tested in preliminary studies. This, as well as the use of natural deep eutectic solvents (NADES) are other potential options that should be tested for exception of toxic organic solvents effects.

Our work here on the chia oil solid phase residue reveals that it contains other plant cell components such as protein and fibers. Additional purification steps may be necessary in order to obtain pure chia phospholipids following Folch extraction and additional studies on the detailed

composition of this solid residue needs to be done, including peptides, antioxidant molecules and others.

One question we did not cover in this thesis research was oxidation status of lipids, neither directly after extraction, neither in nanotechnological formulations. It is necessary to study in case of possible loss of nutritional value or for example, formation of oxidized metabolites of PUFA which can have additional scientific interest.

We have demonstrated that these chia-based NL and NE show little to no cell toxicity in a number of cell types, from liver to neurons and astrocytes. Astrocytes showed more sensibility to chia-derived NL than other cell types which could be explained by cell-specificity. As potential direction of research we could highlight the examination of metabolism levels in different cell types, kinetics of toxicity in time. One of possible way to obtain cytotoxicity in cells are free FA, some additional tests are required.

The next step is to determine the bioavailability of chia-based NE and NL: what is the effect of these soft nanoparticles on lipid status in the different cells? Lipid accumulation in cells is interesting direction of research. How is cell membrane FA composition modified? What are the effects of chia-based NE and NL *in vivo* with respect to prevention of lipid-related disorders, and how does this compare to studies using chia seeds or chia oils?

Chia seeds are also known for their antioxidant properties, and interestingly, our preliminary tests suggest that the chia-based NE and NL have some antioxidant activity *in vitro* using the Hepa1-6 cell model. Therefore, potential bioactivities of the chia-based NL and NE may be based on the presence of antioxidants. Additional studies are needed to pursue this line of investigation to identify the antioxidant components that are present in the chia seed lipid fraction and the resulting soft nanoparticles. Other types of biological tests for anti-oxidative activities can be performed to avoid disadvantages as possible inhibition of cholesterol esterase activities or possible entrapping of hydrogen peroxide by NL.

One of the possible development directions is also recycling of oil-cake, utilization of mucilage, protein and other valuable by-products which can be found in chia seeds.

In conclusion, our studies indicate that PUFA-rich stable NL and NE can be prepared from chia lipid, including phospholipid fractions. Further investigation is merited since these nanoparticles represent potential bioactive vectors for use in both preventive and therapeutic applications in human health.

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## COMMUNICATIONS

1. «*Study of the physicochemical composition of raw ingredients based on chia seeds (Salvia hispanica L.)*». XLVI Scientific and educational-methodical conference of ITMO University. St. Petersburg, Russia, 2017 (In Russian).

2. «*Chia seeds (Salvia hispanica L.) and products of their processing in the food industry*» (In Russian) and «*The possibility of using chia seeds (Salvia hispanica L.) in the food industry*». VI Congress of young scientists (CMU). St. Petersburg, Russia, 2017.

3. «*Prospects for using chia seeds (Salvia hispanica L.) in the composition of therapeutic and prophylactic products on dairy-plant basis*». All-Russian scientific and practical conference «Young researchers – for the development of the dairy industry». Volodga, Russia, 2017 (In Russian).

4. «*Comparative analysis of various varieties of chia seeds (Salvia hispanica L.)*». VIII International Conference «Refrigeration and Food Technologies in the 21st Century». St. Petersburg, Russia, 2017 (In Russian).

5. «*Study of quality indicators of chia seeds oil (Salvia hispanica L.) obtained by different methods*». XLVII Scientific and educational-methodical conference of ITMO University. St. Petersburg, Russia, 2018 (In Russian).

6. «*Development of the functional foodstuff of the complex raw material composition using chia seeds (Salvia hispanica L.)*». International Scientific and Practical Conference «Scientific Innovations for Agrarian Production» dedicated to the 100th anniversary of the Omsk State Agrarian University. Omsk, Russia, 2018 (In Russian).
7. «*Investigation of the chia seeds (Salvia hispanica L.) effect on the coagulation proteins process in milk-plant mixture*». VII Congress of young scientists (CMU). St. Petersburg, Russia, 2018 (In Russian).
8. «*Comparative analysis of chia seeds (Salvia hispanica L.) oil obtained by different methods*». 9<sup>th</sup> International Conference «Biosystems Engineering 2018». Tartu, Estonia, 2018.
9. «*Enzymatic extraction as an alternative method of chia seed (Salvia hispanica L.) oil obtaining*». XLVIII Scientific and educational-methodical conference of ITMO University. St. Petersburg, Russia, 2019 (In Russian) (in absentia).
10. «*Non-traditional sources of nutrients for Russian consumers and prospects of their use*». Orazov A., Nadtochii L., Baranenko D.A., **Kuznetcova D.V.**, Lepeshkin A.I., Melchakov R.M. The Third ASRTU Sino-Russian Bilateral Symposium on Innovation Food Science and Equipment. Harbin Institute of Technology, Harbin, China, 2018 (in absentia).
11. «*Natural and “green” nanoemulsions from chia seeds (Salvia hispanica L.) as potential active vectors*». International Congress on Biotechnology «Biocon 2019». Paris, France, 2019.
12. «*Prospects for the use of microgreens and plant green parts as a source of biologically active substances in the development of functional nutrition*». Nadtochii L.A., **Kuznetcova D.V.**, Proskura A.V., Muradova M.B. IX International Conference «Refrigeration and Food Technologies in the 21st Century». St. Petersburg, Russia, 2019 (In Russian) (in absentia).

## PRESENTATION SYNTHETIQUE DE LA THESE

### «Développement et caractérisation des produits dérivés de chia (*Salvia hispanica* L.) en tant que composés naturels bioactifs»

Les acides gras polyinsaturés (AGPI) oméga-3 sont importants pour le maintien de la nutrition et de la santé chez l'homme. Ils jouent un rôle essentiel dans l'homéostasie des lipides en raison de leurs fonctions de modulation de la fluidité et du fonctionnement de la membrane cellulaire, de la signalisation cellulaire et de la régulation transcriptionnelle de l'expression des gènes. De nombreuses études ont démontré la relation entre les carences en AGPI et le risque de maladies, notamment le syndrome métabolique y compris, l'insulinorésistance, les maladies cardiovasculaires et neurodégénératives.

Les acides gras oméga-3 sont présents dans les organismes marins et les plantes. Puisque les sources marines peuvent être limitées en approvisionnement ou peuvent même présenter un problème en raison de leur allergénicité ou de la possibilité de contamination par des polluants, l'intérêt s'est porté sur les produits d'origine végétale comme alternative intéressante et source prometteuse d'AGPI.

Les graines de chia (*Salvia hispanica* L.) sont un ingrédient alimentaire utilisé depuis des siècles, à commencer par les tribus Aztèques, Incas et Mayas, qui utilisaient ces graines à des fins médicinales, entre autres. L'intérêt mondial récent pour les graines de chia et les études de composition de l'huile obtenue à partir de cette culture, révèlent une teneur élevée en AGPI dans les graines de chia (environ 80%), et en particulier en acide alpha-linolénique (ALA) (oméga-3), qui représente environ 60% de la teneur totale en lipides, l'une des plus élevées enregistrées parmi les sources végétales connues.

Le degré élevé d'insaturation des huiles réduit leur stabilité oxydative, ce qui pourrait entraîner une perte de valeur nutritionnelle lors du stockage, ainsi que la formation éventuelle de produits toxiques indésirables. Par conséquent, l'amélioration de la stabilité de l'huile de graines de chia et la préservation de l'AGPI contre la dégradation sont particulièrement importantes pour son utilisation dans des applications de nutrition et de santé, telles que les aliments fonctionnels ou les traitements thérapeutiques.

L'utilisation des nanotechnologies permet de modifier les caractéristiques traditionnelles des huiles comestibles, d'améliorer leur qualité et leur sécurité, d'accroître leur biodisponibilité et d'élargir le champ d'application. Dans cette recherche de thèse, nous avons choisi de préparer et de caractériser les nanoémulsions (NE) et les nanoliposomes (NL) comme formes de vecteurs actifs à base de lipides de la graine de chia.

Les études des graines de chia comme source de substances biologiquement actives au cours des 10 dernières années ont suscité un grand intérêt de la part de la communauté scientifique en raison de leurs avantages potentiels pour la santé. Etant riche en AG de type oméga-3, l'utilisation de l'huile de graines de chia dans différentes formulations présente un grand potentiel. Cependant, il n'existe pas à ce jour les études systématiques dans la littérature sur les lipides dérivés de la chia, y compris la fraction phospholipidique et leur utilisation dans le développement de vecteurs tels que les nanoparticules (NE et NL). Le développement de telles particules serait utile pour étudier les effets de l'huile dérivée de la graine de chia et des produits riches en ALA sur différents processus métaboliques.

**L'objectif principal de ce projet** de thèse est d'évaluer la biodisponibilité des extraits de lipides de la graine de chia (ChSL) sous différentes formes et d'évaluer leur bioactivité en se focalisant sur le traitement potentiel des troubles liés aux lipides et des maladies associées.

Pour ce projet, nous avons choisi d'utiliser des graines de chia (*Salvia hispanica* L.) de la variété ORURO qui ont été adaptées pour la croissance et la culture en France. Étant donné que la

composition des substances biologiquement actives dans les graines de chia peuvent varier en fonction de la région de croissance, il est particulièrement important d'évaluer la graine de chia de la variété ORURO, et plus particulièrement, la composition lipidique de cette variété.

**Les objectifs spécifiques** de la thèse "Développement et caractérisation des produits dérivés du chia (*Salvia hispanica* L.) en tant que composés bioactifs naturels" sont :

**Objectif 1** - Extraction et caractérisation de la ChSL;

**Objectif 2** - Préparation et caractérisation physico-chimique de NE et NL en utilisant de la ChSL;

**Objectif 3** - Étude de la biodisponibilité et de la bioactivité des NE et NL dérivés de graines de chia à l'aide de modèles cellulaires (cellules d'hépatome, astrocytes et neurones).

L'extraction des lipides par la méthode Folch modifiée a été choisie comme la méthode d'extraction cible à utiliser pour les graines de chia. Cette méthode utilise un mélange chloroforme:méthanol (2:1, v:v) qui permet l'extraction de lipides polaires, y compris des phospholipides, composants essentiels pour la préparation de nanoliposomes. On a constaté que le rendement en huile était de  $29,37 \pm 0,97$  % de la masse totale. Ce résultat est inférieur de 17 % à celui obtenu par la méthode utilisant l'appareil Soxhlet et un mélange éther diéthylique / éther de pétrole (1:1, v:v), ce qui a permis d'obtenir un rendement en huile de  $35,25 \pm 1,42$  % de la masse totale.

L'analyse par chromatographie en phase gazeuse de l'huile de graines de chia a révélé 10 espèces d'acides gras, les niveaux les plus élevés étant les acides ALA, linoléique, palmitique, oléique et stéarique, par ordre décroissant. La quantité d'ALA était légèrement mais significativement plus élevée avec la méthode Folch par rapport aux méthodes Soxhlet. L'analyse de la teneur en lipides polaires de l'huile par chromatographie sur couche mince n'a révélé aucune différence significative entre celle obtenue après extraction Folch ( $2,21 \pm 0,33\%$ ) ou Soxhlet ( $2,34 \pm 0,61\%$ ). L'étape d'évaporation du solvant après extraction des lipides par la méthode de

Folch a révélé la présence d'un résidu solide sur les parois du flacon avec un rendement de  $13,97 \pm 0,25\%$  de la masse totale.

Des analyses de la phase huileuse obtenue par la méthode Folch modifiée ont également été effectuées avant et après l'évaporation du solvant afin de déterminer l'influence de cette étape sur la récupération des lipides polaires. Les résultats de la chromatographie sur couche mince (Iatroscan) ont montré une diminution significative de la teneur en lipides polaires après élimination du solvant pour les fractions d'huile de chia. Les analyses des teneurs en protéine (méthode de Kjeldahl) et en fibres ont révélé la présence à la fois de ces deux composants ; les niveaux de glucides étant relativement mineurs. Sur la base de ces analyses, nous proposons que ce résidu solide soit constitué d'un complexe naturel riche en lipides polaires contenant de l'humidité, des protéines et des fibres, et potentiellement d'autres composants, ce qui pourrait expliquer la perte significative de la teneur en lipides polaires dans la phase huileuse suite à l'évaporation après extraction par Folch.

Nous avons ensuite analysé la composition des phospholipides avant et après l'élimination du solvant par chromatographie liquide à haute performance avec spectrométrie de masse. Les résultats ont révélé que la teneur totale en phospholipides de l'huile de graines de chia était en moyenne de 7,5 % avant l'élimination du solvant et de moins de 0,3 % dans la phase liquide de la fraction lipidique après l'évaporation du solvant. Cela confirme la perte significative de phospholipides dans l'huile à la suite de cette étape, qui se trouve très probablement dans le résidu.

Six classes de phospholipides ont été identifiées dans l'huile de graines de chia d'ORURO. Dans l'échantillon avant évaporation, les phospholipides étaient dans l'ordre décroissant : 30,87% de phosphatidylinositol (PI), 20,20% d'acide phosphatidique, 19,15% de phosphatidyléthanolamine (PE), 12,66% de phosphatidylglycérol (PG), 11,82% de phosphatidylcholine et 5,3% de lysophosphatidyl-choline (Lyso-PC). Une analyse des données a ensuite été effectuée pour déterminer la composition en acides gras des classes de phospholipides

de la graine de chia. La quantité de phospholipides contenant des AGPI ALA a été entre 45 et 68 % dans les différentes classes de phospholipides, la quantité la plus élevée se trouvant dans le PI et la plus faible dans le PC.

Dans le cadre de cette étude, des NE provenant de l'huile de graines de chia et du résidu solide riche en phospholipides, et des NL provenant du résidu solide riche en phospholipides ont été préparés et testés, en utilisant les méthodes déjà établies par le laboratoire LIBio. L'indice de polydispersité (IP), qui est une mesure de l'agrégation, de la taille et du potentiel zêta des particules, a été déterminé. Les valeurs de la DJP de toutes les particules se sont avérées être  $< 0,3$ , indiquant des solutions monodispersées et aucune agrégation de particules. La taille a été déterminée en utilisant la diffusion dynamique de la lumière, étant légèrement supérieure à 100 nm, donc à l'échelle de nanomètres. La stabilité des préparations de la NL et de la NE a également été démontrée par leur faible charge négative (potentiel zêta).

Nous avons mesuré la stabilité de la NL et de la NE en mesurant la taille des particules chaque jour pendant 5 jours après leur préparation, alors qu'elles étaient stockées à 4°C sous N<sub>2</sub>(g) dans des flacons hors lumière. Aucune variation significative n'a été observée au cours de cette période, qui correspond à la période de stockage pour leur usage dans les études *in vitro* sur les cultures cellulaires. La microscopie électronique à transmission a été effectuée pour évaluer les propriétés morphologiques des particules. Les images ont montré la nature multicouche et la morphologie sphérique des préparations de chia NL et NE. Enfin, l'analyse à l'aide d'un kit enzymatique a révélé une teneur de  $3,4 \pm 1,04$  mg/mL et de  $3,8 \pm 1,26$  mg/mL de phospholipides contenant de la choline dans le NL et le NE, respectivement, ce qui confirme la présence de phospholipides dans le résidu solide utilisé pour préparer ces nanoparticules.

Ces NL et NE stables étant préparés à partir de lipides de chia, notre prochain objectif était d'investiguer le niveau de leur biodisponibilité dans l'organisme. Pour cela, il faut l'évaluer dans différents tissus périphériques, y compris le foie (principal tissu de régulation de l'homéostasie des

lipides), ainsi que le système nerveux central (SNC). Nous avons commencé par une étude de cytotoxicité de NL ou de NE de chia, en utilisant différents modèles cellulaires : Hepa-1-6 (hépatome murin), astrocytes (murin) exprimant 3 isoformes d'apoE, et cultures primaires de neurones corticaux embryonnaire de rat. Le test de viabilité cellulaire MTT a montré peu ou pas de toxicité cellulaire après une incubation de 24 h avec des concentrations en allant de 1 à 150  $\mu\text{g/mL}$  de NL ou NE dans les Hepa 1-6 (hépatome de souris). Par contre une diminution significative de viabilité cellulaire a été remarquée lors d'une incubation de 24 h des astrocytes exprimant l'isoforme apoE2 avec les astrocytes exprimant l'isoforme apoE2. Très peu d'effet significatif des NL ou des NE a été observé dans les neurones.

En conclusion, les résultats de ce projet de thèse démontrent la faisabilité de l'utilisation des lipides du chia pour la préparation de NL et NE. En tant que riche source d'AGPI oméga-3, le développement de produits dérivés de la chia est d'un grand intérêt pour la santé et la nutrition, et ce travail a révélé le potentiel de l'utilisation de l'huile et des lipides du chia. En effet, des méthodes d'extraction "vertes", telles que l'extraction par presse et l'extraction enzymatique aqueuse, ont également été testées dans le cadre d'études préliminaires, et semblent prometteuses. L'utilisation de solvants eutectiques naturels profonds (NADES) est aussi une autre option potentielle qui méritent être testées.

Notre travail ici sur le résidu en phase solide de l'huile de chia révèle qu'il contient d'autres composants de cellules végétales tels que des protéines et des fibres. Des étapes de purification supplémentaires peuvent être nécessaires afin d'obtenir des phospholipides purs après l'extraction de Folch.

Nous avons démontré que ces cellules NL et NE à base de chia présentent peu ou pas de toxicité cellulaire dans un certain nombre de types de cellules, du foie, aux neurones et aux astrocytes. La prochaine étape consiste à déterminer la biodisponibilité des NE et NL à base de chia : quel est l'effet de ces nanoparticules molles sur le statut lipidique dans les différentes

cellules? Comment la composition de l'AF de la membrane cellulaire est-elle modifiée? Quels sont les effets *in vivo* de la NE et de la NL à base de chia en ce qui concerne la prévention des troubles liés aux lipides, et comment l'efficacité, est-elle par rapport aux études utilisant des graines ou des huiles de chia?

Les graines de chia sont également connues pour leurs propriétés antioxydantes, et il est intéressant de noter que nos tests préliminaires ont révélé un effet antioxydant des NL et des NE à base de chia *in vitro* chez les cellules Hepa1-6. Les bioactivités potentielles de la NL et de la NE à base de chia pourraient donc être basées non seulement sur leur teneur en AGPI de type oméga-3, mais aussi sur la présence d'antioxydants. Des études supplémentaires sont nécessaires pour poursuivre ces observations afin d'identifier les composants antioxydants qui sont présents dans la fraction lipidique des graines de chia et les nanoparticules molles qui en résultent.

En conclusion, nos études indiquent que les NL et NE stables riches en AGPI peuvent être préparés à partir des lipides du chia, y compris les fractions phospholipidiques. Des recherches plus approfondies sont nécessaires, car ces nanoparticules représentent des vecteurs bioactifs potentiels pouvant être utilisés dans des applications préventives et thérapeutiques en santé humaine.

## APPENDIX I

For this thesis project, some preliminary additional data was obtained, which are presented below. The table below shows the values following analysis of chia seed composition, as compared to the manufacturer's information that was provided on the ORURO variety used in this project.

Appendix Table 1. Preliminary nutritional value of ORURO chia seeds per 100 g of seeds

<b>Index</b>	<b>Value for chia seed varieties ORURO according to our studies*</b>	<b>Manufacturer data</b>
Moisture content, %	4.18±0.12	n / a
Ash content, %	5.16±0.8	n / a
Protein content, %	23.00±1.48	23.8
Oil yield**, %	29.63±3.1	29.1
Carbohydrates content***, %	38.03	37.5
Total energy value ***, kcal	510.79	441

\* average ± SD

\*\* Chia seed oil obtained by the Folch-based method

\*\*\* estimated value

A pilot study was performed to determine the fatty acid composition of chia seeds lipid fraction obtained after cold-pressing.

Appendix Table 2. Preliminary fatty acid composition of chia seeds lipid fraction obtained by cold-pressing

<b>Index</b>	<b>Chia oil cold-pressed, %</b>
<b>IS*, C19</b>	1.38
C16:0	7.23
C18:0	3.24
<b>SFA</b>	<b>10.47</b>
C16:1	0.23
C18:1 n-9	6.42
C18:1 n-7	0.76
C18:1 n-5	0.12
<b>MUFA</b>	<b>7.53</b>
C18:2 n-6	18.50
C18:3 n-6	0.23
C18:3 n-4	0.24
C18:3 n-3	61.32
<b>PUFA</b>	<b>80.29</b>
SFA/MUFA	1.39
SFA/PUFA	0.13
Ratio n-6/n-3	0.31

\*internal standard

Analysis using the Iatroscan showed recovery of 4.54% of polar lipid content in chia seed lipids after cold-pressing. Additional investigation is required to determine the feasibility of this method for use in NL and NE preparation.

## ABSTRACT

Les acides gras polyinsaturés (AGPI) oméga-3 sont importants pour la nutrition et la santé, en raison de leur importance dans l'homéostasie des lipides, et du fait que les carences en AGPI peuvent augmenter le risque de syndrome métabolique, de maladies cardiovasculaires et neurodégénératives. Les graines de chia (*Salvia hispanica* L.) sont riches en AGPI (80%) et contiennent les plus hauts niveaux connus dans les plantes de l'acide gras essentiel oméga-3, l'acide alpha-linolénique (ALA). Un degré élevé d'insaturation dans les huiles comestibles peut réduire la stabilité oxydative, entraînant une perte de valeur nutritionnelle. Les nanotechnologies peuvent être utilisées pour améliorer la qualité et la sécurité des huiles de graines de chia, augmenter la biodisponibilité et élargir le champ des applications. Dans cette optique, notre objectif était de préparer et de caractériser les lipides des graines de chia sous forme de nanoliposomes (NL) et de nanoémulsions (NE), et d'évaluer leur utilisation potentielle en tant que produits bioactifs. Dans une première étape, les fractions de lipides ont été caractérisées. Les lipides ont été extraits de la variété française ORURO de graines de chia en utilisant la méthode Folch modifiée. Dix espèces d'acides gras et six classes de phospholipides (PL) ont été identifiées dans l'extrait de lipides de graines de chia par chromatographie en phase gazeuse et LC-MS, respectivement, le niveau le plus élevé d'acides gras étant l'ALA (62%). La présence d'un résidu solide a été détectée suite à l'évaporation du solvant pour obtenir l'huile de graine de chia. Les analyses par chromatographie sur couche mince et par LC-MS ont démontré que ce résidu contenait les lipides polaires, y compris des PL, qui n'étaient plus présents dans l'huile de chia après l'élimination du solvant par évaporation. Les NE ont été préparés à partir de l'huile de chia et du résidu solide riche en PL du chia, et les NL à partir du résidu solide riche en PL. La caractérisation physicochimique, y compris l'analyse de l'indice de polydispersité (PDI), de la taille et du potentiel zêta, a indiqué que les NE et NL étaient des solutions monodisperses de particules stables (faible potentiel zêta négatif) d'une taille entre 104 et 118 nm. Ces préparations étaient sphériques et multilamellaires (microscopie électronique à transmission), et restaient stables même 5 jours après leur préparation. De plus, un test enzymatique a confirmé la présence des PL dans les NE et les NL à la base de chia. Le test de viabilité cellulaire MTT a montré peu ou pas de toxicité cellulaire (jusqu'à 150 µg/ml NE ou NL) après incubation de 24 heures avec des hépatocytes, des neurones et des astrocytes cultivés. En conclusion, ces résultats démontrent la faisabilité de l'utilisation des lipides du chia pour la préparation de NL et NE de chia, stables et non toxiques, riches en AGPI oméga-3, qui représentent des vecteurs bioactifs potentiels pour des applications préventives et thérapeutiques en santé humaine.

## SUMMARY

Omega-3 polyunsaturated fatty acids (PUFA) are important for nutrition and health, by virtue of their importance in lipid homeostasis, and the fact that PUFA deficiencies can increase risk of metabolic syndrome, cardiovascular and neurodegenerative diseases. Chia (*Salvia hispanica* L.) seeds are rich in PUFA (80%) and contain the highest known levels in plants of the essential omega-3 fatty acid, alpha-linolenic acid (ALA). High degree of unsaturation in edible oils can reduce oxidative stability causing a loss of nutritional value. Nanotechnology can be used to improve chia seed oil quality and safety, increase bioavailability, and expand the scope of applications. Towards this goal, our objective was to prepare and characterize chia seed lipids in the form of nanoliposomes (NL) and nanoemulsions (NE), and to evaluate their potential use as bioactive products. The first step was to characterize lipid fractions. Lipids were extracted from the French ORURO variety of chia seeds using modified Folch method. Ten fatty acid species and six phospholipid (PL) classes were identified in chia seed lipid extract by gas chromatography and LC-MS, respectively, with the highest level of fatty acids being ALA (62%). The presence of a solid residue was detected following evaporation of the solvent to obtain chia seed oil. Analyses by thin layer chromatography and LC-MS demonstrated that this residue contained the polar lipids including PL that were no longer in the chia oil after removal of solvent by evaporation. NE were prepared from chia seed oil and the chia PL-rich solid residue, and NL from the PL-rich solid residue. Physicochemical characterization including analysis of the polydispersity index (PDI), size, and zeta-potential indicated that both NE and NL were monodispersed solutions of stable (low negative zeta potential) particles with a size between 104-118 nm. These preparations were spherical and multilayered (transmission electron microscopy) and remained stable even 5 days after preparation. In addition, enzymatic assay confirmed PL content in both NE and NL. MTT cell viability assay showed little to no cell toxicity (up to 150 µg/mL NE or NL) following 24 h incubation with cultured hepatocytes, neurons, and astrocytes. In conclusion, these results demonstrate the feasibility of using chia lipids for the preparation of stable non-toxic omega-3 PUFA rich NL and NE, which represent potential bioactive vectors for both preventive and therapeutic applications in human health.